Environment, endocrinology, and biochemistry influence expression of stress proteins in bottlenose dolphins

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Abstract

Natural and anthropogenic stressors have been reported to impact the health of marine mammals. Therefore, investigation of quantifiable biomarkers in response to stressors is required. We hypothesized that stress protein expression would be associated with biological and health variables in wild and managed-care bottlenose dolphins (*Tursiops truncatus*). To test this hypothesis, our study objectives were to (1) determine if stress proteins in skin, white blood cells (WBCs), and plasma could be measured with an antibody-based microarray, (2) measure stress-protein expression relative to biological data (location, sex, age, environment), and (3) determine if stress-protein expression was associated with endocrine, hematological, biochemical and serological variables and gene expression in bottlenose dolphins. Samples were collected from two wild groups (n=28) and two managed-care groups (n=17). Proteins involved in the HPA axis, apoptosis, proteotoxicity, and inflammation were identified as stress proteins. The expression of 3 out of 33 proteins was significantly ($p<0.05$) greater in skin than plasma and WBCs. Male dolphins had significantly greater expression levels for 10 proteins in skin compared to females. The greatest number of stress-associated proteins varied by the dolphins' environment; nine were greater in managed-care dolphins and 15 were greater in wild dolphins, which may be related to wild dolphin disease status. Protein expression in skin and WBCs showed many positive relationships with measures of plasma endocrinology and biochemistry. This study provides further understanding of the underlying mechanisms of the stress response in bottlenose dolphins and application of a combination of novel methods to measure stress in wildlife.

Key words (8, alphabetical order): conservation; dolphin; microarray; physiology; proteomics; skin; stress; *Tursiops truncatus*

1. Introduction

Marine mammals face a wide array of potential stressors that include, but are not limited to, the effects of climate change, disease, pollution (including persistent organic pollutants, heavy metals, and other contaminants), fishery activities, noise, and habitat degradation (Simmonds, 2018). The physiological stress response of marine mammals to natural and anthropogenic stressors is thought to be similar to terrestrial mammals (Atkinson et al., 2015), but with some modification to action resulting from their secondary adaptation to the marine environment. This response begins with the sympathetic nervous system (SNS) stimulating the release of epinephrine and norepinephrine from the adrenal medulla and is quickly followed by the activation of the hypothalamus-pituitary adrenal (HPA) gland axis. Activation of the HPA axis then initiates the release of a cascade of hormones, including corticotropin releasing factor (CRF) and subsequently adrenocorticotropic hormone (ACTH; Sapolsky et al., 2000). This is followed by the release of glucocorticoids (GCs), such as cortisol and corticosterone, and mineralocorticoids such as aldosterone from the adrenal cortex (Romano et al., 2004; St. Aubin et al., 1996). In bottlenose dolphins (*Tursiops truncatus*), it has been reported that chronic stress increased adrenal mass, cortex to medulla ratio, and epinephrine-producing cells within the medulla (Clark et al., 2006). During capture and restraint, plasma concentrations of epinephrine, ACTH, and cortisol were also found to increase in dolphins (Champagne et al., 2018; Fair et al., 2014). Furthermore, the concentration of aldosterone increased in response to anthropogenic sounds in bottlenose dolphins (Romano et al., 2004) and both cortisol and aldosterone increased

when bottlenose dolphins were exposed to a stressful event such as encirclement by capture nets (St. Aubin et al., 1996).

In addition to the endocrine responses described above, mammals also express a variety of stress-associated proteins involved in other physiological processes such as oxidative stress, inflammation, and proteotoxicity. For the purpose of this study, stress-related proteins were categorized into four functional groups based on their roles in the stress response: (1) HPA, (2) apoptosis and cell cycle (ACC), (3) cellular stress and proteotoxicity (CSP), and (4) oxidative stress and inflammation (OSI). The HPA group includes arginine vasopressin (AVP), which increases water retention and vasoconstriction (Sapolsky et al., 2000). Caspase proteins were included in the ACC group as these cysteine proteases are involved in apoptosis (Taylor et al., 2008). The CSP group includes the highly conserved heat shock proteins (HSPs; classified by molecular mass), which are involved in protein folding and repair (Kim and Yenari, 2017). Lastly, the OSI group includes members of the superoxide dismutase family which are antioxidant enzymes that catalyze the initial breakdown of superoxide radical anions (Pisoschi and Pop, 2015).

Antibody-based protein microarrays are a recent technique that can be used to detect and measure the expression of such proteins. Antibody microarrays enable the measurement of the expression of multiple proteins in small sample volumes with high sensitivity (Chen et al., 2018). The ability of multiplex protein measurement is crucial for understanding the biological roles of proteins and their expression, as their function is often associated with other proteins, ligands, and receptors (Kingsmore, 2006). These assays have been developed for applications in cancer research (Ingvarsson et al., 2008), drug development (Lee et al., 2004), and reproductive physiology (Gao et al., 2016). It is evident that the expression of such proteins play important

93 roles in responding to stress and maintaining homeostasis in mammalian species; however, very
94 few studies have utilized this technique to assess the physiological stress response of wildlife
95 (Carlson et al., 2016) and the application of this technique to marine mammals has not yet been
96 explored.

97 This study aimed to determine stress-associated protein expression in bottlenose dolphins
98 using a protein microarray that consists of 33 antibodies that were selected from a large panel
99 (>250) of broadly reactive commercial antibodies that specifically cross-react with mammalian
100 skin proteins (Carlson et al., 2016). While plasma and white blood cell samples can be collected
101 during routine capture and release health assessments, remote sampling of skin may provide a
102 useful tool to assess stress and monitor the health of wild dolphin groups, since it does not
103 require capture and restraint of individuals. Therefore, we hypothesized that stress-associated
104 protein expression could be determined using the microarray technique and that protein
105 expression would be associated with certain biological and clinical health parameters. To test this
106 hypothesis, the objectives of this study were to (1) determine whether the microarray could
107 reliably measure stress proteins in skin, white blood cells (WBCs; consisting of neutrophils,
108 eosinophils, basophils, lymphocytes and monocytes), and plasma through the isolation and
109 concentration of proteins in these matrices, (2) measure stress-associated protein expression in
110 skin and WBCs relative to biological data (location, sex, age, environment), and (3) determine if
111 stress-associated protein expression in skin and WBCs was associated with clinical health
112 parameters such as mRNA transcript abundance, and endocrine, hematological, biochemical, and
113 serological variables as these factors are often associated with the stress response in other
114 species. To our knowledge, this is the first study to identify and measure stress-associated
115 proteins in dolphins and relate the changes in protein expression to biological and clinical health

parameters. Furthermore, these methods could be applied to samples collected from wild populations that experience various anthropogenic pressures and these data can be used to aid in the interpretation of those results.

2. Material and methods

2.1. Study Animals

Male and female bottlenose dolphins in wild and managed-care environments were the subjects of this study. Samples were collected from four groups: 1) wild males (n=13) and females (n=3) from Indian River Lagoon (IRL), Florida, USA, 2) wild males (n=11) and a female (n=1) from Charleston Harbor (CHS), South Carolina, USA, 3) managed-care males (n=4) and females (n=6) housed at the Georgia Aquarium (GA), Georgia, USA, and 4) managed-care males (n=3) and females (n=4) housed at the U.S. Navy Marine Mammal Program (MMP), San Diego, California, USA. The IRL population was comprised of adult males (ages 5.5-25 years) both juvenile (ages 5-7 years) and adult (ages 10-15 years) females, while CHS dolphins were adult males (ages 25-33 years) and adult females (ages 9.5-35 years). All female dolphins were non-pregnant during the sampling period. The age ranges for dolphins housed at GA and MMP are listed in (Fair et al., 2017). A detailed description of demographic data for each dolphin group, study sites, health assessment methods, blood and tissue sampling, and release was described previously (Fair et al., 2017, 2013). The number of samples collected for each tissue type and comparison is listed in Supplementary file 1: Table S2. Age was determined in IRL and CHS dolphins by examining the post-natal dentine layers of an extracted tooth (Hohn et al., 1989) and by birth records for GA and MMP dolphins. Samples from IRL and CHS dolphins were collected under National Marine Fisheries Permit Nos. 998-1678 and 14352 (permit dates

from 2009-2014) issued to Dr. Gregory Bossart and approved by the Florida Atlantic Institutional Animal Care and Use Committee (IACUC) under Protocol #A10-18. Samples were collected from MMP dolphins under a protocol approved by the IACUC of the Naval Information Warfare Center (NIWC) Pacific and the Navy Bureau of Medicine and Surgery.

2.2. Isolation and concentration of proteins from skin, WBCs and plasma

Since collection of skin samples is a more invasive procedure compared to blood collection, particularly in managed-care dolphins, we attempted to modify our technique to expand the applicability of the microarray to blood matrices. Whole blood samples were collected from all dolphins, from which plasma and white blood cells (WBCs containing primarily leukocytes and platelets) were separated and stored at -80°C as described previously (Fair et al., 2017). Managed-care blood samples were collected as part of routine preventative health examinations. The endocrine, hematological, biochemical, and serological data was acquired from the same samples in the present study (see methods in Fair et al., 2017). Skin samples were collected only from wild dolphins (IRL [n=16] and CHS [n=12]) using a surgical biopsy procedure (Fair et al., 2006) and stored at -80°C. Frozen skin biopsy samples were ground to powder under liquid nitrogen using a pre-chilled mortar and pestle. Proteins were isolated from ground skin (100-200 mg), WBC and plasma samples (1-2 ml) from individual dolphins by adding lysis buffer (10 ml/g tissue for skin, or 10 ml/ml for WBCs or plasma) containing 50 mM HEPES pH 7.0, 5 mM EDTA, 50mM NaCl, 10 mM sodium pyrophosphate, 50 mM NaF, 10 mM sodium orthovanadate, 1% Nonidet P-40, and complete protease inhibitor (Roche, Toronto, ON, Canada) and incubating for 15 min on ice. In addition, pooled skin, WBC and plasma standards for the protein microarray (see Section 2.3) were prepared from eight IRL dolphins where sufficient tissue and blood matrices were available from the same animals, and

lysed as above. Lysed samples were centrifuged for 20 min at 5000 *g* and 4 °C. Supernatants were collected and concentrated using centrifugal filters (Millipore Amicon Ultra, Sigma-Aldrich, Toronto, ON, Canada). Protein concentrations were determined in protein isolates using a modified (Lowry et al., 1951) assay (DC protein assay, BioRad, Hercules, CA, USA). These concentrations were used to standardize the amount of protein loaded into each microarray spot.

2.3. Protein microarray

An antibody-based protein microarray developed for a terrestrial mammal (Carlson et al., 2016) was used to simultaneously determine relative expression levels of 33 stress-associated proteins in skin, WBC and plasma samples collected from dolphins. Proteins analyzed using the microarray were categorized into four functional groups based on their roles in the stress response (Table 1). Detailed information regarding each stress-associated protein, including commercial antibody suppliers and catalogue numbers is provided in Carlson et al. (2016). In addition to the 31 antibodies described previously (Carlson et al., 2016) and listed in Table 1, two additional antibodies were included in the protein microarray for the current study: corticosteroid-binding globulin (CBG; Chow et al., 2010) and an additional HSP90 antibody that recognizes both constitutive and induced HSP90 forms (Sigma H1775; Sigma-Aldrich, Toronto, ON, Canada).

Full procedural details and validation steps for the protein microarray are provided elsewhere (Carlson et al., 2016). Briefly, each microarray consisted of 33 antibodies and three control spots printed in six replicate 6 × 6 grids on individual nitrocellulose-coated glass microscope slides (First Phase Technologies, Tempe, AZ, USA). Control spots consisted of a negative control (print buffer) and positive controls for the cyanine fluorescent dyes used to label proteins (Cy3 and Cy5; GE Healthcare, Mississauga, ON, Canada). Proteins isolated from

individual dolphins were labeled with Cy5, and pooled standards (consisting of equal quantities of protein from n=8 IRL dolphins) were labeled with Cy3. Equal quantities of protein (80 µg) from the dye-labeled individual dolphin samples and the pooled standard for each sample type (skin, WBCs, plasma) were combined and added to each of three arrays on a slide (i.e., individual samples were run in triplicate). The reaction was incubated for 1 h with agitation, then slides were rinsed and dried under a gentle stream of N_2 before fluorescence scanning.

Microarray scanning was conducted using a GenePix 4000B microarray scanner (Molecular Devices, Sunnyvale, CA, USA). Scanned fluorescence values for each of the 33 stress-associated proteins from each individual dolphin sample run in triplicate on the microarray were standardized using GenePix Pro 6.1 software (Molecular Devices) by dividing by the fluorescence value obtained from the pooled standard for a given sample type (skin, WBC, or plasma). Thus, each individual dolphin sample produced triplicate values for the expression of each stress-associated protein in relation to the same standard sample. The triplicate values were averaged to provide a single relative protein expression value used for statistical analyses.

