

**MOLECULAR SIGNATURE OF PHYSIOLOGICAL STRESS IN DOLPHINS
BASED ON PROTEIN EXPRESSION PROFILING OF SKIN**

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ABSTRACT

Global climate change and the growing impact of human activities have contributed to an increase in environmental stress in most ecosystems¹. The consequences for wildlife may be profound, but remain poorly understood. A major problem is that any impact is only noticed when a population suffers rapid decline, by which time it may be too late to implement conservation strategies². Here we describe a new technique for detecting a molecular signature of physiological stress, based on the expression profiles of 40 proteins known to be associated with stress. Profiles were obtained for 93 reference individuals with known health status, representing 8 species of mammal and a wide range of stressors. Diagnostic stress-associated changes could be readily identified using antibody staining of the skin. Using high throughput profiling we then surveyed cellular stress in 868 wild spotted dolphins sampled from a natural population potentially stressed by commercial tuna fishing operations³. The protein panel identified in this study provides a new tool for defining the proteomics of physiological stress responses. The integration of high throughput profiling linked to ecological data provides an effective approach to assess physiological stress in free living wild populations, and has further applications in medicine.

INTRODUCTION

Dolphins and whales encounter diverse forms of environmental and anthropogenic stress including climate variability, biotoxins, infectious diseases, habitat alteration, industrial pollution, and incidental entrapment in fishing gear. Some cetacean populations, including the gray whales and the North Atlantic right whales, have been declining due to unexplained wasting conditions and low reproduction rates^{4,5}. Spotted dolphins in the eastern tropical Pacific have been extensively chased and captured during commercial tuna fishing operations,³ and it is necessary to determine whether the involvement caused physiological stress, and triggered incipient changes in the health and reproduction fitness of the population.

In cetaceans, monitoring population size cannot provide early warnings of increased stress impact because of the long generation times². Thus, new tools are needed to help guide proactive conservation and inform management strategies. Furthermore, the test tissue must be readily and non-invasively sampled. The only practical choice is the skin, which can be repeatedly collected by projectile biopsy, and for which large archives already exist.

Is there a generic molecular change that could indicate exposure to stress? Studies in a variety of organisms suggest that the common physiological outcome of different types of stress is perturbation of the physiological homeostasis, manifested as systemic cellular stress, and the counter-acting molecular stress response⁶⁻¹³. Cellular stress is characterized by physical and chemical perturbations in the cellular microenvironment including ionic, pH and redox changes, and heat dissipation. Following a localized stress impact such as a closed head injury, cellular stress spreads rapidly to distant tissues

including skin¹⁴. Skin produces and responds to several homeostatic processes that provide stress defense, including oxidative stress repair, cellular detoxification, control of cell growth and cell death, cell adhesion, immunological signaling and neuroendocrine regulation. During continual stress such as disease, cellular stress persists in the whole organism¹¹⁻¹³. Stress response is necessary but when prolonged it overburdens the organism, and instead of restoring health it can contribute to disease pathogenesis, impair reproduction and accelerate aging¹¹⁻²¹.

An independent scientific peer review of this work was administered by the Center for Independent Experts located at the University of Miami. Responses to reviewer's comments can be found in the Appendix.

METHODS

Stress response proteins (SRP)

A panel of 40 stress response proteins (SRP, Table 1) was identified based on several conceptual and experimental criteria: (1) Documented function in mammalian cellular stress/molecular stress response pathways during prolonged stress. (2) Documented expression in human keratinocytes. (3) Detectable in the formalin-fixed cetacean skin using a commercial antibody. (4) Baseline expression localized to the epidermis. (5) A stress-associated increase, rather than a decrease, in the expression level within the epidermis.

Proteins were chosen as the most suitable proxy molecules to detect cellular stress in the skin for several reasons. (1) Proteins, unlike RNA messages, are directly involved in the stress response pathways. (2) Proteins are better preserved in the chemically fixed skin (the majority of our specimens) than mRNA. (3) Protein expression is less likely to be altered by post-mortem events than other molecules used for detecting stress, such as lipids or mRNA.

Reference specimens

Reference specimens from individuals with known, contrasting differences in cellular stress were necessary to characterize the SRP expression in the cetacean skin, and to assess the performance of the SRP panel as a stress marker. Based on a review of medical literature we assumed that live, apparently healthy individuals had nominally normal levels of cellular stress, and that individuals with clinical diseases, or individuals that were severely physiologically distressed, had abnormal levels of cellular stress. A detailed description of 42 individuals classified as normal, and 51 individuals classified as stressed, is in Table 2. To focus on general differences between the normal and stressed groups, the reference specimens were from 7 cetacean species and from humans, and represented a variety of physiological and pathological conditions, both genders, and different ages.

The reference individuals classified as normal may have experienced some acute stress due to sampling. Potential contribution of sampling stress would have been eliminated from the comparative expression analysis during determination of the relative expression levels (see below).

The cetacean specimens were obtained by opportunistic sampling and therefore, in most cases, the precise skin sampling locations were not known, and were supposed to represent random sites on the dorsum and the head. The original skin samples were preserved in several different ways (Table 2). To assure consistent immunohistochemical reactivity with the selected panel of anti-SRP antibodies (see below), sub-specimens (typically, 5x5x5-10mm) were placed into 10% normal buffered formalin for at least 24 hours before embedding in the paraffin blocks.

Multi-specimen slides

Formalin-fixed skin specimens were used to prepare multi-specimen histological slides. The concept of multi-tissue slides was developed in our laboratory to allow simultaneous immunohistochemical staining of a large number of specimens, and thus provide the basis for quantitative comparisons among specimens. Furthermore, the use of multi-specimen slides substantially decreased the cost of the histological processing and the immunohistochemical staining.

The multi-specimen slides were produced in the Histology Laboratory, UCSD Medical Center, San Diego, using our instructions (Fig. 1). Six different skin fragments were embedded in one paraffin block. Thin sections (5 microns) from 3 blocks were placed on one slide (18 specimens). Positive-charged slides were used to facilitate adhesion of the tissue sections. The multi-specimen slides were stained as described in the standard immunohistochemical staining procedure (see below), using 0.5ml of each immunohistochemical reagent per slide. Up to 25 slides were processed simultaneously in one staining experiment, providing 450 highly comparable staining results.

Immunohistochemistry

Antibodies. An overview of the primary antibodies to individual SRP antigens is in Table 3. All the antibodies were commercially available, well characterized reagents except for the 1D3 antibody against extra-mitochondrial cytochrome c that was developed and provided as a gift by Dr. R. Jemmerson, Department of Microbiology, University of Minnesota, Minneapolis.

We chose only such anti-SRP antibodies that were robust and compatible, in order to assure that a simple immunohistochemical staining procedure would be generally applicable to all the SRP antigens. The anti-SRP antibodies were chosen based on following criteria: (1) Mouse or rabbit IgGs. (2) Identical reaction conditions. (3) Non-specific binding blocked by pre-incubation in 7% goat serum. (4) Documented reactivity with formalin-fixed, paraffin-embedded antigens. (5) Antigen retrieval required or harmless. (6) Documented cross-reactivity with several mammalian species including human and mouse.

All the antibodies were titrated using the human and cetacean skin to establish optimal dilutions (Table 3). We aimed to find optimal dilutions at which the baseline expression was detected as a low staining intensity, in order to provide maximal resolution for detecting an increased expression.

Non-specific staining was efficiently blocked as determined using negative control antibodies (commercial non-immune rabbit sera and epitope-matched mouse IgGs). Tissue preservation was assessed using the expression of keratin (a ubiquitous keratinocyte protein). Specimens with inconsistent, variable keratin expression were eliminated from further analysis. Inter- and intra-experimental reproducibility of staining was assessed using several positive control sections included in each staining experiment.

Detection of cetacean SRP using cross-reactive antibodies. We selected only such antibodies that were designed to recognize highly conserved SRP epitopes, as documented by actually observed, specific cross-reactivity against homologous SRP antigens in different tissues of humans and mice (and other species, in many cases). When possible, we have chosen rabbit polyclonal antibodies that recognized multiple epitopes, rather than monoclonal antibodies, to further increase the probability of specific cross-reactivity with homologous cetacean proteins. The antibody design and the empirically validated cross-reactivity of these antibodies strongly suggested that a homologous protein would be recognized in cetaceans. Specific reactivity of all the anti-SRP antibodies with the cetacean skin was shown experimentally, using the reference specimens of dolphin and whale skin, and the human skin as a positive control.

Potential non-specific cross-reactivity was eliminated by antibody titration using the skin from 7 species of cetaceans, and the human skin as a positive control.