2.4 Real-Time PCR analyses

Total RNA was extracted from individual dolphin skin samples (n=12) using RNeasy® plus mini kit (QIAGEN, Toronto, ON, Canada) per manufacturer's instructions. RNA quality and quantity were measured, respectively, with a Nanodrop™ ND-2000 spectrophotometer (Thermo Fisher Scientific, Mississauga, ON Canada), and a Bio-Rad Experion™ electrophoresis station using the RNA StdSens Analysis kit (Bio-Rad, Mississauga, ON, Canada). Total RNA (1 µg) was reverse transcribed using the QuantiTect® Reverse transcription kit (QIAGEN, Toronto, ON, Canada) as per manufacturer's instructions. Quantitative RT-PCR analyses were performed on a CFX96 Touch® real-time PCR detection system using SsoFast™ EvaGreen® Supermix

(Bio-Rad, Mississauga, ON, Canada) with a final concentration of 300 nM for each primer in a total reaction volume of 13 µL. The PCR conditions were as follows: 95°C for 30s, followed by 40 cycles of 95°C for 5s and 60°C for 15s. Primers were designed using Primer-BLAST (http://ncbi.nlm.nih.gov/tools/primer-blast/) based on the corresponding *T. truncatus* sequence in Genbank. Data were normalized using mRNA levels of two reference genes with the most stable transcription level across experiments according to geNorm algorithm: glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) and phosphoglycerate kinase1 (*pgk*). Gene names, symbols and accession numbers as well as primer-specific amplification efficiencies, sequences and length of amplification products are detailed in Supplementary file 1: Table S1. Each reaction was run in technical duplicate and the mean of six independent biological replicates was calculated.

2.5. Statistical analysis

In order to compare stress-associated protein expression in skin, WBCs, and plasma samples using the microarray, protein expression was evaluated as (1) individual proteins, (2) cumulative protein expression within each of the four functional groups (HPA, ACC, CSP, and OSI), and (3) total cumulative protein expression. We initially analyzed these data for normality, homoscedasticity and outliers, and then applied natural log or square-root transformations to achieve normality. Parametric statistical comparisons, using repeated measures analysis of variance (ANOVA) or linear mixed modeling of protein expression in skin, WBCs and/or plasma among the groups of dolphins were then conducted with a significance level of (α) 0.05. Repeated measures analysis of variance utilized tissue (plasma, skin, WBC) as the repeated measure, with location (IRL, CHS) as the factor, and age was included as a covariate. Repeated measures were used because of the lack of independence of skin, WBC, and blood plasma

231 measurements within animals. Means and standard errors are based on the observed values and
232 were not adjusted for effects of sex, age, and/or tissue-type.

233 To test the hypothesis that biological data were associated with stress-associated protein
234 expression, we used a linear mixed model analysis with a stepwise backward selection approach
235 starting with a global model containing all potential predictor variables. Potential predictor
236 variables for biological data and health parameters are listed in Table 2. Age was used as a
237 covariate and dolphin ID was included as a random effect. The small-sample extension of
238 Akaike's Information Criterion (AICc) was used to sequentially eliminate least important
239 variables. The global model (all predictor variables included) was reduced by one variable at a
240 time to arrive at the most parsimonious model with the lowest AICc score. Two-way interactions
241 were not evaluated when developing these models.

242 In order to determine the associations between relative gene transcription and protein
243 expression in skin and white blood cells, Pearson's correlation (r) was used to identify
244 significant ($P \leq 0.05$) linear associations and Spearman's correlation (ρ) was used to detect
245 potential non-linear associations. Associations between the relative gene transcription and
246 protein expression were analyzed using the following related genes: apoptosis inducing factor
247 (AIF), heat shock protein 90 (HSP90), heat shock protein 90 Stressgen (HSP90sg), heme
248 oxygenase 2 (HO2), and superoxide dismutase 1 (SOD1). Visual evaluation of scatterplots was
249 used to visualize the association between the response variable (expression of a specific protein)
250 and the potential predictor variable (relative gene transcription for the same specific protein).
251 The construction and visual evaluation of scatterplots was used to potentially identify curvilinear
252 or non-linear associations that may not be detected using Pearson's or Spearman's correlation

methods. All statistical analyses were completed using R statistical package (R Development Core Team, 2013) with statistical significance deemed to occur when $P \leq 0.05$.

3. Results

3.1. Stress-associated proteins in plasma, skin, and WBCs

The microarray recognized all 33 stress-associated proteins in dolphin skin samples; sufficiently concentrating proteins from WBCs and plasma also enabled measurement of all proteins in blood matrices. Comparison of mean expression values between plasma, skin, and WBCs revealed several proteins with significant differences among tissue type and age of dolphins (Table 3). The expression levels of protein functional groups between plasma, skin, and white blood cells (WBCs) in eight wild dolphins at Indian River Lagoon are shown in Figure 1. Tissue type had an effect on the mean relative expression of 12 stress-associated proteins including AVP, CBG, POMC, AIF, Annex4, caspase1, caspase3, caspase6, Cox2, eNOS, SOD-1, SOD-2. The mean relative expression of caspase6, eNOS, and SOD-2 was greater in skin compared to plasma and WBCs but was not different between plasma and WBC samples. The mean relative expression of POMC in skin was greater than in WBCs and the relative expression of AIF and SOD-1 was less than in WBC samples.

Age of animals also had an effect on the mean relative expression of individual proteins, including: CBG, POMC, GRP78/BIP, HSP110, HSP40, HSP90(Stressgen), eNOS, and SOD-2. Age had an effect on the cumulative protein expression of the CSP and the OSI group; however, there was no tissue effect on these two groups. Generally, as age increased the mean expression of the CSP and OSI group also increased (Figure 2). The expression of CBG, POMC, eNOS, and SOD-2 was associated with both tissue type and age. In both plasma and WBC samples, the

276 expression of these individual proteins increased as age increased. However, in skin samples, the
277 expression of these proteins slightly decreased or remained at similar levels as age increased.
278 The comparison of stress-associated protein relative expression between skin and WBCs
279 in two populations of wild dolphins demonstrated that tissue type, location, and age had effects
280 on mean expression (Supplementary file 1: Table S3). Tissue type had an effect on the mean
281 relative expression of CBG, POMC, prolactin, Annex2, caspase3, caspase6, GAPDH,
282 cytokeratin, GRP78/BIP, HSP110, HSP27, HSP40, HSP70(i), HSP90, HSP90(Stressgen), CCR5,
283 eNOS, iNOS, PRDX3, SOD-1, and SOD-2. The expression of the majority of stress proteins was
284 greater in skin samples compared to WBC. The mean relative expression of CBG, POMC,
285 caspase6, GAPDH, GRP78/BIP, HSP110, HSP27, HSP40, HSP70(i), HSP90, HSP90(Stressgen),
286 CCR5, eNOS, iNOS, PRDX3, and SOD-2 was greater in skin samples compared to WBC, while
287 the expression of Annex2, caspase3, cytokeratin, and SOD-1 was greater in WBC samples.
288 Tissue type also had an effect on the mean relative expression of the CSP group, with expression
289 greater in skin. The mean relative expression of GR, caspase1, and cytokeratin was greater in
290 dolphins from the IRL group, while the mean relative expression of CRF, POMC, caspase3, and
291 HSP70(i) was greater in dolphins from the CHS group. Lastly, age influenced the mean relative
292 expression of CRF, Annex2, Annex4, caspase3, Cox2 and SOD-1 as well as the mean relative
293 expression of the ACC and OSI groups.
294 *3.2. Stress-associated proteins in skin and WBCs relative to biological data*
295 The comparison of stress protein relative expression in skin between wild dolphins
296 sampled at IRL and CHS revealed statistically significant differences within individual protein
297 expression; however, there were few trends among protein groups and total protein expression
298 (Table 4). Location influenced the mean relative expression of ACTH, AVP, GR, prolactin,

caspase1, caspase6, cytokeratin, GRP78/BiP, HSP27, HSP40, HSP70, HSP70(i), HSP90, HSP90(Stressgen), SOD-1, and SOD-2 (Figure 3). The mean relative expression of AVP, GR, prolactin, caspase1, caspase6, cytokeratin, HSP70, HSP90, and SOD-1 was greater in dolphins from IRL, while the mean relative expression of ACTH, GRP78/BIP, HSP27, HSP40, HSP70(i), HSP90(Stressgen), and SOD-2 was greater in dolphins from CHS. The mean relative expression of ten proteins, including AVP, CRF, GR, prolactin, AIF, caspase6, cytokeratin, HSP70, Cox2, and HO-2, were influenced by sex, all of which were greater in the skin of male dolphins compared to females. Age had an effect on the expression of caspase6, cytokeratin, and iNOS as well as on the relative expression of the ACC group.

Statistically significant differences were observed among individual proteins in comparison of stress-associated protein relative expression in WBCs between wild (IRL and CHS) and managed-care dolphins (Table 5). Environment (wild or managed-care) influenced the mean relative expression of ACTH, AVP, GR, POMC, prolactin, all individual proteins in the ACC group (Table 1), HSP110, HSP60, HSP70, HSP90, HSP90(Stressgen), CCR5, Cox2, PRDX3, SOD-1, and SOD-2 (Figure 4). Wild dolphins also showed greater expression of ACC related proteins compared to managed-care dolphins. The relative expression of AVP, GR, prolactin, AIF, Annex2, Annex4, caspase1, caspase3, E-Cadherin, HSP110, HSP90(Stressgen), Cox2, PRDX3, and SOD-1 was greater in wild dolphins, while expression of ACTH, POMC, caspase6, GAPDH, HSP60, HSP70, HSP90, CCR5, and SOD-2 was greater in managed-care dolphins. Sex had an effect on the expression of GAPDH and CCR5 in males compared to females.

3.3. Stress-associated proteins in skin and WBCs relative to endocrine, hematological, biochemical, and serological parameters

Models describing the association between skin protein expression level, as the response variable, and sex, location, age, and endocrine concentrations, as potential predictor variables, revealed associations in wild dolphins captured for health assessment at IRL and CHS (Supplementary file 1: Table S4). The plasma concentration of ACTH, cortisol, total T3, and free T4 was associated with in CBG, GRP78/BIP, HSP110, and PRDX3 in skin. Furthermore, CBG, GRP78/BIP, HSP110, HSP27, HSP40, were associated with the greatest number (≥4) of endocrine predictor variables (Figure 5). The plasma concentration of ACTH, cortisol and free T4 was also associated with ACTH expressed in skin. The plasma concentration of solely free T4 was associated with skin expression levels of AVP, cytokeratin, CCR5, and eNOS. Furthermore, the plasma concentration of ACTH, cortisol, and total T4 and free T4 was related with the skin expression levels of HSP40 and HSP90(Stressgen). While the plasma concentrations of ACTH, cortisol, total T4, total T3, and free T4 were significantly associated with skin protein expression levels, the plasma concentrations of estradiol, progesterone, and testosterone showed very few to no associations with skin protein expression. For example, the plasma concentration of estradiol was associated with the skin expression levels of HSP27 and the plasma concentration of progesterone was associated with iNOS. Together, the plasma concentrations of ACTH, cortisol, estradiol, total T3, and free T4 best predicted the skin expression levels of HSP27 and the plasma concentrations of progesterone, total T4, and free T4 best predicted the skin expression levels of iNOS.

There were significant associations between WBC protein expression level, as the response variable, and sex, environment, age, and endocrine concentrations, as potential predictor variables, in dolphins sampled at IRL, CHS, and GA (Supplementary file 1: Table S5). However, there were fewer associations than in skin. ACTH was the only protein in WBC

samples to be associated with more than one endocrine variable, total T3 and free T4. The plasma concentration of total T4 was associated with WBC expression levels of AVP, Cox2, and HO-2. Additionally, the plasma concentration of total T3 was associated with WBC expression of ACTH, POMC, GRP78/BIP, HSP110, HSP27, HSP40, HSP70(i), HSP90(Stressgen), PRDX3, and SOD-2. The plasma concentration of free T4 was correlated with WBC expression of ACTH, CBG, CRF, and iNOS.

Models describing the association between skin protein expression level, as the response variable, and sex, location, age, and hematological variables, as potential predictor variables, in wild IRL and CHS dolphins demonstrated significant associations among sex, location, and hematological variables (Supplementary file 1: Table S6). The correlation of the expression of stress-related proteins in skin and WBCs associated with at least two hematological predictor variables is shown in Figure 6. Five hematological variables showed associations with skin protein expression levels: RBC, neutrophil, N:L ratio, monocytes, and eosinophils. RBC was a suitable predictor of the skin expression levels of caspase6, E-Cadherin, and eNOS. Neutrophils were associated with the skin expression levels of cytokeratin and Cox2, while the N:L ratio was also related with cytokeratin and Cox2 in addition to HSP90 and iNOS. Monocytes were associated with the expression level of POMC and eosinophils with levels of ACTH, AVP, prolactin, caspase1, HSP90 and Cox2.

Investigation into the association between WBC protein expression level, as the response variable, and sex, environment, age, and hematological variables, as potential predictor variables, in dolphins sampled at IRL, CHS, and GA revealed significant associations with almost all predictor variables (Supplementary file 1: Table S7). Five hematological variables were associated with the WBC expression levels of particular proteins. Hemoglobin was associated

with the WBC expression levels of the greatest number of proteins: POMC, GRP78/BIP, HSP40, HSP70(i), HSP90(Stressgen), PRDX3, and SOD-2, while RBC was only associated with the WBC expression of CRF. Neutrophils were associated with the WBC expression of HSP40, HSP70(i), and PRDX3. N:L ratio was associated with the WBC expression of POMC, caspase6, cytokeratin, HSP27, and eNOS. Eosinophils were associated with the WBC expression of AVP, Cox2, and HO-2.

Models describing the association between skin protein expression level, as the response variable, and sex, location, age, and biochemical variables, as potential predictor variables, in wild IRL and CHS dolphins demonstrated several significant associations (Supplementary file: Table S8a). When considering the first group of plasma biochemical variables (Table 2), only four were associated with the expression of certain proteins in skin. The correlation of the expression of stress-related proteins in skin associated with at least two biochemical predictor variables in the first group is shown in Figure 7. Potassium was related with the skin expression of Annex4, caspase1, and HO-2, while chloride was associated with the expression of GAPDH. Phosphorus was associated with the skin expression of the greatest number of individual proteins, which included ACTH, AVP, Annex2, GRP78/BIP, HSP27, HSP40, HSP70(i), HSP90(Stressgen), PRDX3, and SOD-2. Magnesium was associated with the expression of AVP, Annex2, Annex4, caspase1, HSP40, HSP90(Stressgen), HO-2, and SOD-2.

When considering the second group of biochemical variables (Table 2), ALT, TIBC, TSAT, and lipemia were associated with the expression of individual proteins in skin (Supplementary file 1: Table S8b). ALT was associated with the expression of caspase6, HSP60, and HSP70. TIBC was associated with the expression of Annex2 and caspase1, while TSAT was associated with the expression of HSP70. Lipema was associated with the expression of GAPDH

391 in skin. No stress-related proteins in skin were associated with at least three biochemical
392 predictor variables in the second group; HSP70 was associated with two variables, ALT and
393 TIBC.

394 When considering serological variables (Table 2) as potential predictor variables, five
395 were associated with the expression of individual proteins in skin (Supplementary file 1: Table
396 S8c). Albumin was associated with the expression of HSP60, while albumin-2 was associated
397 with the expression of SOD-1. Alpha-1 globulin was associated with the expression of caspase6,
398 cytokeratin, and HSP70, while alpha-2 globulin and beta-1 globulin were associated with the
399 expression of SOD-1. The expression of SOD-1 in skin was the only protein that was associated
400 with three serological predictor variables, Albumin-2, alpha-2 globulin, and beta-1 globulin.

401 Models describing the association between WBC protein expression level, as the
402 response variable, and sex, environment, age, and biochemical variables, as potential predictor
403 variables, in dolphins sampled at IRL, CHS, and GA revealed several significant associations
404 (Supplementary file 1: Table S9a). However, there were fewer associations in WBC samples
405 compared to skin samples. Each stress-related protein in WBC samples was associated with only
406 one biochemical predictor variable in the first group. Potassium was associated with the WBC
407 expression of GR, while the Na:K ratio was linked with the WBC expression of CBG. Chloride
408 was associated with the WBC expression of GRP78/BIP and SOD-2 and phosphorus was
409 associated with the WBC expression of caspase6, cytokeratin, and HSP90. Lastly, Mg was
410 associated with the WBC expression of AVP and eNOS.

411 Seven biochemical variables in the second group (Table 2) were significantly associated
412 with the WBC expression levels of specific proteins (Supplementary file 1: Table S9b). The
413 correlation of the expression of the top five stress-related proteins in white blood cells associated

with at least three biochemical predictor variables in the second group is shown in Figure 8. Creatinine was associated with the WBC expression of Annex4 and eNOS, while glucose was only associated with the WBC expression of HO-2. AP was associated with the WBC expression of AVP, AIF, Annex2, Annex4, caspase3, E-Cadherin, HSP60, and Cox2. Iron was associated with the WBC expression of ACTH, CBG, GRP78/BIP, HSP90(Stressgen), and iNOS. TIBC was associated with the WBC expression of CBG, CRF, POMC, AIF, Annex2, Annex4, caspase1, E-Cadherin, HSP60, HSP70(i), and HO-2. TSAT was associated with the WBC expression of the greatest number of proteins, which included CRF, POMC, Annex2, caspase1, caspase3, E-Cadherin, HSP110, HSP27, HSP40, HSP60, HSP70(i), PRDX3, and SOD-2. Finally, the lipemia index was associated with the WBC expression of AIF, Annex2, Annex4, E-Cadherin, HSP60, and Cox2.

Models describing the association between WBC protein expression level, as the response variable, and sex, environment, age, and serological variables, as potential predictor variables revealed several significant associations (Supplementary file 1: Table S9c). The correlation of the expression of stress-related proteins in white blood cells associated with at least two serological predictor variables is shown in Figure 9. Albumin was associated with the WBC expression level of E-Cadherin, while albumin-2 was associated with the WBC expression of GRP78/BIP, HSP27, HSP90(Stressgen), and SOD-2. Total globulin was associated with the WBC expression of caspase1 and HSP70(i). Alpha-2-globulin was associated with the WBC expression of CBG, CRF, POMC, Annex2, and HO-2. Beta-1-globulin was associated with the WBC expression of CBG, HSP110, HSP70(i), iNOS, PRDX3, SOD-1, and SOD-2. Gamma-globulin was associated with the WBC expression of only one protein, Annex2. Lastly, the albumin to globulin ratio-2 was associated with the WBC expression of CRF, caspase3,

cytokeratin, and HSP90. A summary of the individual proteins with significant associations to potential predictor variables in skin and WBC samples is shown in Supplementary file 1: Table S10. The number of stress-related proteins with significant associations to all predictor variables is shown visually in Supplementary file 1: Figure S1.

There were very few significant associations between relative gene transcription and protein expression in skin and WBCs in wild dolphins captured and handled at CHS (Supplementary file 1: Table S11). However, apoptosis-inducing factor (AIF) gene expression was positively associated with AIF protein expression in WBCs.

3.4. Comparison of stress-associated proteins in skin and WBCs relative to endocrine, hematological, biochemical, and serological parameters

A summary of the individual proteins with significant associations to potential predictor variables in skin and WBC samples is shown in Supplementary file 1: Table S10. When considering endocrinology as potential predictor variables, there were a greater number of associations with the expression of stress-associated proteins in skin compared to WBCs. For example, ACTH, cortisol, estradiol, progesterone, total T4, total T3, and free T4 were related with the expression of stress proteins in skin, while only total T4, total T3, and free T4 were associated with the expression of stress proteins in WBCs. The expression of GRP78/BIP, HSP110, HSP27, and PRDX3 in both skin and WBCs was associated with total T3. Additionally, ACTH, CBG, and iNOS were correlated with free T4 in both skin and WBC samples.

The number of select hematological variables with significant associations with stress protein expression was the same in both skin and WBC samples. RBC, neutrophils, N:L ratio, and eosinophils were associated with the expression of individual proteins in both skin and WBCs, while hemoglobin was only linked with proteins in WBCs and monocytes were only

associated with proteins in skin samples. The expression of cytokeratin was associated with N:L ratio in both skin and WBCs and the expression of AVP and Cox2 was associated with eosinophils in skin and WBCs.

Seven plasma biochemical variables were associated with individual stress proteins in both skin and WBCs (i.e., K, Cl, P, Mg, TIBC, TSAT, and lipemia). ALT was the only plasma biochemical variable with associations with the expression of stress proteins in skin, while Na:K ratio, creatinine, glucose, AP, and iron were associated with stress proteins solely in WBCs. The expression of AVP was associated with Mg in both skin and WBCs. The expression of Annex2 and caspase1 was related with TIBC.

Four serological variables were associated with individual stress proteins in both skin and WBCs. Alpha-1 globulin was only associated with proteins in skin, while total globulin, gamma-globulin, and the albumin:globulin ratio-2 were only associated with stress proteins in WBCs. The expression of SOD-1 was linked with beta-1 globulin in both skin and WBC samples.

4. Discussion

Capture-release health assessments are typically performed to gather baseline clinical data on dolphin groups and to determine if anthropogenic factors are impacting their health (Fair et al., 2017, 2014). During these assessments, blood is routinely sampled from individual dolphins. Therefore, this study focused on (1) evaluating the applicability of the microarray to dolphin skin biopsy specimens, and (2) determining whether the microarray could reliably measure stress proteins in white blood cells (consisting of neutrophils, eosinophils, basophils, lymphocytes and monocytes) and plasma collected during routine blood sampling of dolphins. Stress-associated proteins could be measured using the microarray technique in different

matrices, and proteins in these matrices demonstrated very few different expression levels based on tissue type. Other major findings of this study in bottlenose dolphins were that stress-associated protein expression was associated with biological data such as location, sex, and environment and that the majority of stress-associated protein expression appears to be influenced by certain clinical health parameters such as endocrinology (in skin samples) and plasma biochemical variables (in WBC samples). To our knowledge, this is the first time such a proteomics approach has been achieved in marine mammals.

The tissue type had a statistically significant effect on the mean relative expression of 12 stress-associated proteins. For three proteins, the mean relative expression of individual proteins in plasma and WBC were similar, while the values in skin were greater than both. The differences in protein expression among skin, WBCs and plasma were protein-specific with no clear trends for differences among matrices. This is not surprising since the relative importance and roles of specific proteins likely differ among matrices (Svobodová et al., 2011). Therefore, while plasma and WBC samples collected during routine capture and release health assessments can be used to determine stress levels in individuals, remote sampling of skin may provide a useful tool to assess stress and help monitor the health of wild dolphin groups, since it does not require capture and restraint of individuals. Skin not only provides a protective barrier from the environment, but also has the ability to respond to environmental stressors in order to maintain cutaneous homeostasis (Slominski et al., 2000, 2018), making skin a model organ for assessing the neuro-endocrine-immune response to stress (Arck et al., 2006). It is thought that skin has an independent and functional neuroendocrine system that expresses proteins, such as corticotropin-releasing hormone (CRH), POMC, and ACTH, which control the activity of the HPA axis (Slominski et al., 2000; Szöllősi et al., 2017). Furthermore, skin systems are able to communicate

with the brain about changes in the epidermal environment and initiate other responses from coordinating systems through neurotransmission, resulting in the management of global homeostasis (Slominski et al., 2012). Thus, it is not surprising that changes in stress-associated proteins in skin would respond rapidly to acute stressors, similar to classic HPA activation. In bottlenose dolphins, circulating ACTH, cortisol, and aldosterone in serum, blubber, and feces have been reported to increase in response to an acute stressor, such as blood draws, blubber biopsies, and remaining out of water for 2 hours (Champagne et al., 2018) and the concentration of cortisol in serum and blubber appeared to be related (Champagne et al., 2017). Skin, as well, may be a well-suited matrix for monitoring stress in marine mammals and wildlife in general (Carlson et al., 2016). However, it is important to acknowledge that the dermal dynamics of skin in aquatic mammals is not necessarily the same (e.g., high degree of daily shedding in dolphins) as that in terrestrial mammals. Therefore, there may be potential differences in the role of skin between aquatic and terrestrial mammals when applying this method to monitor stress in wildlife.

The effect of location was assessed in wild dolphins from two groups, IRL and CHS, of which both groups are considered to have poor health (Reif et al., 2008). These dolphin populations are affected by complex infectious and neoplastic diseases often associated with immunologic disturbances and may be associated with anthropogenic influences (Bossart et al., 2017; Reif et al., 2017). Elevated concentrations of organochlorine contaminants (Genov et al., 2019), perfluoroalkyl substances (Houde et al., 2005), mercury (Stavros et al., 2008, 2007), and other organic contaminants (Fair et al., 2010, 2007) have been identified in bottlenose dolphins; however, concentrations of these different contaminants are population specific. Furthermore, the estuarine sediment from Charleston Harbor was found to have high levels of perfluoroalkyl

529 substances (White et al., 2015) and other organic contaminants (Long et al., 1998). Land and
530 watershed use in this area (Adams et al., 2014, 2008) have been associated with greater
531 concentrations of organic contaminants (Fair et al., 2007; Houde et al., 2005) in dolphins from
532 CHS compared to IRL. Furthermore, skin from adult males in the CHS group have greater
533 concentrations of trace elements, while the adult males from IRL tended to have greater
534 concentrations of total mercury and methylmercury in skin (Stavros et al., 2007). Greater
535 concentrations of As, Mn, and Se in blood were found in CHS dolphins compared to those at
536 IRL, while the concentrations of Cu, THg, and Zn were greater in dolphins from IRL (Stavros et
537 al., 2008). Both IRL and CHS groups suffer from mucocutaneous lesions (Bossart et al., 2015)
538 and genital tumors that are related to papillomaviruses and herpesviruses (Bossart et al., 2008;
539 Rehtanz et al., 2012, 2006), resulting in impaired immune function. Additionally, the adaptive
540 immunity of IRL dolphins has become impaired due to cases of lobomycosis (Reif et al., 2009).
541 Similarly, dolphins from the IRL group with positive morbillivirus antibody titers showed
542 impaired cell-mediated adaptive immunity and an increased humoral immune response (Bossart
543 et al., 2011, 2010). Lastly, chlamydiaceae infections have been found in both groups, which
544 impact overall health (Bossart et al., 2014).