Detailed molecular identification of the cross-reactive antigens in cetacean skin was not carried out for several reasons. (1) Within the scope of this study, knowledge of the precise molecular identity of the cross-reactive cetacean antigens was not required. It was sufficient to establish empirically that a cross-reacting cetacean antigen was useful for discriminating between healthy and diseased individuals. (2) Precise molecular characterization of the cross-reactive cetacean antigens was not technically possible with the majority of the reference specimens because formalin and DMSO fixed tissues were not suitable for Western blotting or cDNA analysis. (3) The limited scope of this study precluded the development and the use of cloned cultured cetacean cells as an alternative route to characterize the SRP antigens in cetaceans.

Antibody cocktail. The goal of the mandated study was a high throughput molecular analysis of stress in a large number of specimens. To compress the staining procedure, and still retain the broad functional potential of multiple stress markers, we implemented a cocktail of all the individual antibodies. We expected that the cocktail would visualize a cumulative change in the expression of all the SRP. To promote additive change, we have chosen only such SRP that were preferentially upregulated, rather than downregulated, in the diseased individuals (see above).

The use of cocktail antibodies requires a careful design and empirical validation of specific reactivity. Cocktail antibodies are used routinely in medical diagnostics (see Appendix), for example for a rapid identification of HIV-positive blood in the VA

Hospital in San Diego (Abbott Diagnostics HIV-1, 2 kit). Based on the experience from our previous studies²³, only highly compatible primary antibodies (see below) were used to design SRP cocktails. The optimal dilutions of antibodies in the cocktail were established empirically, starting with the optimized concentrations of the individual antibodies.

At first we designed a universal cocktail, applicable to all reference specimens. The universal cocktail contained all anti-SRP antibodies at concentrations that were 40-fold lower concentrations than the optimized single antibody concentrations (Table 3). This cocktail was used to analyze the reference specimens.

For high throughput profiling of spotted dolphins we designed a new, spotted dolphin specific cocktail (Table 3). Twelve versions of the spotted dolphin cocktail were tested by staining all the available 868 spotted dolphin specimens (see below), to identify antibody concentrations that provided optimal visual display of the broad range of SRP expression levels in that large specimen group.

Staining procedure. The immunohistochemical staining was performed in our laboratory, as described previously²². Briefly, slides were de-waxed using 3 changes of xylene, 3 changes of absolute ethanol, 3 changes of graded ethanol (95-80-60%), and transferred to water. After antigen retrieval (full boil for 10 minutes in 10mM sodium citrate buffer), primary antibodies were applied at 4°C (all the anti-SRP antibodies), or at a room temperature (the anti-cytokeratin antibody), for 16 hours. The primary antibodies were detected by consecutive application of several reagents. (1) Biotinylated antibody (concentrated mouse or rabbit links, 1:20 dilution, 1 hour, a room temperature, or a 1:1 mixture of mouse and rabbit links at 1:20 dilutions for cocktail antibody detection; BioGenex, San Ramon, CA, USA). (2) Alkaline phosphatase-conjugated streptavidin (concentrated AP label, 1:15 dilution, 30 minutes, a room temperature, BioGenex). (3) Fuchsin chromogen (5 minutes color development, Dako, Carpinteria, CA, USA). (4) Mayer's hematoxylin (1 minute, BioGenex). The red signal produced by Fuchsin was visualized using a light microscope connected to a computerized image analysis system (see below).

Quantitative analysis of SRP expression using computerized image analysis (CIA)

We could measure and compare SRP expression levels in different specimens because the SRP expression levels were visualized under approximately identical conditions (identical immunohistochemical reagents and procedure, simultaneous processing using the multi-specimen slides).

SRP expression levels (EL) were quantified based on the immunohistochemical staining intensity, which was determined using an improved CIA technique. The staining intensity value was computed as the product of the mean optical density of the stained cells (MOD), and the percentage of the stained area (PA). MOD and PA were determined using a new procedure developed in our laboratory (see Color File and Outlining File below). A semi-automated method for computing EL was based on a new software program developed in our laboratory (Visual Basic). Three microscopic images captured

at 100x magnification (see Image capture below) were used for each specimen, to provide a mean EL value.

The CIA-based quantification was generally applicable to all the SRP proteins regardless of their individual sub-cellular distributions within keratinocytes because the EL value was computed based on the summary distribution within the entire epidermis. The epidermis was the common site where all the SRP proteins were expressed because when designing the SRP panel, we only chose such proteins that were predominantly expressed in the epidermis.

Color File. MOD selection was performed based on the RGB color cube model that describes the color spectrum as a cube of intensity along red, green, and blue axes. Each separate value in the color cube model can be selected independently, which allows very different colors in an image to be included in the same color class. This model is especially appropriate for use with images that are difficult to segment. Using the eyedropper tool, varying intensities of red signal (all SRP antigens were visualized as a red pigment) were selected empirically from representative specimens and saved in a color file. That color file was then applied to all images, ensuring that the same range of red signal was analyzed each time. Application of the color file is illustrated in Fig. 2.

Outlining File. Precise PA selection was performed using the free-form outlining tool. The outlining tool enabled us to precisely select the area of image in which staining needed to be quantified (the epidermis, in this study), and allowed the exclusion of artifacts such as tissue tears, folds, and non-specific staining, thus increasing the accuracy and precision of signal quantification. The outlining file for each image was saved and archived. Application of the outlining tool is illustrated in Fig. 2.

Image Capture. All images were captured using the same microscope and camera settings. The condenser, aperture, field iris diaphragm, and exposure time remained constant. All images were taken at 100x magnification, and an LBD color-balancing filter was used to even out the background and any hotspots present in the lens. A light-preset button, which sets the light intensity at a pre-selected level regardless of the position of the voltage lever, was engaged for all images captured, and images were taken during the same time of day to control for changes in ambient light. Three images representing about 20-30% of the entire skin section were captured. All images were saved as 48-bit color 8 MB tiff files, and archived.

CIA system description. Gateway E-4200 computer and VX900 monitor, with the display set to 32-bit True Color. Images were taken with the DVC digital camera 1310C, which is an RGB color high-resolution 10-bit digital camera outputting 1300(h) x 1030(v). The camera was mounted on an Olympus System Light Microscope Model BX50, and was linked to the computer via a pixelYNX image processor board (Scientific Instrument Company, Sunnyvale, CA). The analysis software package used was the commercially available Image-Pro Plus version 4.1 for Windows 95/98/NT 4.0 (Media Cybernetics, Silver Spring, MD).

SRP profiling of reference specimens

The reference specimens were stained using the same immunohistochemical conditions. Some specimens were stained repeatedly, to assess staining and scoring reproducibility. Each staining experiment involved internal negative and positive controls to monitor for staining specificity (non-immune rabbit sera, control rabbit antisera, isotype-matched control mouse antibodies), efficiency (serial SOD Mn antibody dilutions applied to sections from the same positive specimen), and tissue preservation (cytokeratin).

Classification of SRP expression profiles. SRP expression profiles in the reference specimens were classified based on changes in the SRP staining intensity relative to the average staining intensity in the reference normal specimens (the baseline expression level). Changes in the staining intensity were evaluated using a CIA-based scale (Fig. 3). We found that the normal specimens had very similar expression levels (<2-fold differences) of SRP. In the stressed specimens, the staining intensity ranged from 3-fold decreased to 30-fold increased, relative to the normal pool. Only 3-fold changes in the staining intensity, relative to the normal pool, were considered significant. The resulting staining intensity scores were -3, 0, 3, 9 and 27.

For most applications, such as statistical analysis of expression profile variability, ratio measurements are most naturally processed in a log space²⁴. Therefore, we computed a relative expression level (REL) score, as a log 3 transformed value of the staining intensity score. Base 3 was chosen because 3-fold differences in the staining intensity were considered significant. The resulting REL scores, corresponding to the staining intensity scores, were -1, 0, 1, 2 and 3 (Fig. 3).

Each skin specimen was assigned a SRP expression profile composed of 40 REL values corresponding to the individual SRP. In addition, each SRP was assigned an expression profile composed of 93 values corresponding to the individual specimens.

Perturbation index (PI). A new variable was defined to evaluate the quantitative variability in SRP expression profiles. PI values were defined to range from 0% (a normal SRP profile) to 100% (the maximal observable change in a SRP profile, if all REL = 3, not detected in this study). PI was a percentage ratio of the sum of the absolute values of the actual RELs, and the sum of maximal expected RELs (if all REL=3).

To assess the variability across the SRP, PI^a was computed for each specimen. Example: For the specimen ID#43, $PI^a = (5 \times 1) + (35 \times 0) / 40 \times 3 = 5/120 = 4\%$. To assess the variability across the specimens, PI^b was computed for each SRP. Example: For SOD Mn, $PI^b = 72/141 = 51\%$.