545 Overall, there were several sex differences in protein expression, although this was
546 limited by the relatively small number of females sampled. The expression of 10 out of 33
547 proteins varied by sex, with the expression of all proteins being greater in male dolphins
548 compared to females. Individual proteins belonged to all four protein functional groups, four
549 proteins were from the HPA group, two from the ACC group, two from the CSP group, and two
550 from the OSI group. Several trace element concentrations have also been shown to vary between
551 males and females, which is likely due to differences in diet (Stavros et al., 2008, 2007). In

552 humans, oxidative stressors appear to have less of an effect on females compared to males
553 because of differences in estrogen concentration and NADPH-oxidase activity. Both are related
554 to immune function, as estrogen is believed to be a potential antioxidant, while NADPH-oxidase
555 generates superoxide to combat invasive microorganisms (Kander et al., 2017).

556 The greatest number of stress-associated proteins (24) varied by the dolphins'
557 environment, 15 of which were greater in wild dolphins and 9 of which were greater in managed-
558 care dolphins. Fair et al. (2017) found similar results when comparing wild and managed-care
559 dolphins; wild individuals had an upregulated immune system, likely due to increased exposure
560 to pathogens, compared to managed-care-dolphins (Fair et al., 2017). Additionally, several
561 differences were consistent with lower pathogenic antigenic stimulation in managed-care
562 dolphins (Fair et al., 2017). Ultimately, the environment appears to play an important role in the
563 immune and endocrine responses of dolphins in these groups (Fair et al., 2017) as well as their
564 circulating thyroid hormone concentrations (Fair et al., 2011). Although individual protein
565 expression values were significantly associated with multiple predictor variables as described
566 above, cumulative protein expression within functional categories and total cumulative protein
567 expression were not as consistently associated with predictor variables.

568 Protein expression in skin and WBCs showed many consistent associations with
569 measures of plasma endocrinology, hematological, and biochemical variables; this was
570 particularly true for plasma endocrinology in skin and plasma biochemical variables in WBCs.
571 The expression of individual proteins in skin was associated with 70% of endocrine variables,
572 compared to 40-50% of the other parameters. The expression of stress-associated proteins in
573 WBCs was associated with 60% of the plasma biochemical variables, compared to 30-58% of
574 the remaining parameters. The difference in associations among these parameters suggests that

these variables play an important role in the expression of stress-associated proteins in bottlenose dolphins. Specifically, plasma cortisol, ACTH, and thyroxine were associated with the expression of several proteins in skin. Activation of the HPA axis initiates the release of CRF and subsequently ACTH, which is followed by the release of cortisol (Sapolsky et al., 2000). Therefore, it is not surprising that these endocrine variables would be associated with the expression of individual proteins. Thyroxine was associated with the expression of almost half of all individual stress proteins tested in skin. The hypothalamic-pituitary-thyroid (HPT) axis regulates the release of thyroid hormones (T4 and T3) by the thyroid gland into the bloodstream (Gilbert and Zoeller, 2010), which play important roles in development and metabolism (Ikegami and Yoshimura, 2017). In a previous study, T3 and T4 concentrations in bottlenose dolphins varied significantly by geographic location, perhaps due to an adaptive response to a cold water environment (Fair et al., 2011). The plasma concentrations of T3 and T4 did not differ between wild and managed-care dolphins in a previous study (Fair et al., 2017), suggesting that other variables such as season (water temperature) may be associated with these hormones. Therefore, T4 may be involved in the response to seasonal and/or natural environmental stressors. In the present study, plasma phosphorus was associated with the expression of individual stress-associated proteins across all functional groups in skin. Plasma phosphorus has also been linked with increased cell viability, antioxidant capacity, and energy generation in other aquatic animals under stress (Ye et al., 2016). Similarly, magnesium was associated with the expression of several individual stress-associated proteins in skin, including CCR5, HO-2, and SOD-2 in the oxidative stress and inflammation group. It has been reported that a deficiency in magnesium often results in increased levels of oxidative stress markers and an impaired antioxidant response (Zheltova et al., 2016). In WBCs, total iron-binding capacity and transferrin saturation were

associated with the greatest number of stress-associated proteins. Transferrin is able to bind potentially toxic free iron, believed to play a role in the physiological response to oxidative stress and may even reflect states of oxidative stress (Elsayed et al., 2016).

Hematological variables were also tested as potential predictor variables in this study; however, there were few associations with the expression of stress-associated proteins. Neutrophils and eosinophils were associated with the expression of 5 and 9 individual proteins among skin and WBC samples, respectively. The percentage of neutrophils and eosinophils have been reported to differ between wild and managed-care dolphins (Asper et al., 1990). Eosinophil levels have been found to be lower in managed-care dolphins, which may be linked with less opportunity to encounter infectious agents, particularly parasitic infections (Asper et al., 1990; Fair et al., 2017). There were also no consistent associations between protein expression and mRNA transcript abundance in skin and WBCs, although this was limited by the number of comparisons that could be made. However, apoptosis-inducing factor (AIF) gene expression was positively associated with AIF protein expression in WBCs, which is likely of biological significance due to the rapid turnover (high rate of apoptotic cell death) of WBCs. The few associations may be explained by the lack of association between mRNA and protein expression (Cutler, 2003), which may be due to differing dynamics of mRNA and protein expression in response to stress (Cheng et al., 2016).

5. Conclusions

In combination with previous and on-going research being conducted on bottlenose dolphin groups, this study provides unique insight into stress-responses in multiple matrices. The present work involved using a novel antibody-based protein microarray to determine expression

levels of 33 stress-associated proteins in small skin biopsy, plasma, and white blood cell samples collected from bottlenose dolphins. While there were limitations to this study, such as the high number of predictor variables tested and small sample size, particularly of the managed care population, the microarray was able to measure the expression of proteins associated with four key aspects of the stress response: HPA axis, apoptosis/cell cycle, proteotoxicity/cellular stress, and oxidative stress/inflammation in bottlenose dolphins. The majority of elevated stress protein expression was influenced by location, sex, environment, and plasma endocrinology. This research provides further understanding of the underlying mechanisms of the stress response in bottlenose dolphins. Additionally, this novel method is a well-suited technique for monitoring stress in wildlife and combines recent advances in cutaneous neuroendocrinology to the emerging field of conservation physiology.

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Conflict of interest statement

The authors have declared no conflict of interest.

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Figure captions

Figure 1. Expression levels of protein functional groups between plasma, skin, and white blood cells (WBCs) in eight wild dolphins at Indian River Lagoon.

Abbreviations: Hypothalamus-pituitary-adrenal axis (HPA), Apoptosis and cell cycle (ACC), Cellular stress and proteotoxicity (CSP), Oxidative stress and inflammation (OSI)

Figure 2. Mean expression levels of cellular stress and proteotoxicity (CSP) and oxidative stress and inflammation (OSI) proteins across ages of wild dolphins at Indian River Lagoon (IRL).

Figure 3. Mean relative expression of stress proteins in skin between wild dolphins sampled at Indian River Lagoon (IRL) and wild dolphins sampled at Charleston, SC (CHS). Nine proteins were significantly ($P \leq 0.05$) greater in IRL dolphins and seven proteins were greater in CHS dolphins.

Abbreviations: Adrenocorticotropic hormone (ACTH), arginine vasopressin (AVP), glucocorticoid receptor (GR), glucose regulated protein 78 (GRP78/BIP), heat shock proteins (HSP), inducible (i), Stressgen (S), superoxide dismutase (SOD)

Figure 4. Mean relative expression of stress proteins in white blood cells (WBCs) between wild and managed-care dolphins. Nine proteins were significantly ($P \leq 0.05$) greater in managed-care dolphins and 15 were greater in wild dolphins.

Abbreviations: Hypothalamus-pituitary-adrenal axis (HPA), Apoptosis and cell cycle (ACC), Cellular stress and proteotoxicity (CSP), Oxidative stress and inflammation (OSI)

Figure 5. Correlation of the expression of the top five stress-related proteins in skin associated with at least four endocrine predictor variables. No stress-related proteins in white blood cells were associated with at least four endocrine predictor variables.

Abbreviations: total T3 (triiodothyronine), free T4 (thyroxine), heat shock proteins (HSP), corticosteroid-binding globulin (CBG), glucose regulated protein 78 (GRP78/BIP)

Figure 6. Correlation of the expression of stress-related proteins in (A) skin and (B) white blood cells (WBCs) associated with at least two hematological predictor variables.

Abbreviations: Segmented neutrophils (neutro), neutrophil-to-lymphocyte ratio (N:L ratio), eosinophils (eosino), cyclooxygenase-2 (Cox2), heat shock proteins (HSP), C-terminal proopiomelanocortin (POMC), peroxiredoxin-3 (PRDX3)

Figure 7. Correlation of the expression of stress-related proteins in skin associated with at least two biochemical predictor variables in the first group. No stress-related proteins in white blood cells were associated with at least two biochemical predictor variables in the first group.

Abbreviations: Potassium (K), phosphorus (P), Magnesium (Mg), annexin II (Annex2), annexin IV (Annex4), arginine vasopressin (AVP), C-C chemokine receptor 5 (CCR5), heme oxygenase-2 (HO-2), heat shock proteins (HSP), superoxide dismutase (SOD)

Figure 8. Correlation of the expression of the top five stress-related proteins in white blood cells associated with at least three biochemical predictor variables in the second group. No stress-related proteins in skin were associated with at least three biochemical predictor variables in the second group.

Abbreviations: Apoptosis inducing factor (AIF), annexin II (Annex2), annexin IV (Annex4), epithelial (E)-cadherin (E-Cadherin), heat shock proteins (HSP)

Figure 9. Correlation of the expression of stress-related proteins in white blood cells associated with at least two serological predictor variables. The expression of SOD-1 in skin was associated with three serological predictor variables.

Abbreviations: annexin II (Annex2), corticosteroid-binding globulin (CBG), corticotropin releasing factor (CRF), heat shock proteins (HSP), superoxide dismutase (SOD)

Tables

Table 1. A total of 33 stress-related proteins were measured in bottlenose dolphins using the antibody microarray.

Functional Group	**Proteins**
Hypothalamus-pituitary-adrenal axis (HPA)	Adrenocorticotropic hormone (ACTH), arginine vasopressin (AVP), corticosteroid-binding globulin (CBG), corticotropin releasing factor (CRF), glucocorticoid receptor (GR), C-terminal proopiomelanocortin (POMC), prolactin
Apoptosis and cell cycle (ACC)	Apoptosis inducing factor (AIF), annexin II (Annex2), annexin IV (Annex4), caspase1, caspase3, caspase6, epithelial (E)-cadherin (E-Cadherin), glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
Cellular stress and proteotoxicity (CSP)	Cytokeratin, glucose regulated protein 78 (GRP78/BIP), heat shock proteins (HSP27, HSP40, HSP60, HSP70, HSP70 inducible (HSP70i), HSP90, HSP90 Stressgen (HSP90S), HSP110)
Oxidative stress and inflammation (OSI)	C-C chemokine receptor 5 (CCR5), cyclooxygenase-2 (Cox2), endothelial nitric oxide synthase (eNOS), heme oxygenase-2 (HO-2), inducible nitric oxide synthase (iNOS), peroxiredoxin-3 (PRDX3), superoxide dismutase (SOD) 1, SOD-2

Table 2. Potential predictor variables of stress-associated protein expression

Category	**Predictor Variables**
Tissue Type	Plasma, white blood cells (WBC), skin
Biological data	Location, sex, age, environment
Endocrine variables	ACTH, cortisol, estradiol, progesterone, testosterone, total T4 (thyroxine), total T3 (triiodothyronine), free T4
Hematological variables	Packed cell volume (PCV), hemoglobin, red blood cell (RBC), segmented neutrophils (neutro), lymphocytes (lympho), neutrophil-to-lymphocyte ratio (N:L ratio), monocytes (mono), eosinophils (eosino)
Biochemical variables	Group 1: Sodium (Na), potassium (K), Na:K ratio, chloride (Cl), bicarbonate (HCO_3^-), anion gap, calcium (Ca), phosphorus (P), magnesium (Mg) Group 2: Creatinine (creat), glucose (gluc), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP), γ-glutamyltransferase (GGT) cholesterol (chol), iron, total iron-binding capacity (TIBC), transferrin saturation (TSAT), lipemia index (lipemia)
Serological variables	Total protein (TP), albumin (Alb), albumin-2 (Alb2), total globulin (Glob), alpha-1 globulin (α-1), alpha-2 globulin (α-2), beta-1 globulin (β-1), beta-2 globulin (β-2), gamma-globulin (γ), albumin:globulin ratio (A:G), albumin:globulin ratio-2 (A:G2)

Table 3. Comparison[a] of stress protein relative expression between plasma, skin, and white blood cells (WBC) collected from eight wild dolphins at Indian River Lagoon in June 2011. Significant P-values (≤0.05) are presented in bold red type-face.