High throughput SRP profiling of spotted dolphins

Several technical innovations were integrated to support a rapid, accurate and inexpensive survey of cellular stress in 868 spotted dolphins. The innovations included multi-specimen slides for simultaneous analysis of 450 specimens (Fig. 1), a spotted dolphin-adapted SRP antibody cocktail for simultaneous SRP display (Table 3), and a semi-automated computerized image analysis technique for quantification of SRP expression levels (Fig. 2). The exclusive use of the SRP cocktail, instead of using the cocktail as well as the individual antibodies, was dictated by the limited scope of the mandated study. Profiling of the individual SRP would have provided much more accurate and precise determination of the cellular stress. However, the required increase in time, personnel and budget was not available.

Spotted dolphin specimens. Skin samples were obtained from wild spotted dolphins as described in detail elsewhere²⁵. Three specimen sets were analyzed. (1) Dolphins drowned in tuna fishing nets during 1988-91 (450 jaw skin specimens preserved in formalin for several years and then stored in 70% isopropyl alcohol). (2) Dolphins sampled by projectile biopsy during 1998-99; these dolphins were bow-riding voluntarily near research vessels (250 dorsal skin specimens, stored frozen). (3) Dolphins sampled by manual biopsy during the Chase Encirclement Stress Studies (CHESS) Cruise in 2001²⁶ (234 specimens of dorsal fin skin, and 22 specimens of dorsal skin, stored frozen).

Small portions of the original specimens (5x5x3 mm³) were fixed in 10% normal buffered formalin for at least 24 hours before embedding in paraffin. Multi-specimen paraffin blocks (6 specimens per a block) and slides (18 sections per slide) were produced in the Histology Laboratory, UCSD Medical Center, to our specifications. Detailed records describing the original specimens, the blocks and the slides were kept as a hard copy.

Immunohistochemical staining. Each specimen set (450 skin sections) was stained with the spotted dolphin-adapted SRP antibody cocktail (Table 3). The CHESS specimens were randomly mixed with 200 previously analyzed bow-riding specimens to assess the reproducibility of SRP profiling (see below). The staining was repeated 2-3 times, using slightly different dilutions (1.5-fold variations) of the optimized antibody cocktail, to optimize the visual display of the SRP profiles.

Classification of SRP profiles. A majority of the specimens (707/868 = 81%) had profiles resembling closely the normal or the altered profiles found in the reference specimens (Figs. 4a, b). In 161 spotted dolphins, novel types of altered SRP profiles were detected. The novel profiles had a "banded" appearance due to discrete horizontal layers with high or low SRP expression levels (Figs. 4c-f). The banded profiles could not be explained as artifacts for several reasons: (1) The same types of the banded profiles were found in 161 dolphins that were sampled randomly (different body sites, different sampling dates, different primary preservation procedure). (2) The banding was consistent in several adjacent sections stained with serial dilutions of the antibody cocktail. (3) Cytokeratin expression was either uniform or showed only limited banding.

Because of the banded profiles, classification of the SRP profiles in the spotted dolphins could not be based only on the quantity of the SRP expression, as in the reference specimens, but also on the spatial distribution of the SRP expression within the epidermis.

The spatial distribution of the SRP expression was assessed subjectively. An objective, image analysis-based method for evaluation of the spatial distribution could not be developed due to the limited scope of the study. Spatial distribution of SRP was classified as normal or altered based on the visual similarity with the SRP expression in the reference specimens.

The quantity of the SRP expression was measured objectively using CIA, as a continuous range of EL values (EL=0-33). A normal quantitative level of SRP expression was defined as the average EL in the spotted dolphins with a normal spatial SRP distribution (normal EL=mean EL \pm 2 SD).

SRP profiles were classified as “normal” if they had a normal spatial distribution, and a normal quantitative level of SRP expression. All other SRP profiles were classified as “altered”. During the final scoring, 278 poor quality specimens (too small, folded, torn), were identified by a specific code, and recommended for elimination from further analysis²⁵.

Blinding. The multi-tissue slide production, the immunohistochemical staining, CIA, visual inspection and the scoring of SRP profiles were performed blind, without knowledge of the fishing activity level associated with individual specimens. However, the identity of historical fishery dolphins and bow-riders, as groups, was known (except in the combined CHESS/bow-riders set that was assembled blind). The blind data were exported to the Access database for statistical analysis²⁵.

Reproducibility. A high reproducibility of the SRP profile classification was demonstrated using results from a repeated classification of 158 bow-riders. The bow-riders were analyzed at first in the context of 250 other bow-riders, and then in a blinded combination with 250 CHESS specimens. The original and the repeated classifications correlated significantly (137/158=87% duplicated classification, 21/158=13% different classification, Spearman rank correlation test $r_s=0.999$).

RESULTS AND DISCUSSION

Development of SRP profiling

To detect stress-associated molecular changes in the cetacean skin, we identified 40 proteins (stress response proteins, SRP) with documented roles in molecular stress responses of humans and other mammals, and known expression in the human skin (Table 1). The SRP panel represents several principal homeostatic pathways and provides the potential for broad-spectrum recognition of diverse changes triggered by different types of stress. Reference specimens were obtained from wild dolphins and whales with

known health status in order to characterize stress-associated changes in the SRP expression in cetaceans. To reveal general manifestations of stress, the reference specimens represented 7 species of cetaceans and different types and severity of physiological perturbations (Table 2). Human specimens were included to provide both a positive control, and to allow further generalization of the stress-associated changes. Live, apparently healthy individuals were classified as normal, with baseline levels of stress (n=42, Table 2a). Among 51 individuals classified as stressed (Table 2b), 23 individuals with documented clinical diseases provided a framework within which to compare individuals with more complex health histories. Those included 15 belugas trapped in a closing ice hole, 4 belugas killed in a subsistence hunt, 8 spotted dolphins drowned in fishing nets, and one accidentally drowned dolphin (Table 2b).

Stress-associated changes in the SRP expression were first explored qualitatively, to provide a basic characterization of the SRP expression in the skin of healthy and diseased cetaceans. SRP were detected using immunohistochemical staining of skin sections, in order to visualize the quantitative and the spatial distribution of the SRP expression within the skin. Fig. 5 illustrates a typical expression profile, here for the mitochondrial superoxide dismutase, SOD Mn.

In healthy cetaceans, the baseline expression of SOD Mn was widespread in the skin (Fig. 5a), with greatest concentrations in the basal and suprabasal keratinocytes of the epidermis (Figs. 5 a-c). The expression of the other SRP was similar, except for metallothionein, strictly localized in the basal keratinocytes, and laminin, detected also in the vascular endothelium and the extracellular matrix of the dermal papillae. The baseline expression levels appeared invariable within the same species and similar among species. The elevated expression in the germinal and immature keratinocytes may be due to intrinsic physical and chemical stresses in the cellular microenvironment, triggered by the high metabolic rate of those cells. The invariability of the baseline expression profiles indicates that the SRP expression in the skin is essential and highly conserved.

In diseased cetaceans, the SOD Mn expression was variable, ranging from the baseline expression profile to altered profiles characterized by increased expression levels in both immature and mature keratinocytes (Figs. 5 d-e). All SRP had altered expression profiles in some of the stressed individuals. The altered expression profiles probably indicated a generalized increase in the cellular stress in the skin.

Differential expression analysis, based on quantitative characterization of SRP expression in the reference specimens, was used to validate the use of the SRP panel as an effective stress marker. Fig. 6 provides an overview of SRP expression profiles in the reference specimens, using a relative expression level (REL) scale (Fig. 3). The 42 individuals classified as normal were used to define a normal profile of the SRP expression (REL=0 for all SRP). Among 51 individuals classified as stressed, the majority (47/51=92%) had altered profiles of the SRP expression, relative to the normal profile. The altered profiles involved changes in the expression levels of many individual SRP (5-38 SRPs, mean=27). The relative changes in the SRP expression levels ranged 30-fold (mean = 2.8-fold, S.D. = 1.5-fold change).

To describe and compare the relative extent of quantitative changes in altered SRP profiles, we introduced a new variable termed the perturbation index that measures

the relative change in the profile for each specimen, and each protein (PI, see Methods). PI for the individual SRPs ranged from 11-57% (Fig. 6), showing that all the SRP were sensitive to some manifestations of stress, and ranking the SRP based on their variability across the specimen panel. PI for the individual specimens ranged from 0-61% (Fig. 6), illustrating the wide range of stress manifestations in the reference specimens. The least perturbed profiles were in belugas killed by subsistence hunters (PI=0, green IDs, Fig. 6), and in the accidentally drowned dolphin (PI=4%, gray ID). The most perturbed profiles were in clinically diseased individuals (mean PI= 36%, purple IDs). Spotted dolphins killed in fishing nets (yellow IDs), and belugas entrapped in the closing ice hole (blue IDs) displayed a wide range of perturbations showing that the same type and level of stress can have different individual impact. The results suggest that PI-based ranking could be used to identify predominant homeostatic processes that drive stress response in particular individuals, and provide a measure of severity of stress-induced health changes.

We found that the more extensively altered SRP profiles (PI \geq 14%) could also be revealed using a cocktail of the individual anti-SRP antibodies (Fig. 6). Based on the PI ranking, the cocktail was a more sensitive universal stress marker (PI=65%) than any of the individual antibodies (PI=11-57%). The reaction mechanism of staining with the SRP antibody cocktail is not known. However, the empirical results of this and other studies²² demonstrate that staining with an antibody cocktail provides rapid, efficient and inexpensive means for sorting expression profiles into normal and altered, which can be then analyzed in detail using the individual antibodies.

SRP expression profiles were highly conserved within macroscopic areas of the skin as demonstrated by the persistence of the profiles in adjacent as well as remote sections, up to 5 cm apart. The expression of a ubiquitous epidermal protein keratin was consistent in all the reference specimens, demonstrating that the observed changes in the SRP expression were not due to trivial differences such as preservation or staining artifacts.

In summary, we have shown that SRP profiling of the skin can efficiently identify wild cetaceans with a history of prolonged physiological stress. Full interpretation of SRP profiles as markers of cellular stress awaits characterization of SRP expression dynamics and kinetics, and rigorous evaluation of sampling variables.

High throughput SRP profiling of spotted dolphins

SRP profiling was applied to gain insight into the physiological impact of commercial tuna fishing on a population of wild spotted dolphins. In the eastern tropical Pacific, yellowfin tuna (*Thunnus albacares*) habitually swim below schools of pantropical spotted dolphins (*Stenella attenuata*), and tuna fishermen have used dolphins to catch tuna since the 1950s. By the late 1980s, incidental drowning in tuna nets depleted the northeastern offshore population of spotted dolphins to 27% of its original size³. Since then, a change in fishing gear facilitates the release of dolphins from nets. However, millions of spotted dolphins are still chased and captured during commercial

tuna fishing each year, and the impact of this involvement on the dolphin population remains unclear.

Characterization of SRP profiles in spotted dolphins. Skin specimens were obtained from 868 wild spotted dolphins with different levels of involvement in commercial tuna fishing. Dolphins that approached research vessels voluntarily were presumed to have a limited experience with the tuna fishing boats (bow-riders, n=202). In contrast, dolphins that were chased and captured during commercial tuna fishing in areas with frequent fishing operations, were presumed to show a range of impacts (historical fishery, n=424, and CHESS²⁶, n=242). Details of the sampling and the estimation of exposure to commercial tuna fishing are provided by Dizon et al²⁵. High throughput SRP profiling was performed using multi-specimen slides, a SRP antibody cocktail, and semi-automated CIA. The resulting SRP profiles were classified as normal or altered based on the quantity and the spatial distribution of the SRP expression in the epidermis (see Methods).

Among the 868 specimens, 231 had normal SRP expression profiles and 637 had altered profiles (Fig. 4). The normal SRP profiles resembled the baseline expression in the reference cetaceans (Fig. 4a). Most of the altered profiles (476/637=75%) had a uniform distribution of SRP staining within the epidermis, resembling the altered SRP profiles in the diseased reference cetaceans (“uniform profiles”, Fig. 4b). The other 161 altered profiles had a non-uniform distribution of SRP staining, with discrete bands of very high and very low SRP levels (“banded profiles”, Figs. 4 c-f). The banded profiles were preferentially found in spotted dolphins exposed to commercial tuna fishing (historical fishery 103/424=24%, CHESS 45/242=19%, bow-riders 13/202=6%). To model the dynamics, we considered that the banded profiles may contain a dynamic stress signature, similar to outgrowing tree rings, which may have been induced by isolated periods of fishing-related cellular stress. In contrast, the uniform profiles may have been induced by continual cellular stress associated with diseases or prolonged periods of frequent fishing-related stress. In a molecular model, the banded appearance of the SRP distribution in the epidermis could be explained as outgrowing layers of keratinocytes with suppressed or elevated SRP expression, which might have been induced during different phases of the stress response. Full interpretation of the SRP profiles observed in the spotted dolphins awaits elucidation of the SRP expression mechanism during stressful stimulation.

Altered SRP profiles were significantly more frequent in spotted dolphins sampled during the historical fishery or CHESS than in the bow-riders ($\chi^2=79$, $P<0.001$): 83% in the historical fishery (354/424), 86% in the CHESS (208/242) and 37% in the bow-riders (75/202). The increased frequency of the altered SRP profiles in the dolphins sampled during the historical fishery and CHESS may be due to the involvement in commercial tuna fishing, and/or potential effects of geographical, biological and sampling variables. The potential effects of gender and the geographical sampling location were excluded statistically²⁵. We concluded that the death per se did not affect SRP profiles because the frequency of altered profiles was similar between dolphins sampled after they died in nets (historical fishery, 83%) and dolphins sampled alive in

nets (CHESS, 86%). In addition, only minimal changes in the expression of the individual SRP were detected in healthy cetaceans that died due to subsistence hunting and accidental drowning (IDs 63-66, and 43, respectively, Fig. 6). However, we could not exclude the effects of the age, the reproductive status and the anatomical sampling site (the dorsum was sampled in bow-riders, the dorsum and the dorsal fin in CHESS, and the jaw in the historical fishery).

The specimens from the historical fishery were directly comparable (identically preserved jaw skin) and enabled us to further investigate the effects of commercial tuna fishing in the absence of the sampling site effect. In a blinded study, a significant positive correlation was documented between the frequency of altered SRP profiles, or the frequency of the banded SRP profiles, and our index of involvement in commercial tuna fishing (Mann-Whitney U test, $P < 0.001$). Detailed statistical analysis of the relationships is provided by Dizon et al²⁵. The increased proportion of altered SRP profiles was found only in dolphins exposed to repeated captures for at least 10 days (and up to 70 days) prior to sampling. Dolphins exposed to captures for < 10 days before sampling did not have significantly higher proportions of altered profiles. This empirical observation suggests that the increase in altered SRP profiles was induced by an accumulated impact of many tuna fishing operations, rather than immediately following one or a few captures. A significant correlation was also found between the type of the altered profile and the level of involvement in the fishery. The banded and the uniform profiles were significantly associated with lower and higher levels of involvement, respectively (Mann-Whitney U test, $P < 0.001$). These empirical results further support the models described above, whereby the banded SRP profiles might be driven by isolated stress events, and the uniform profiles, by continual stress.

SRP profiles in spotted dolphins recaptured during CHESS. 12 dolphins were captured twice or three times within 5 days during CHESS²⁶. In these recaptured dolphins the proportions of altered profiles were lower following the recaptures as compared to the first capture (altered/normal profiles: 1st capture 10/1; 2nd capture 7/4; 3rd capture 0/1). In four of the 12 repeatedly captured dolphins, altered profiles were detected following the first capture, and normal profiles during recaptures. This observation appeared to be in contrast with a conclusion based on the historical fishery study, suggesting that increased involvement in the fishery was associated with increased proportion of altered SRP profiles (see above). The contrasting results can be explained based on differences between the historical fishery and CHESS specimens. The differences may involve both the level and duration of stress due to recaptures, and also the type of stress, if the CHESS recaptures had a different impact than the commercial tuna fishery.

Regarding the differences in the level/duration of stress, the historical fishery specimens constituted two categories based on the relationship between altered SRP profiles and the involvement in the fishery (see above). Only the dolphins which were frequently involved in the fishery during > 10 days before sampling showed a relationship between the frequency of altered SRP profiles and the involvement in the fishery. The dolphins involved less frequently, for < 10 days, did not show the relationship. The

CHESS specimens, and the SRP profiling results, were similar to the latter category of the historical fishery specimens (a few captures in <10 days).