Stress Proteins	Mean relative expression ± SE			Tissue effect (*P*)	Age effect (*P*)
	Plasma	Skin	WBC		
1) Hypothalamic-pituitary-adrenal axis (HPA)					
ACTH	0.64 ± 0.072	0.94 ± 0.038	0.64 ± 0.067	0.924	0.823
AVP	1.18 ± 0.058	1.04 ± 0.075	1.24 ± 0.081	**0.038**	0.238
CBG	0.70 ± 0.116	1.03 ± 0.047	0.71 ± 0.099	**0.037**	**0.008**
CRF	0.85 ± 0.049	0.92 ± 0.052	0.85 ± 0.067	0.136	0.458
GR	1.29 ± 0.066	1.01 ± 0.062	1.35 ± 0.089	0.088	0.488
POMC	0.57 ± 0.076	0.78 ± 0.048	0.47 ± 0.065	**0.005**	**0.021**
Prolactin	1.33 ± 0.087	1.03 ± 0.064	1.38 ± 0.094	0.137	0.853
HPA group mean	0.94 ± 0.040	0.96 ± 0.048	0.95 ± 0.039	0.441	0.512
2) Apoptosis and cell cycle (ACC)					
AIF	1.28 ± 0.076	1.03 ± 0.061	1.41 ± 0.118	**0.019**	0.322
Annex2	1.23 ± 0.073	1.02 ± 0.057	1.37 ± 0.096	0.105	0.721
Annex4	1.20 ± 0.061	1.00 ± 0.055	1.30 ± 0.101	**0.020**	0.236
Caspase1	1.29 ± 0.063	1.02 ± 0.060	1.44 ± 0.063	**0.035**	0.600
Caspase3	1.16 ± 0.080	0.94 ± 0.051	1.20 ± 0.086	**0.048**	0.292
Caspase6	0.91 ± 0.065	1.52 ± 0.067	0.80 ± 0.084	**0.001**	0.144
E-Cadherin	1.21 ± 0.065	0.93 ± 0.046	1.32 ± 0.087	0.125	0.982
GAPDH	0.44 ± 0.057	0.51 ± 0.082	0.45 ± 0.074	0.226	0.751
ACC group mean	1.09 ± 0.039	1.00 ± 0.041	1.16 ± 0.058	0.134	0.658
3) Cellular stress and proteotoxicity (CSP)					
Cytokeratin	1.00 ± 0.084	1.73 ± 0.106	0.95 ± 0.111	0.061	0.511
GRP78/BIP	0.74 ± 0.117	1.02 ± 0.055	0.67 ± 0.102	0.114	**0.008**
Hsp 110	0.77 ± 0.108	1.00 ± 0.055	0.68 ± 0.086	0.227	**0.049**
Hsp 27	0.69 ± 0.114	0.89 ± 0.062	0.62 ± 0.097	0.455	0.248
Hsp 40	0.68 ± 0.091	1.00 ± 0.050	0.64 ± 0.086	0.136	**0.024**
Hsp 60	1.05 ± 0.116	0.90 ± 0.037	1.26 ± 0.177	0.228	0.721
Hsp 70	0.84 ± 0.091	1.45 ± 0.141	0.79 ± 0.060	0.441	0.546
Hsp 70 (i)	0.64 ± 0.101	0.89 ± 0.046	0.60 ± 0.063	0.489	0.590
Hsp 90	0.98 ± 0.103	1.53 ± 0.086	0.96 ± 0.072	0.205	0.975
HSP 90 (Stressgen)	0.75 ± 0.081	1.06 ± 0.045	0.68 ± 0.086	0.073	**0.009**
CSP group mean	0.79 ± 0.056	1.11 ± 0.037	0.76 ± 0.053	0.135	**0.021**
4) Oxidative stress and inflammation (OSI)					
CCR5	0.55 ± 0.065	0.68 ± 0.059	0.53 ± 0.079	0.260	0.571
Cox2	1.08 ± 0.047	1.01 ± 0.059	1.23 ± 0.112	**0.015**	0.135
eNOS	0.64 ± 0.074	0.91 ± 0.034	0.61 ± 0.076	**0.008**	**0.011**
HO-2	0.94 ± 0.041	0.95 ± 0.054	0.96 ± 0.062	0.104	0.448
iNOS	0.95 ± 0.122	1.09 ± 0.048	0.78 ± 0.075	0.137	0.109
PRDX3	0.77 ± 0.112	1.00 ± 0.045	0.68 ± 0.084	0.108	0.014
SOD-1	1.31 ± 0.077	0.99 ± 0.062	1.47 ± 0.105	**0.035**	0.517
SOD-2	0.53 ± 0.079	0.96 ± 0.042	0.55 ± 0.069	**0.025**	**≤0.001**
OSI group mean	0.85 ± 0.047	0.95 ± 0.041	0.85 ± 0.033	0.335	**0.033**
5) Total stress protein	29.2 ± 0.94	32.2 ± 1.28	29.6 ± 1.11	0.677	0.122

[a] **Method of comparison:** Repeated measures analysis of variance with tissue (plasma, skin, WBC) as the repeated measure, and with age included as a covariate. Means and standard errors are based on the observed values and have not been adjusted for effects of sex, age, and/or tissue-type.

Table 4. Comparison[a] of stress protein relative expression in skin between wild dolphins sampled at Indian River Lagoon (IRL; 3 females + 13 males) in June 2011, and wild dolphins sampled at Charleston, SC, (CHS; 1 female + 11 males) in August 2013. Significant P-values (≤0.05) are presented in bold red type-face.

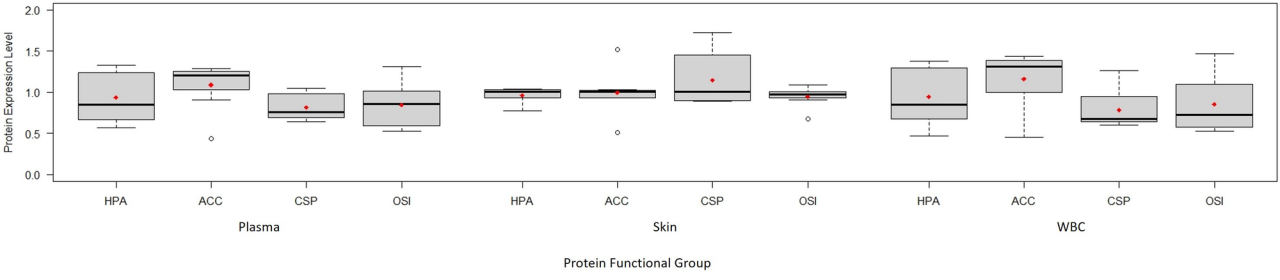
Stress Proteins	Mean relative expression ± SE		Factor or covariate effect (*P*)		
	IRL (*n* = 16)	CHS (*n* = 12)	Location	Sex	Age
1) Hypothalamic-pituitary-adrenal axis (HPA)					
ACTH	0.80 ± 0.046	0.98 ± 0.046	**0.016**	0.547	0.532
AVP	0.98 ± 0.053	0.78 ± 0.024	**0.009**	**0.024 (M > F)**	0.167
CBG	0.93 ± 0.040	0.90 ± 0.045	0.933	0.688	0.142
CRF	0.86 ± 0.039	0.82 ± 0.034	0.789	**0.030 (M > F)**	0.070
GR	1.02 ± 0.051	0.78 ± 0.018	**≤0.001**	**0.032 (M > F)**	0.071
POMC	0.76 ± 0.032	0.86 ± 0.069	0.076	0.898	0.201
Prolactin	1.02 ± 0.051	0.79 ± 0.027	**0.003**	**0.020 (M > F)**	0.147
HPA group mean	0.91 ± 0.037	0.84 ± 0.031	0.404	0.084	0.110
2) Apoptosis and cell cycle (ACC)					
AIF	1.00 ± 0.046	0.92 ± 0.041	0.462	**0.033 (M > F)**	0.080
Annex2	0.99 ± 0.043	0.89 ± 0.043	0.247	0.054	0.200
Annex4	0.98 ± 0.040	1.00 ± 0.040	0.325	0.081	0.062
Caspase1	1.02 ± 0.053	0.80 ± 0.055	**0.018**	0.058	0.254
Caspase3	0.97 ± 0.040	0.98 ± 0.043	0.458	0.390	0.110
Caspase6	1.66 ± 0.083	0.96 ± 0.068	**≤0.001**	**0.037 (M > F)**	**0.032**
E-Cadherin	0.96 ± 0.039	0.91 ± 0.039	0.823	0.151	0.081
GAPDH	0.54 ± 0.047	0.63 ± 0.046	0.054	0.394	0.172
ACC group mean	1.01 ± 0.037	0.89 ± 0.039	0.099	0.061	**0.050**
3) Cellular stress and proteotoxicity (CSP)					
Cytokeratin	1.75 ± 0.087	0.76 ± 0.046	**≤0.001**	**0.010 (M > F)**	**0.035**
GRP78/BIP	0.86 ± 0.052	1.03 ± 0.055	**0.040**	0.876	0.333
Hsp 110	0.89 ± 0.042	0.97 ± 0.049	0.117	0.218	0.062
Hsp 27	0.76 ± 0.050	0.97 ± 0.059	**0.007**	0.811	0.275
Hsp 40	0.83 ± 0.052	1.12 ± 0.056	**0.002**	0.388	0.240
Hsp 60	1.19 ± 0.098	1.32 ± 0.106	0.523	0.177	0.172
Hsp 70	1.42 ± 0.078	0.94 ± 0.064	**≤0.001**	**0.038 (M > F)**	0.156
Hsp 70 (i)	0.82 ± 0.034	1.01 ± 0.054	**0.004**	0.600	0.194
Hsp 90	1.47 ± 0.055	1.20 ± 0.070	**0.011**	0.070	0.262
HSP 90 (Stressgen)	0.94 ± 0.048	1.10 ± 0.060	**0.035**	0.355	0.130
CSP group mean	1.05 ± 0.029	1.02 ± 0.040	0.867	0.190	0.193
4) Oxidative stress and inflammation (OSI)					
CCR5	0.64 ± 0.040	0.73 ± 0.046	0.064	0.884	0.087
Cox2	0.96 ± 0.046	0.82 ± 0.034	0.058	**0.024 (M > F)**	0.098
eNOS	0.95 ± 0.038	0.86 ± 0.041	0.386	0.519	0.074
HO-2	0.89 ± 0.043	0.88 ± 0.028	0.894	**0.034 (M > F)**	0.125
iNOS	1.03 ± 0.036	0.91 ± 0.050	0.311	0.227	**0.019**
PRDX3	0.89 ± 0.039	0.96 ± 0.053	0.152	0.309	0.106
SOD-1	1.00 ± 0.050	0.52 ± 0.076	**≤0.001**	0.121	0.946
SOD-2	0.83 ± 0.044	1.05 ± 0.055	**0.004**	0.349	0.232
OSI group mean	0.90 ± 0.035	0.84 ± 0.034	0.494	0.102	0.095
5) Total stress protein	28.93 ± 1.108	31.12 ± 0.765	0.366	0.079	0.079

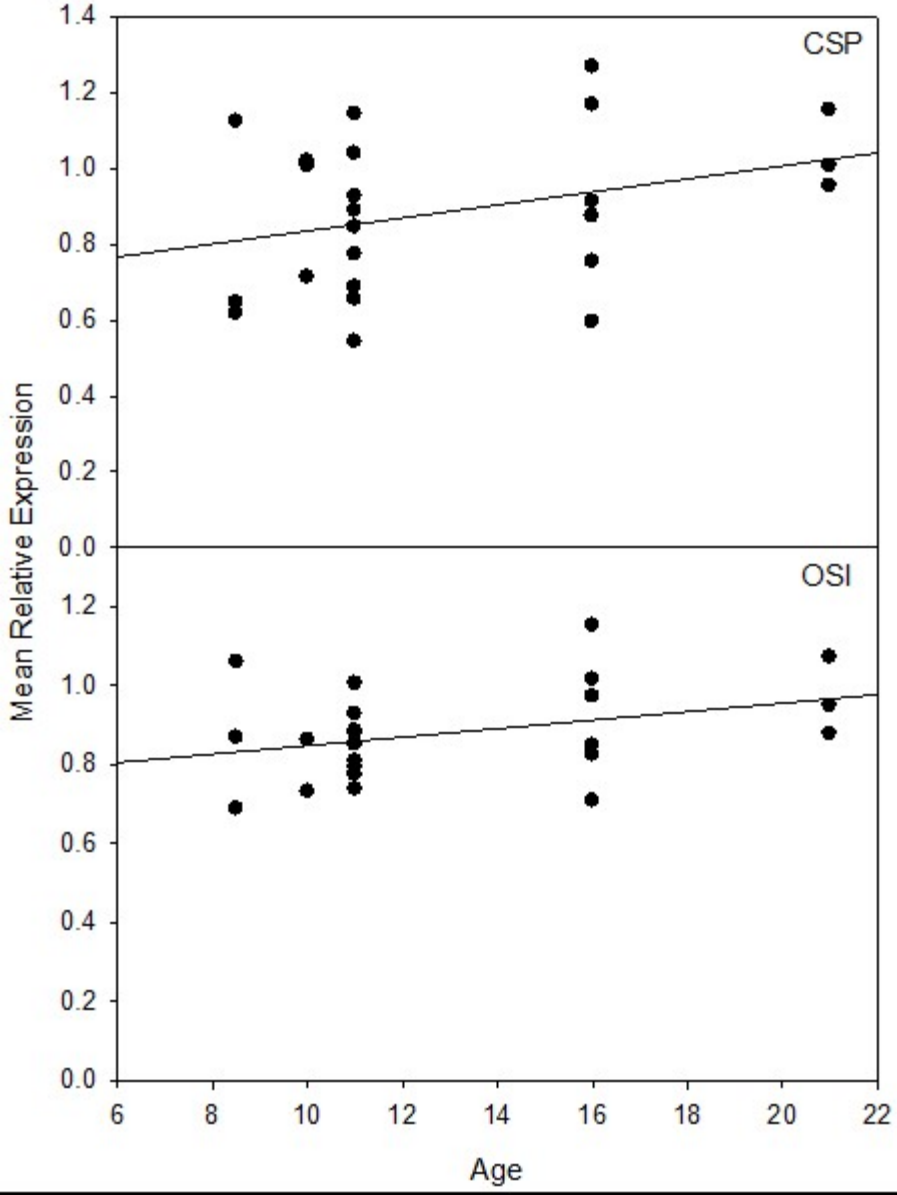
[a] **Method of comparison:** Linear mixed model analysis with location (IRL, CHS) and sex as factors, and age as a covariate. Dolphin ID was included as a random effect.