In a hypothetical model of the SRP expression dynamics, the two categories of specimens may represent two distinct phases of the molecular stress response. The low level/duration category (CHESS recaptures and the similar historical fishery specimens) may represent the initial phase of stress response, involving non-linear (even chaotic) molecular events, with suppressed or unpredictable alteration of the molecular expression. A transient suppression of the molecular stress response, induced by stressful stimuli repeated once a day for 6 days, was described previously, and was found to be mediated by a surge of corticosterone and heat shock proteins^{28,29}. A transient suppression of SRP expression may be manifested as a banded SRP profile, or as a normal-like profile, depending on whether the original profile was normal or uniformly altered. The high level/duration category (the other historical fishery specimens, no similar CHESS counterparts) may represent a later, mature phase of the stress response with quasi-linear dynamics. In the mature phase, stressful stimuli may drive a regularly increasing accumulation of increased SRP expression across the epidermis, manifested as a uniformly altered SRP profile. This hypothetical reconstruction of SRP expression dynamics is consistent with the empirical observations described above but requires experimental validation.

Considering the very limited number of animals (n=12), the potential effects of tagging (the recaptured CHESS animals were tagged with 3 kinds of tags, and no untagged controls were available for repeatedly captured animals), the potential difference between CHESS and commercial tuna fishing, and the complex, unknown dynamics of SRP expression, it may be premature to draw any firm conclusions from the CHESS recaptures.

SRP profiles in the skin from the dorsal fin and the dorsum of CHESS dolphins. Different proportions of altered SRP profiles were found in the skin of CHESS dolphins obtained by different sampling methods (sampling details in Forney et al.²⁶). Most of the CHESS dolphins (n=220) were sampled from the dorsal fin while handled by a researcher and had a high proportion of altered profiles (198/220=90%). A small group (n=22) was sampled using a long pole (dorsal skin biopsies, n=22), and had a lower proportion of altered profiles (10/22=45%).

The high proportion of altered profiles in the dorsal fin skin may be associated with prolonged exposure to stress, or with the constitutive biochemical properties of the fin skin. The proportion of altered SRP profiles in the fin skin was similar to the jaw skin sampled in the historical fishery (90% and 83%, respectively). In the jaw skin, the frequency of altered SRP profiles was not invariable, as expected for a constitutive property. Rather, we found significant differences associated with exposure to the fishery (see above). Similarly, the frequency of altered SRP profiles in the dorsal fin skin may be stress-related rather than constitutive.

In the CHESS specimens obtained by the manual biopsy (n=220), the proportion of altered SRP profiles produced by previous exposures to stress might have been changed during the sampling procedure, due to direct handling of the fin skin, and

systemic stress due to a close contact with humans. Spotted dolphins are known to react anxiously when handled, and the first CHESS capture was most likely the first time these dolphins were touched by humans. Direct handling of the skin during massage in humans resulted in a transient display of altered SRP profiles within 30 minutes (S.O.S. and V. Morhenn, unpublished results).

In the CHESS dolphins sampled by the pole biopsy (n=22), the proportion of altered SRP profiles may have reflected an unidentified previous exposure to stress, that might (transiently) suppressed altered SRP profiles (the effect of isolated stress events, see above). Noticeably, the 22 dolphins were captured together²⁶, indicating a common history that might have affected their SRP profiles.

Any possibility of variability in the SRP profiles relating to anatomical sampling sites, handling and previous stress history will require systematic evaluation in future experiments, using precisely defined skin biopsies.

In summary, SRP profiling was applicable to analysis of physiological stress in a large sample of wild dolphins exposed to commercial tuna fishing activities. High proportions of altered SRP profiles were found in spotted dolphins sampled in the eastern tropical Pacific during 1988-91 and 2001, in areas with frequent commercial tuna fishing. Those results indicate that long-term systemic cellular stress was present in large numbers of spotted dolphins. Based on analogy with humans and other mammals, prolonged systemic cellular stress could have affected the health and the reproduction fitness of individual spotted dolphins, and triggered incipient population level effects. The mechanism whereby frequent involvement in commercial tuna fishing could induce systemic cellular stress in spotted dolphins is currently unknown. It was shown that cellular stress can be triggered by involuntary social disorganization²⁹, which happens during commercial tuna fishing (mother-calf separation³⁰), and by tissue injury during a chase³¹. Full interpretation of SRP profiling results in spotted dolphins awaits characterization of SRP expression mechanism during repeated stress, rigorous evaluation of skin sampling variables, and elucidation of the relationship between altered SRP profiles and health status in the wild spotted dolphins. These questions need to be resolved in further study.

CONCLUSIONS

The high throughput SRP profiling technique described in this study is a new, effective and practical tool for detecting prolonged systemic cellular stress in wild cetaceans. We showed that SRP are expressed widely in the epidermis of the normal skin, and their expression becomes altered due to prolonged physiological stress. Changes in the cutaneous SRP expression can be triggered rapidly (S.O.S. unpublished data), and can persist and accumulate for 10 weeks during continual stress²⁵. The general pattern of the stress-associated changes in SRP expression is highly conserved in mammals, and can be visualized by antibody staining of routinely preserved skin and other tissues (S.O.S. unpublished data). SRP are highly conserved in most vertebrates, and the anti-SRP antibodies identified here were successfully used in other species including elephants and birds (S.O.S. unpublished data), suggesting broad applicability of SRP profiling.

Repeated sampling of the skin could allow long-term monitoring of SRP profiles. Conceptually analogous SRP panels could be designed for most organisms and used for ecosystem-wide studies.

To the best of our knowledge, this is the first report of a large-scale survey of cellular stress in wild, free-ranging cetaceans, and the first report of cellular stress associated with the involvement of spotted dolphins in commercial tuna fishing. The integration of high throughput SRP profiling and linked ecological data has the potential to provide an early warning of a relationship between ecological variables and incipient individual health changes. In medicine, SRP profiling could provide better understanding of diseases that involve cellular stress such as cancer, stroke and AIDS.

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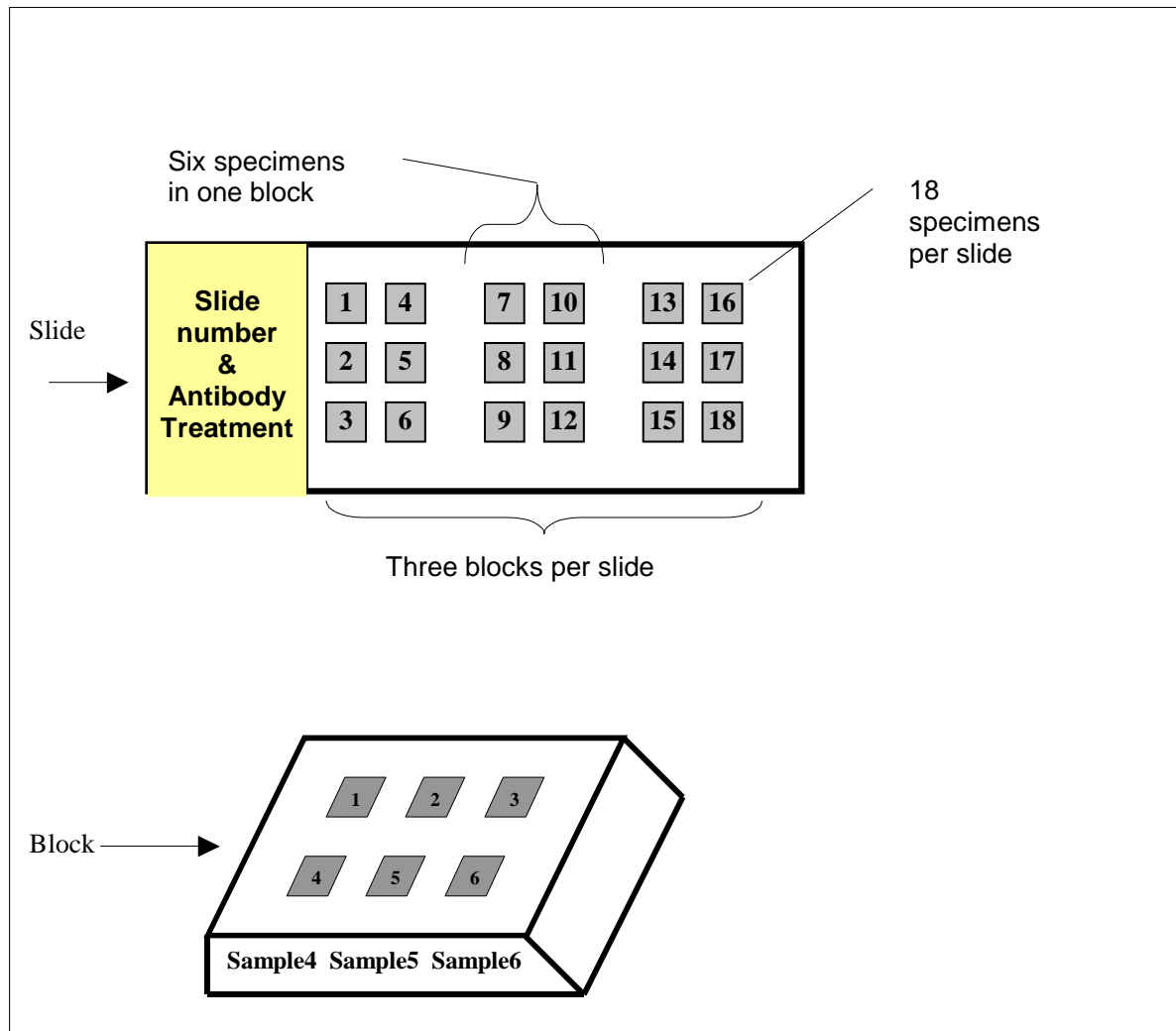


Fig. 1 Diagram of a multi-specimen slide and block. Slides with multiple specimens were prepared using custom-made paraffin blocks with 6 different skin fragments. 25 slides were used in one staining experiment, providing 450 highly comparable staining results. The use of the multi-specimen slides facilitated precise quantitative comparisons between SRP expression profiles in individual specimens, and provided basis for high throughput SRP profiling in large sets of specimens.

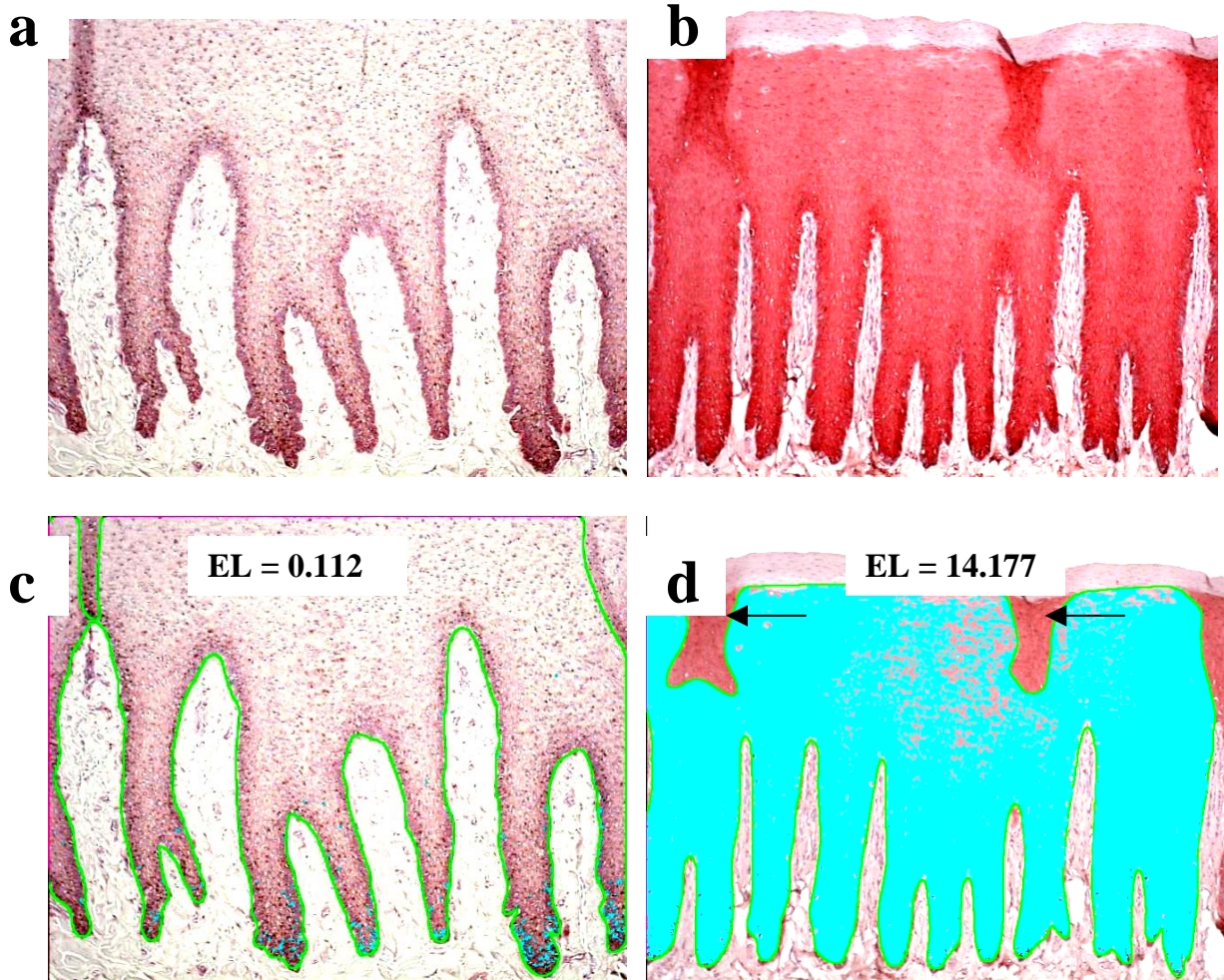


Fig. 2. Quantification of SRP expression levels using computerized image analysis (CIA). SRP expression levels were visualized as a red pigment using immunohistochemical staining with a SRP antibody cocktail. The accumulation of the red pigment was quantified using CIA. Precise selection of the stained area (the PA value) was achieved using the free-form outlining tool (green outlines in c, d). The outlining was also used to exclude processing artifacts (arrows in d). Precise selection of the red color range, used for computing the mean optical density (the MOD value), was performed by empirical optimization, using the eyedropper tool. The selected range of red color is visualized here as an artificial blue color co-localized with the red pigment (c, d). SRP expression levels (EL) were computed as $EL = MOD \times PA$. $EL=0.112$ in (c), and $EL=14.177$ in (d). Original magnifications: x100.

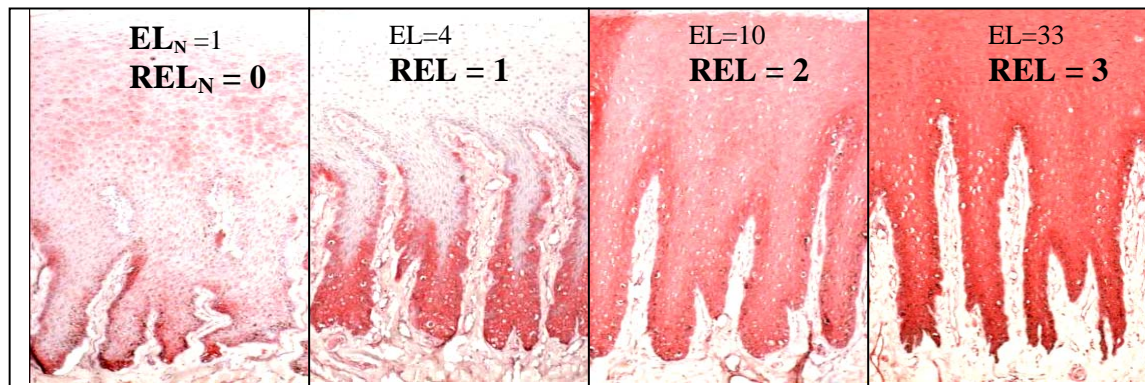


Fig. 3. Relative expression level scale. Relative expression levels (REL) were based on the staining intensity values (EL) quantified by CIA. REL values were computed as a log3-transformed ratio of EL and the mean EL in the pool of normal specimens (here illustrated as EL_N). $REL = EL/EL_N$. Original magnifications x100.