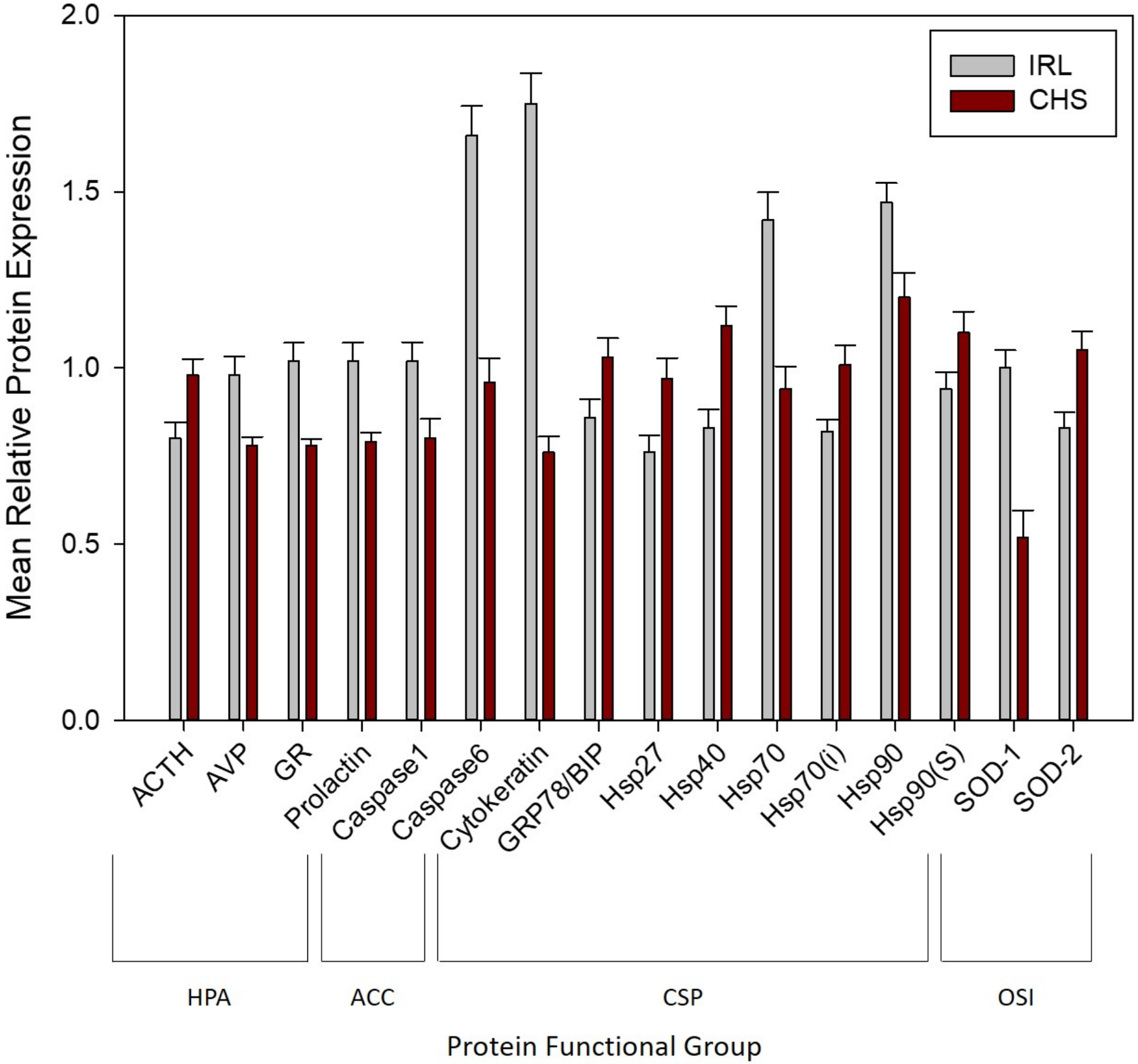
Table 5. Comparison[a] of stress protein relative expression in white blood cells between wild dolphins sampled at Indian River Lagoon (2 females + 7 males) and at Charleston, SC, (1 female + 11 males) and managed-care dolphins sampled at the Georgia Aquarium (6 females + 4 males) and the San Diego Naval facility (4 females + 3 males). Significant P-values (≤0.05) are presented in bold red type-face.

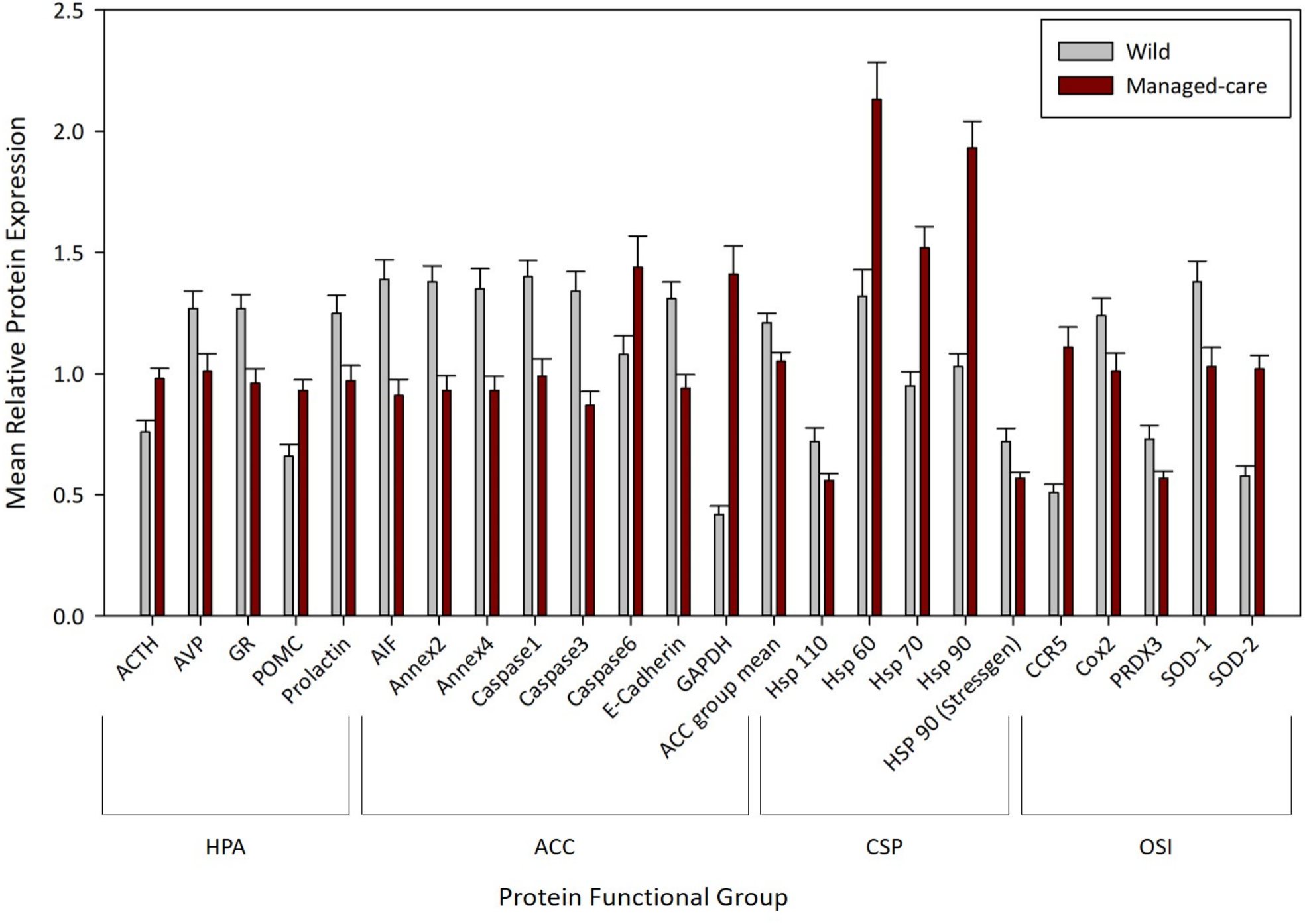
Stress Proteins	Mean relative expression ± SE		Factor or covariate effect (*P*)		
	Wild (*n* = 21)	Managed-care (*n* = 17)	Environment	Sex	Age
1) Hypothalamic-pituitary-adrenal axis (HPA)					
ACTH	0.76 ± 0.047	0.98 ± 0.042	**0.004**	0.503	0.672
AVP	1.27 ± 0.070	1.01 ± 0.073	**0.025**	0.546	0.647
CBG	0.70 ± 0.070	0.63 ± 0.023	0.395	0.568	0.472
CRF	1.07 ± 0.076	0.95 ± 0.073	0.316	0.902	0.510
GR	1.27 ± 0.057	0.96 ± 0.061	**≤0.001**	0.409	0.845
POMC	0.66 ± 0.047	0.93 ± 0.044	**≤0.001**	0.588	0.899
Prolactin	1.25 ± 0.074	0.97 ± 0.064	**0.025**	0.803	0.502
HPA group mean	1.00 ± 0.034	0.92 ± 0.037	0.135	0.684	0.991
2) Apoptosis and cell cycle (ACC)					
AIF	1.39 ± 0.080	0.91 ± 0.066	**≤0.001**	0.408	0.383
Annex2	1.38 ± 0.065	0.93 ± 0.061	**≤0.001**	0.523	0.232
Annex4	1.35 ± 0.083	0.93 ± 0.059	**≤0.001**	0.330	0.368
Caspase1	1.40 ± 0.067	0.99 ± 0.070	**≤0.001**	0.208	0.548
Caspase3	1.34 ± 0.081	0.87 ± 0.056	**≤0.001**	0.079	0.165
Caspase6	1.08 ± 0.076	1.44 ± 0.129	**0.040**	0.883	0.963
E-Cadherin	1.31 ± 0.068	0.94 ± 0.056	**0.003**	0.798	0.487
GAPDH	0.42 ± 0.034	1.41 ± 0.117	**≤0.001**	**0.009 (M > F)**	0.232
ACC group mean	1.21 ± 0.040	1.05 ± 0.039	**0.018**	0.396	0.333
3) Cellular stress and proteotoxicity (CSP)					
Cytokeratin	1.19 ± 0.091	1.50 ± 0.142	0.111	0.508	0.898
GRP78/BIP	0.69 ± 0.055	0.60 ± 0.034	0.440	0.385	0.105
Hsp 110	0.72 ± 0.056	0.56 ± 0.029	**0.020**	0.678	0.277
Hsp 27	0.63 ± 0.048	0.61 ± 0.034	0.837	0.599	0.189
Hsp 40	0.75 ± 0.066	0.59 ± 0.024	0.058	0.508	0.418
Hsp 60	1.32 ± 0.108	2.13 ± 0.155	**≤0.001**	0.911	0.931
Hsp 70	0.95 ± 0.058	1.52 ± 0.087	**≤0.001**	0.381	0.722
Hsp 70 (i)	0.76 ± 0.062	0.64 ± 0.020	0.090	0.154	0.461
Hsp 90	1.03 ± 0.053	1.93 ± 0.112	**≤0.001**	0.878	0.495
HSP 90 (Stressgen)	0.72 ± 0.055	0.57 ± 0.024	**0.045**	0.950	0.593
CSP group mean	0.86 ± 0.036	0.97 ± 0.032	0.091	0.925	0.589
4) Oxidative stress and inflammation (OSI)					
CCR5	0.51 ± 0.034	1.11 ± 0.082	**≤0.001**	**0.046 (M > F)**	0.175
Cox2	1.24 ± 0.072	1.01 ± 0.075	**0.048**	0.472	0.503
eNOS	0.76 ± 0.042	0.93 ± 0.059	0.061	0.874	0.532
HO-2	1.11 ± 0.068	0.97 ± 0.057	0.071	0.279	0.869
iNOS	0.83 ± 0.072	0.72 ± 0.022	0.181	0.479	0.463
PRDX3	0.73 ± 0.056	0.57 ± 0.028	**0.022**	0.605	0.367
SOD-1	1.38 ± 0.084	1.03 ± 0.078	**0.010**	0.365	0.146
SOD-2	0.58 ± 0.040	1.02 ± 0.056	**≤0.001**	0.079	0.702
OSI group mean	0.89 ± 0.029	0.92 ± 0.033	0.784	0.642	0.906
5) Total stress protein	31.51 ± 0.845	30.95 ± 0.774	0.511	0.535	0.900

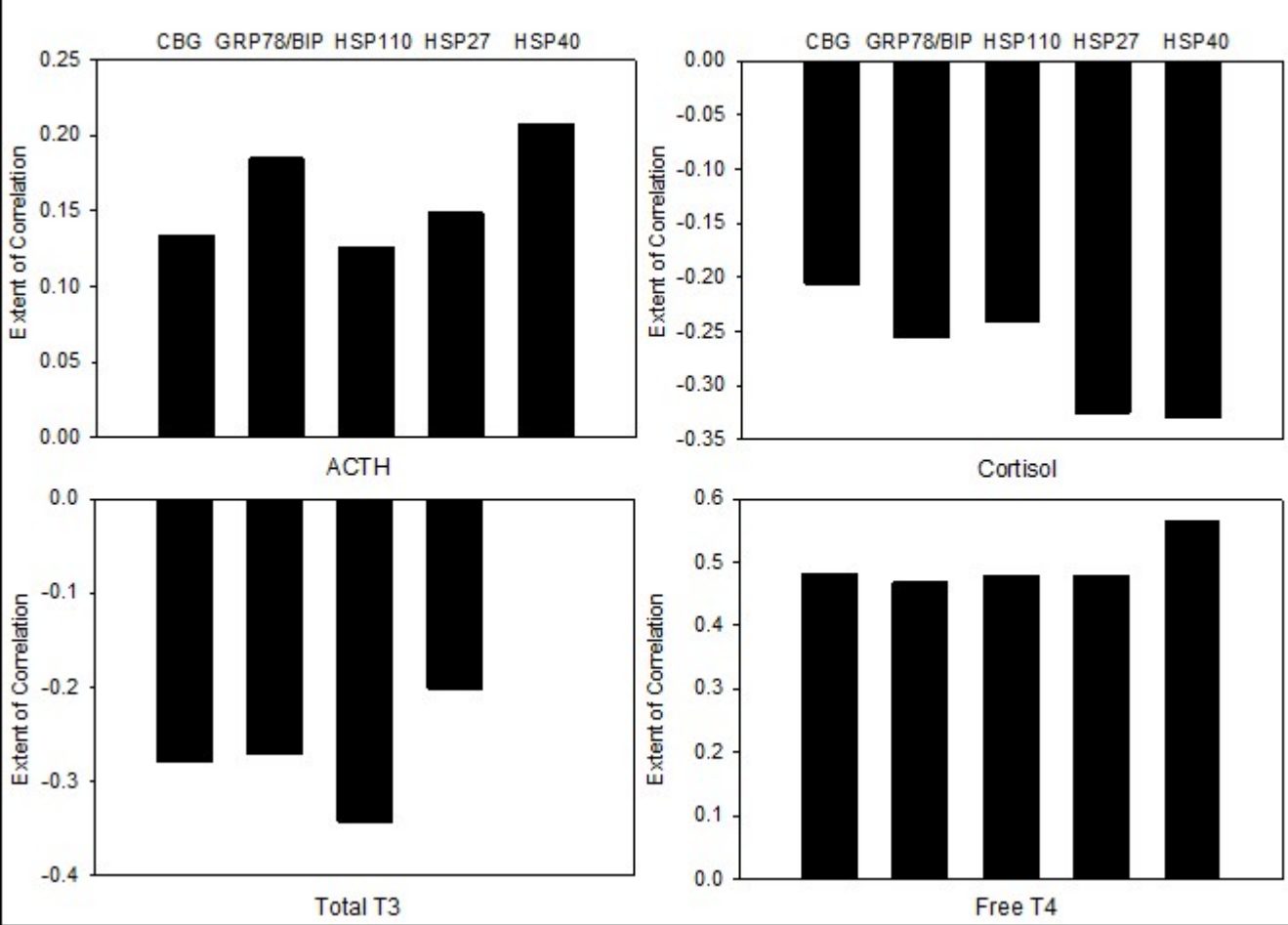
[a] **Method of comparison:** Linear mixed model analysis with environment (wild, managed-care) and sex as factors, and age as a covariate. Dolphin ID was included as a random effect

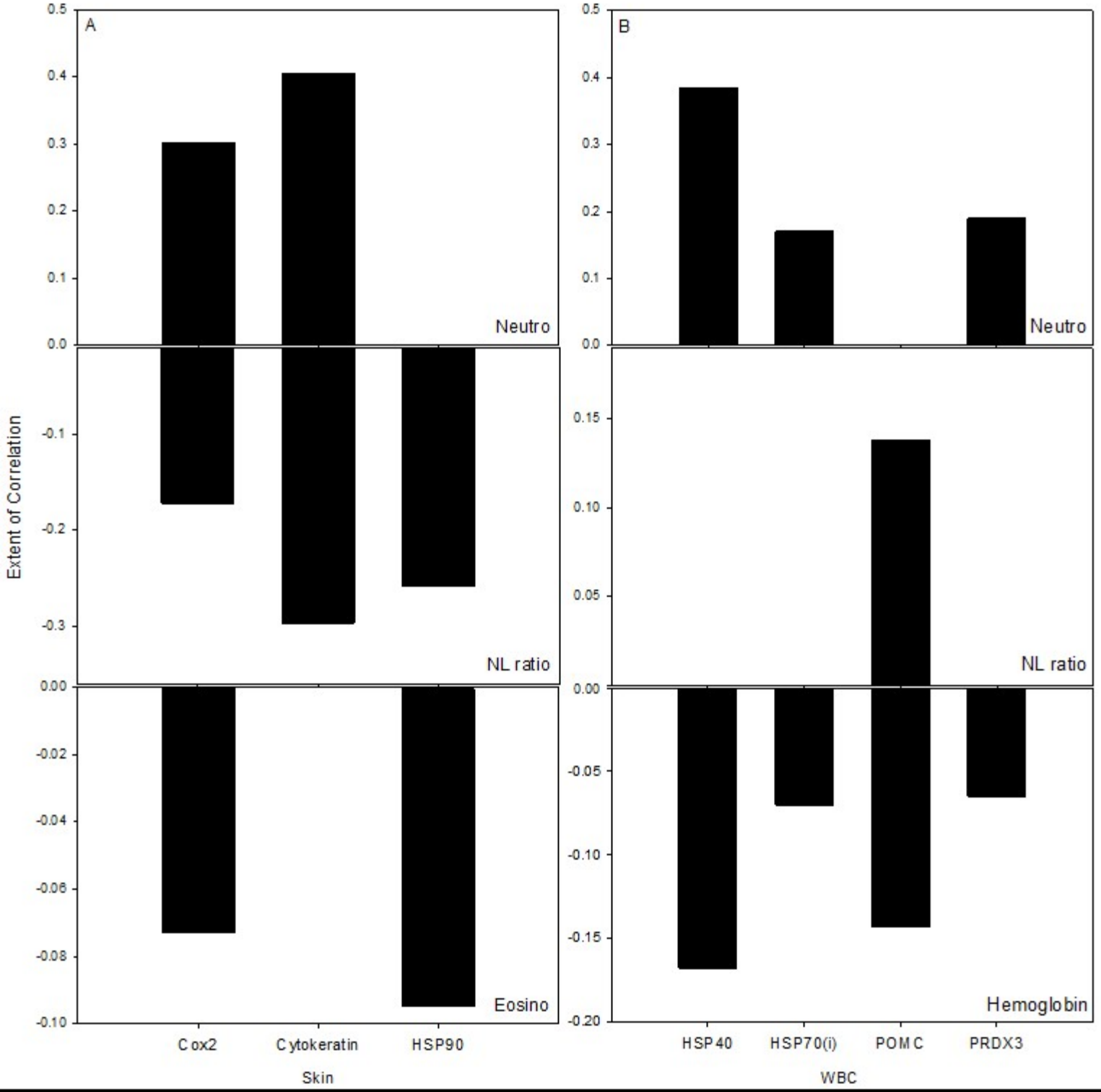

Protein Expression Level
2.0
1.5
1.0
0.5
0.0
HPA
ACC
CSP
OSI
Plasma
Skin
WBC
Protein Functional Group

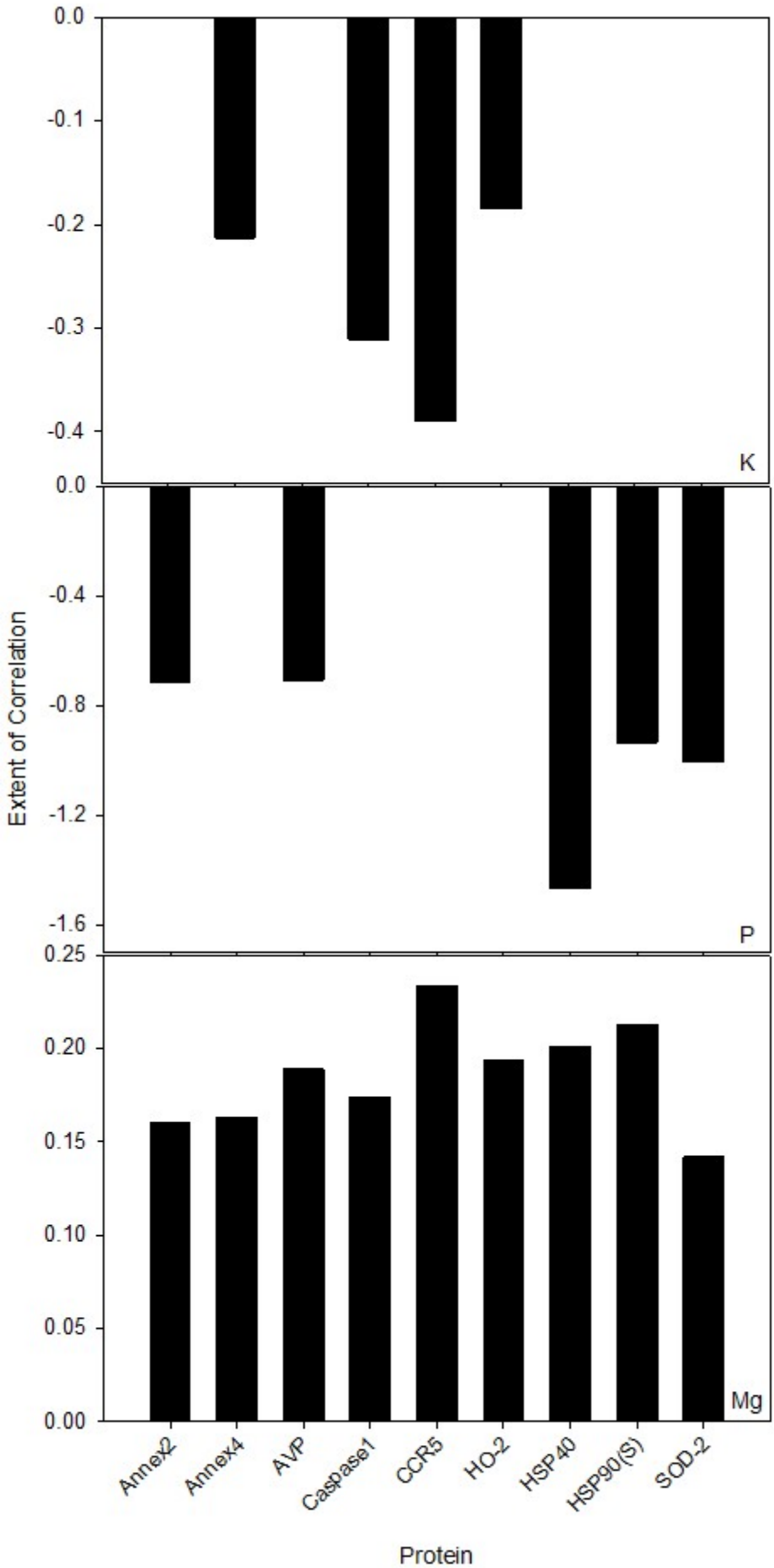

CSP
OSI
Mean Relative Expression
Age
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
6
8
10
12
14
16
18
20
22

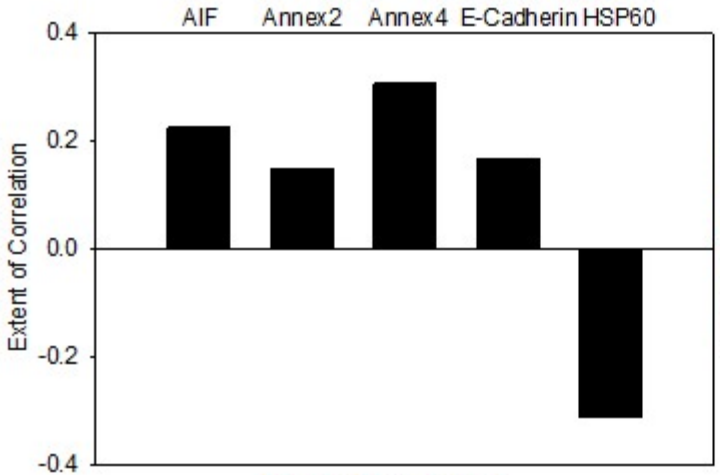

2.0
1.5
1.0
0.5
0.0
Mean Relative Protein Expression
IRL
CHS
ACTH
AVP
GR
Prolactin
Caspase1
Caspase6
Cytokeratin
GRP78/BIP
Hsp27
Hsp40
Hsp70
Hsp70(i)
Hsp90
Hsp90(S)
SOD-1
SOD-2
HPA
ACC
CSP
OSI
Protein Functional Group