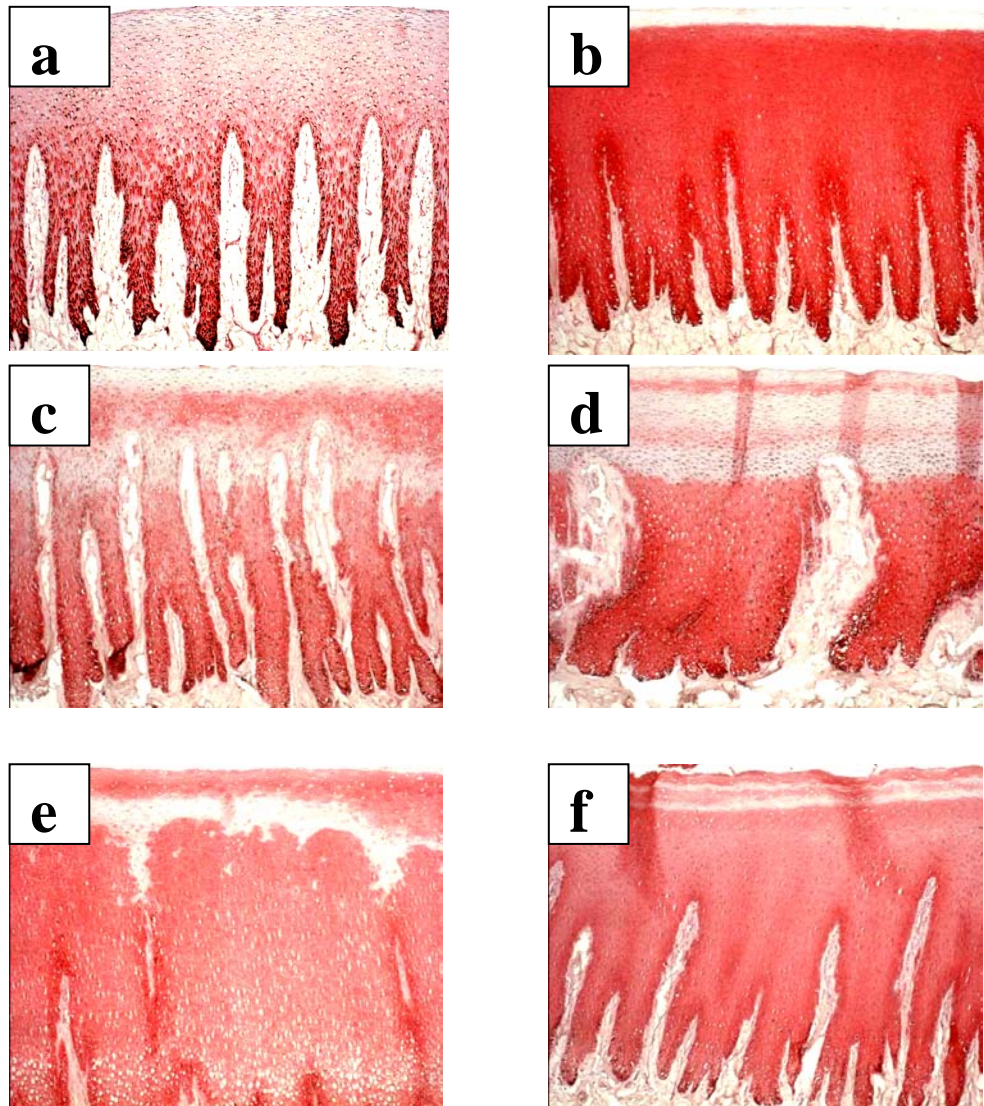


Fig. 4 SRP expression profiles in a large sample of spotted dolphins. SRP profiles were visualized using a SRP antibody cocktail and classified as normal or altered. Normal profiles (a) closely resembled SRP expression in the normal reference cetaceans. Altered profiles (b-f) were more complex than in the reference specimens. The most common altered profile had a uniform distribution of SRP across the epidermis (b), and resembled closely the altered SRP profiles in the diseased reference cetaceans. Novel types of altered profiles, not noted in the reference specimens, had a "banded" appearance due to vertical layers of high and low SRP staining (c-f). Original magnification x100.

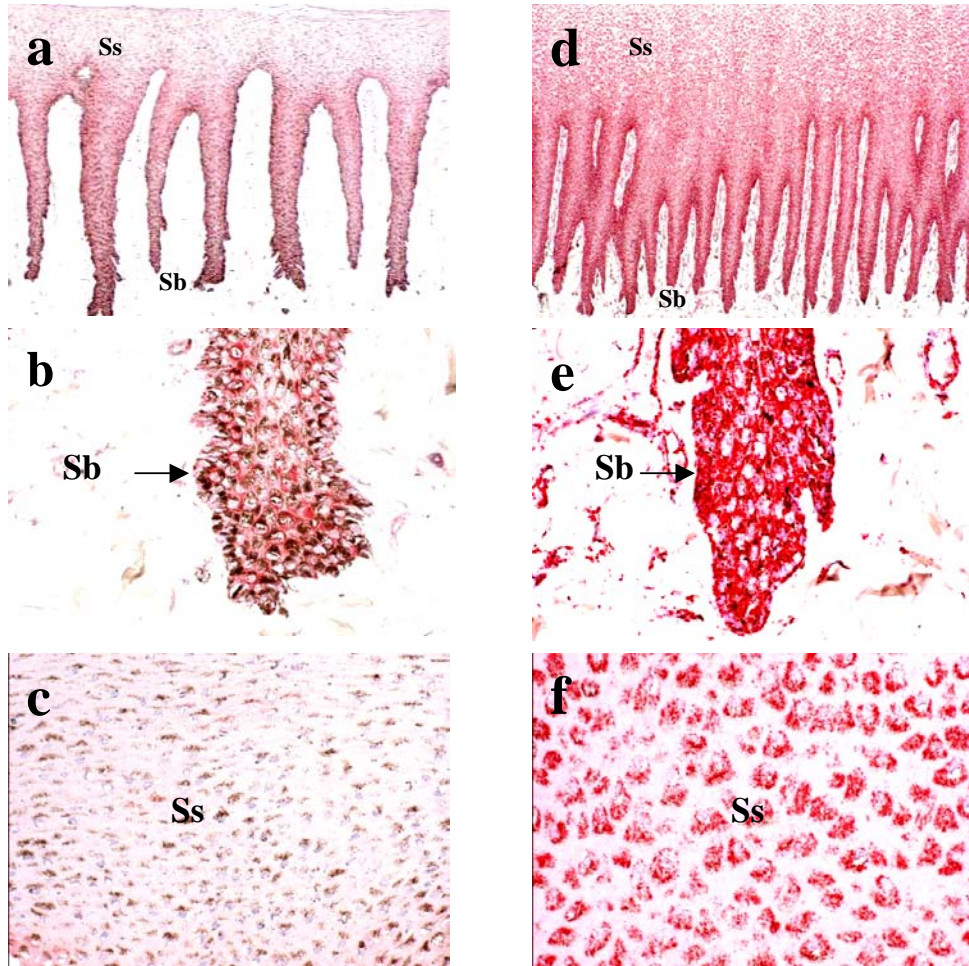


Figure 5 SOD Mn expression in the dolphin skin. SOD Mn was visualized as a red pigment using immunohistochemical staining with a rabbit antibody to human SOD Mn. In a normal bottlenose dolphin (ID# 2), SOD Mn expression was localized to the epidermis, predominantly to the basal and suprabasal keratinocytes (*Stratum basale*, Sb, a, b). SOD Mn was not detected in the mature keratinocytes (*Stratum spinosum*, Ss, c). In a diseased bottlenose dolphin (ID#45), SOD Mn expression remained localized to the epidermis (d) and the expression level was increased both in the immature (e) and the mature keratinocytes (f). Original magnifications: x40 (a, d) and x400 (b, c, e, f).

Homeostatic process	Stress Response Protein	Abbreviation
Oxidative stress repair, cellular detoxification	Superoxide dismutase Mn	SOD Mn
	Superoxide dismutase Cu/Zn	SOD Cu/Zn
	Heat shock transcription factor HSF-1	HSF-1
	Heat shock protein 25	HSP25
	Heat shock protein 40	HSP40
	Heat shock protein 60	HSP60
	Heat shock protein 90	HSP90
	Heme oxygenase-1	HO-1
	Glucose regulated protein Grp58	Grp58
	Glucose regulated protein Grp75	Grp75
	Metallothionein	MT
	Ferritin	Ferritin
	Cytochrome P 450 IIE1	CYP450E
	NADPH-cytochrome P 450 reductase	Cyp450 red
	Glutathione S-Transferase π	GST π
Cell growth control	Cyclin D1	Cyclin D1
	Proto-oncogene c-fos	c-fos
	Proto-oncogene c-jun	c-jun
	Mitogen activated MAP kinase Mek-1	Mek
	Stress-activated MAP kinase Mek-1	Mekk
	Glucocorticoid receptor	GR
	Epidermal growth factor receptor	EGFR
	Leptin receptor	Leptin R
	Transforming growth factors β -1,2,3	TGF- β
	Cyclooxygenase 2	Cox-2
Apoptosis	Caspase 8	Caspase 8
	Cytoplasmic cytochrome c	Cyt c-ID3
	Tumor suppressor p53	p53
Immunological signaling	Interleukin IL-1 β	IL-1 β
	Interleukin IL-6	IL-6
	Interleukin IL-8	IL-8
	Interleukin IL-10	IL-10
	Interleukin IL-12	IL-12
	Nitric oxide synthase II, inducible	iNOS
Neuroendocrine regulation	β -endorphin	β -endorphin
	Substance P	Substance P
	Serotonin	Serotonin
	Serotonin receptor	SerR
	Vasoactive intestinal peptide	VIP
Adhesion	Laminin	Laminin

Table 1. Panel of 40 stress response proteins (SRP). The functional categorization of SRP shown here is simplified. Most SRP are pleiotropic and can participate in multiple homeostatic processes.