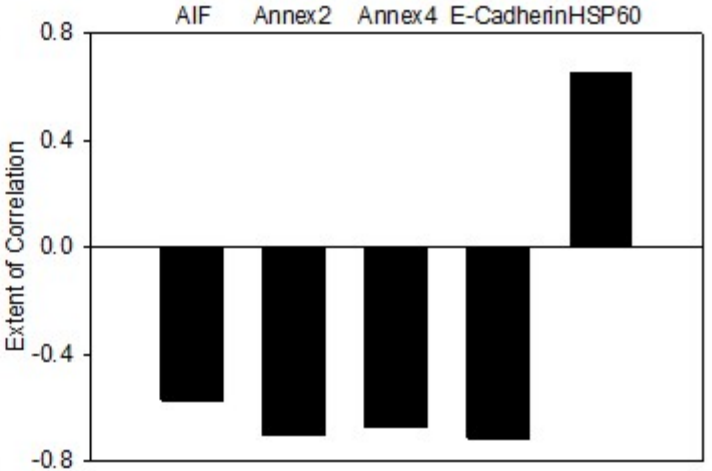

Wild
Managed-care
Mean Relative Protein Expression
2.5
2.0
1.5
1.0
0.5
0.0
ACTH
AVP
GR
POMC
Prolactin
AIF
Annex2
Annex4
Caspase1
Caspase3
Caspase6
E-Cadherin
GAPDH
ACC group mean
Hsp 110
Hsp 60
Hsp 70
Hsp 90
HSP 90 (Stressgen)
CCR5
Cox2
PRDX3
SOD-1
SOD-2
HPA
ACC
CSP
OSI
Protein Functional Group

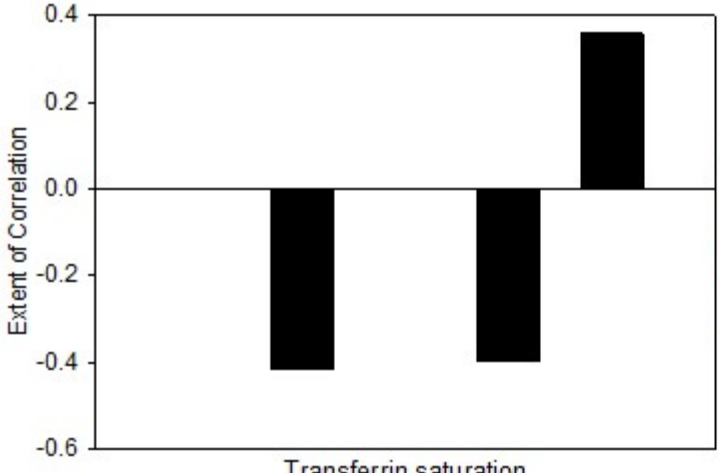

CBG GRP78/BIP HSP110 HSP27 HSP40
Extent of Correlation
0.25
0.20
0.15
0.10
0.05
0.00
ACTH
CBG GRP78/BIP HSP110 HSP27 HSP40
Extent of Correlation
0.00
-0.05
-0.10
-0.15
-0.20
-0.25
-0.30
-0.35
Cortisol
Extent of Correlation
0.0
-0.1
-0.2
-0.3
-0.4
Total T3
Extent of Correlation
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Free T4

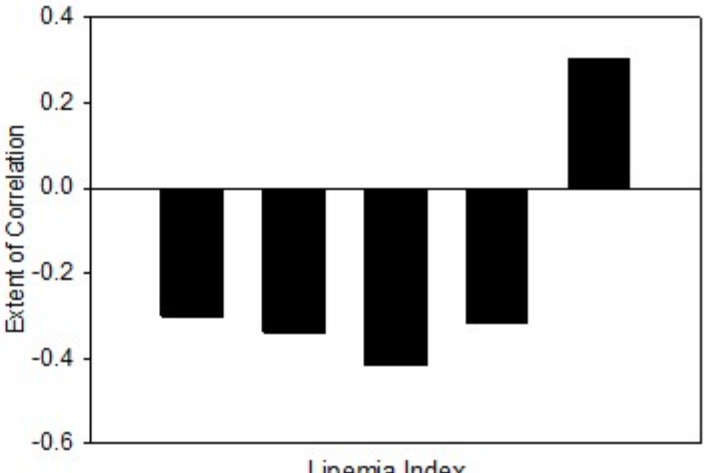

A
B
Neutro
NL ratio
Eosino
Hemoglobin
Extent of Correlation
Cox2
Cytokeratin
HSP90
HSP40
HSP70(i)
POMC
PRDX3
Skin
WBC

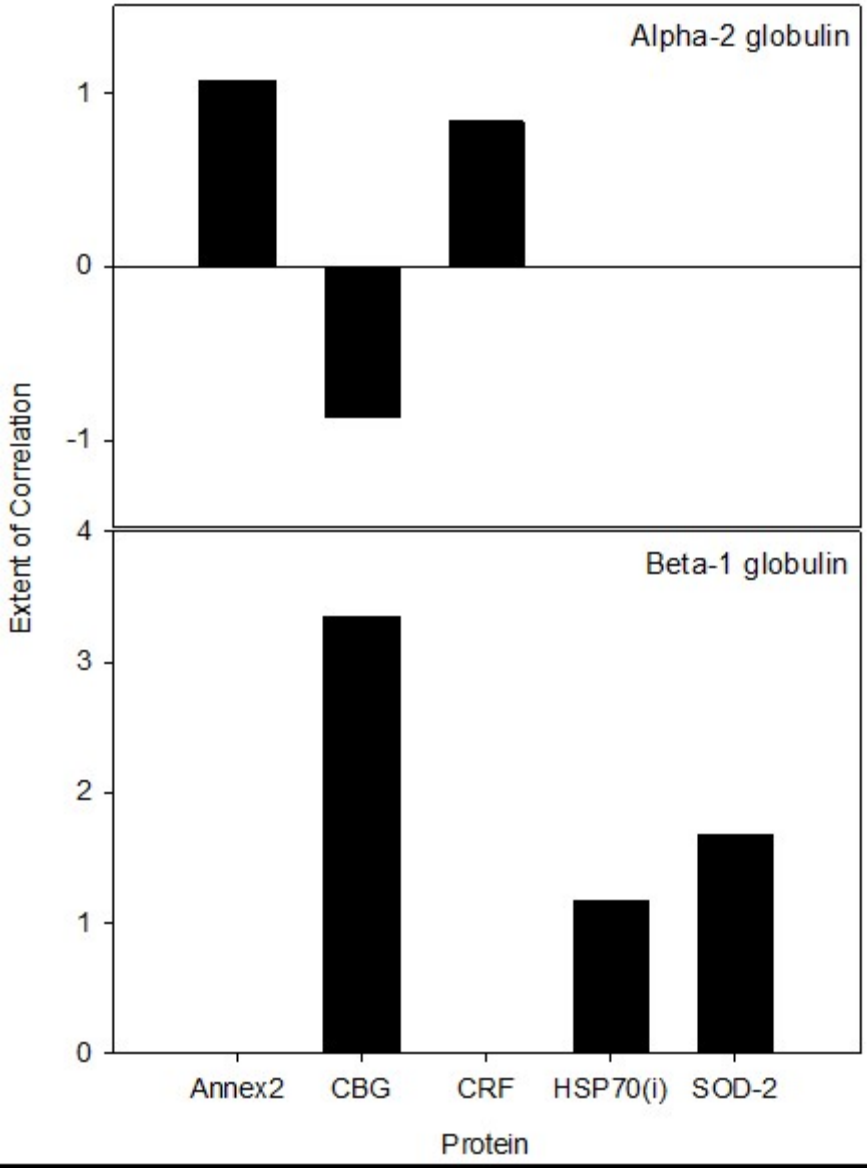

0.0
-0.1
-0.2
-0.3
-0.4
K
0.0
-0.4
-0.8
-1.2
-1.6
P
Extent of Correlation
0.25
0.20
0.15
0.10
0.05
0.00
Mg
Annex2
Annex4
AVP
Caspase1
CCR5
HO-2
HSP40
HSP90(S)
SOD-2
Protein


AIF
Annex2
Annex4
E-Cadherin
HSP60
0.4
0.2
0.0
-0.2
-0.4
Extent of Correlation
Alkaline Phosphatase


AIF
Annex2
Annex4
E-Cadherin
HSP60
0.8
0.4
0.0
-0.4
-0.8
Extent of Correlation
Total iron-binding capacity


0.4
0.2
0.0
-0.2
-0.4
-0.6
Extent of Correlation
Transferrin saturation


0.4
0.2
0.0
-0.2
-0.4
-0.6
Extent of Correlation
Lipemia Index


Alpha-2 globulin
1
0
-1
Beta-1 globulin
4
3
2
1
0
Extent of Correlation
Annex2
CBG
CRF
HSP70(i)
SOD-2
Protein

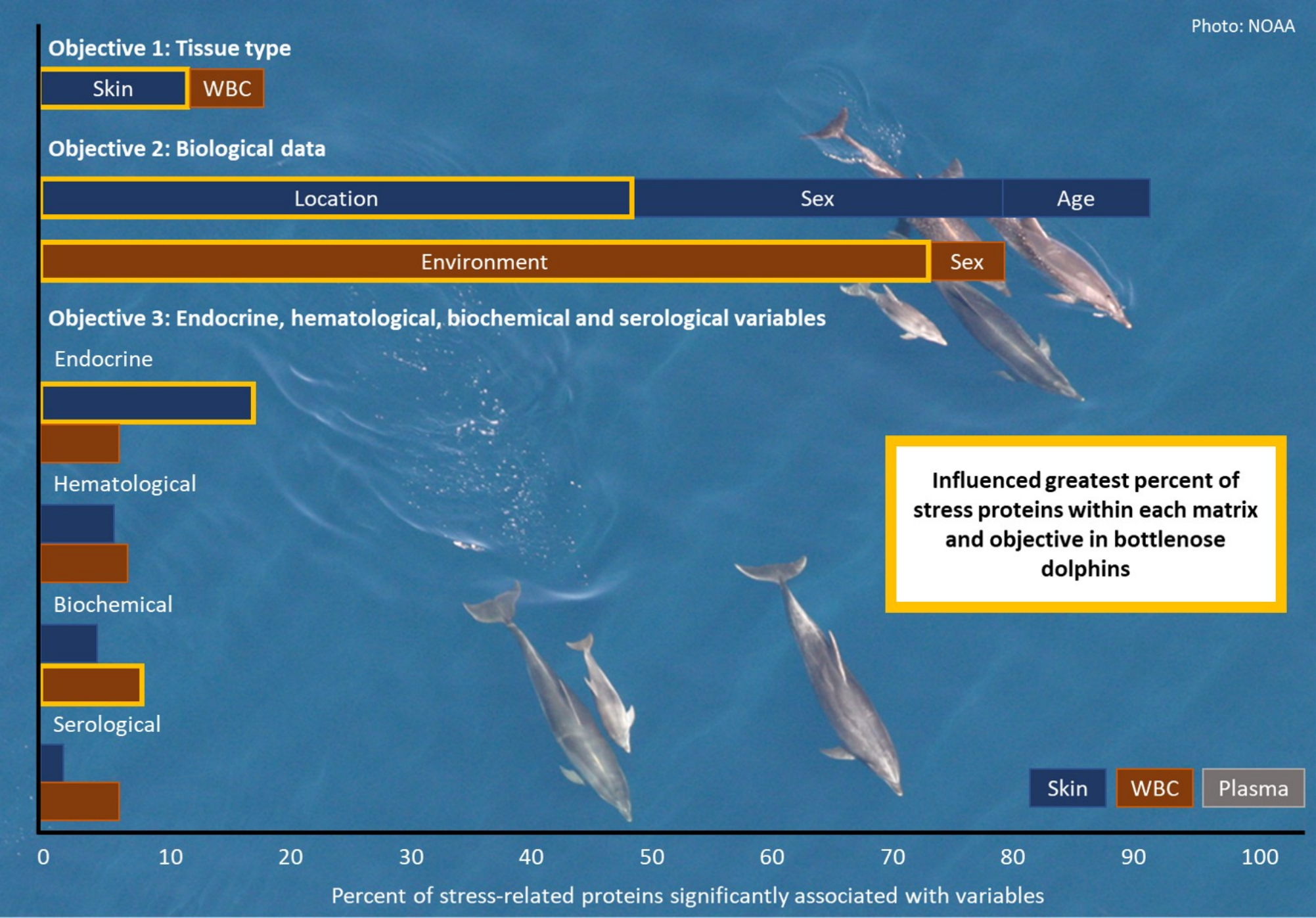


Photo: NOAA
Objective 1: Tissue type
Skin
WBC
Objective 2: Biological data
Location
Sex
Age
Environment
Sex
Objective 3: Endocrine, hematological, biochemical and serological variables
Endocrine
Hematological
Biochemical
Serological
Influenced greatest percent of stress proteins within each matrix and objective in bottlenose dolphins
Skin
WBC
Plasma
0
10
20
30
40
50
60
70
80
90
100
Percent of stress-related proteins significantly associated with variables