Classification	Specimens										
	Species	Health history related to physiological stress	Gender	Age	Lab Code	Sampling Date	Preservation	Provider	ID #		
NORMAL	bottlenose dolphin <i>T. truncatus</i>	Free-ranging wild dolphins, Sarasota Bay, Gulf of Mexico. These dolphins were captured and restrained for blood and skin sampling aboard a small research vessel (1-2hr procedure), before release. The blood sample analysis indicated that all the dolphins w		F	A	FB9	9/6/98	frozen, formalin		1	
				lactating	F	A	FB11			1/6/98	2
					F	A	FB54			2/6/98	3
					M	A	FB58			5/6/98	4
					F	A	FB63			9/6/98	5
				lactating	F	A	FB90			2/6/98	6
				calf of FB90	F	C	FB113			2/6/98	7
				lactating	F	A	FB115			4/6/98	8
					F	A	FB117			9/6/98	9
				calf of FB11	M	C	FB146			1/6/98	10
				calf of FB163 pregnant,	M	C	FB152			4/6/98	11
				lactating	F	A	FB163			4/6/98	12
				calf of FB115	M	C	FB166			4/6/98	13
					M	A	FB174			5/6/98	14
					M	J	FB176			8/6/98	15
					M	J	FB178			9/6/98	16
	pantropical spotted dolphin <i>S. attenuata</i>	Wild spotted dolphins, Eastern Tropical Pacific. The dolphins were sampled by projectile biopsy while bowriding close to a reasearch vessel in eastern tropical Pacific.	Unknown			Z1260	1992	DMSO, formalin		17	
						Z1262	1992			18	
						Z1263	1992			19	
						Z11379	1998	frozen, formalin		20	
						Z11380	1998			21	
						Z11381	1998			22	
	beluga <i>D. leucas</i>	Wild belugas, projectile biopsy, NW Territories, Canada.	M	J	Z11045	1998	DMSO, formalin	SWFSC	23		
			M	J	Z11046	1998			24		
			M	J	Z11047	1998			25		
			F	A	Z11048	1998			26		
			F	A	Z11049	1998			27		
			F	A	Z11050	1998			28		
				F	A	Z11014	1998		29		
		A wild beluga, tagging biopsy, Norway.	M	A	DL99/7	1999	frozen, formalin		30		
		Wild belugas, biopsy/satellite tagging, Cook Inlet, Alaska. The animals were restrained before sampling.	U	J	Z17296	1998	DMSO, formalin		31		
	M		A	Z17297	2000	formalin	32				
	northern right whale <i>E. glacialis</i>	A wild North Atlantic right whale, projectile biopsy, Bay of Fundy.	F	A	Z15112	2000	DMSO, formalin		33		
	gray whale <i>E. robustus</i>	Wild gray whales, projectile biopsy, Eastern Tropical Pacific.	U	U	Z3950	1995			34		
			M		Z3951	1995			35		
			F		Z3949	1995			36		
	short-finned pilot whale <i>G. macrorhynchus</i>	A wild pilot whale, projectile biopsy, Hawaii.	Unknown		Z11480	1998	formalin		37		
	human	healthy volunteer	M	A	NS-1	1996			38		
		healthy volunteer	F	A	NS-2	1998			39		
benign nevus, uninvolved skin		M	A	NS-3	1999	40					

Table 2a. Reference specimens classified as normal. SWFSC: Southwest Fisheries Science Center. DMSO, formalin: stored in DMSO, transferred to formalin. Frozen, formalin: frozen at -80 C, transferred to formalin. DMSO: NaCl saturated with 20% dimethyl sulphoxide.

Class-ification	Specimens												
	Species	Health history related to physiological stress			Gender	Age	Lab Code	Preservation	Provider	Sampling Date	ID #		
		Known diseased	Health problems	Comments									
STRESSED	bottlenose dolphin <i>T. truncatus</i>		Drowning	Semi-domesticated bottlenose dolphins under veterinary care. Necropsy specimens.	M	A	Tt651	formalin	SPAWARs	8/30/95	43		
		YES	Scoliosis and dermatitis		M	A	Tt773			4/28/99	44		
		YES	Pneumonia and old age		F	A	Tg735			1/2/93	45		
		YES	Disseminated histoplasmosis		F	A	Tt001			8/7/95	46		
		YES	Suppurative peritonitis		M	A	Tt775			2/3/99	47		
		YES	Sudden idiopathic calf death		M	C	Tx615c			8/7/99	48		
	YES	F		C	Tt783	12/20/93	49						
	common dolphin <i>D. delphis</i>	YES	Stranded wild common dolphins, high morbillivirus titres; Southern California.			U	A	Z91783	DMSO, formalin		1995	50	
					A	Z91854	1995	51					
					A	Z91963	1996	52					
	pantropical spotted dolphin <i>S. attenuata</i>		Wild spotted dolphins found drowned in nets during purse-seine tuna fishery, eastern tropical Pacific (ETP). The dolphins may have been repeatedly captured in tuna fishing nets before		M	C	Z751	formalin		1992	53		
					F	C	RAB005			1991	54		
				pregnant, lactating	F	A	DAM0019			1991	55		
				pregnant, lactating	F	A	BXR0624			1991	56		
					F	A	BXR0628			1991	57		
					F	A	BXR0633			1991	58		
			pregnant	F	A	BXR0650	1991	59					
			Multiple chases and captures during CHESs, drowned in a net during the last capture.	lactating	F	A	Z 25176	frozen, formalin		Oct-01	60		
		YES	Live stranding, shark bites, died 4 months later in a rehabilitation center, Long Key, Florida.		F	A	Z 4539	DMSO, formalin		12/27/94	61		
		YES	Live stranding, diseased, died next day in a rehabilitation center, Quintana Beach, Texas.		F	A	Z 5092			10/12/95	62		
	beluga <i>D. leucas</i>		Wild, apparently healthy belugas killed in an aboriginal subsistence hunt (5-30 min chase), Canada		F	A	Z4104	frozen, formalin	SWFSC	1989	63		
					F	J	Z4105			1989	64		
					M	A	Z4106			1990	65		
					F	C	Z1599			1993	66		
					F	A	Z6557			1996	67		
					F	C	Z6543			1996	68		
					F	A	Z6552			1996	69		
					M	A	Z6556			1996	70		
					F	C	Z6550			1996	71		
					F	A	Z6555			1996	72		
					F	A	Z6544			1996	73		
					M	A	Z6546			1996	74		
		A pod of wild belugas was cut off by the rapid formation of new ice during their summer migration at Husky Lake, North West Territories, Canada. The belugas were trapped in a progressively closing ice hole (<i>savssat</i>) for over 4 weeks. All the animals were severely emaciated and their skin was discolored. They were all killed together by aboriginal hunters and skin samples were collected.		F	A	Z6547	1996		75				
				M	J	Z6548	1996		76				
				M	A	Z6541	1996		77				
				F	J	Z6553	1996		78				
				F	A	Z6554	1996		79				
				F	A	Z6545	1996		80				
				M	C	Z6549	1996		81				
			YES	A semi-domesticated beluga, chronic pleuritis and fungal infection. Acute fungal lesion biopsy.	F	A	DI99425		formalin	Mystic	1998	82	
			YES	A semi-domesticated beluga, fungal encephalitis. Necropsy specimen.	M	A	DI576			SPAWARs	1999	83	
			northern right whale <i>E. glacialis</i>	YES	Wild NARW, shipstrike trauma, found dead a week later, severely emaciated and infected. Welfleet, Massachusetts.	F	A		Z13086	DMSO, formalin	SWFSC	1999	84
		YES		A wild NARW, dubbed Churchill, was observed entangled in a fishing line, emaciated, with infected wound, near Boston, in June 2001. Biopsied twice during rescue efforts in July. Found dead, discolored, severely emaciated, systemically infected, in September.	U	A	Eg1102-1		frozen, formalin	7/10/01		85	
		YES					Eg1102-2			7/14/01		86	
		gray whale <i>E. robustus</i>	YES	Severe emaciation and encephalitis.	Wild gray whales, live strandings, San Francisco. Necropsy specimens.	M	J		ErC91	formalin		5/11/99	87
			YES	Severe emaciation and gut infection.		M	J		ErC94			6/26/99	88
	short-finned pilot whale <i>G. macrorhynchus</i>	YES	Wild pilot whales, North Atlantic, live strandings followed by rehabilitation in captivity, acute dolphin pox infection. Acute infected lesion biopsy.		M	J	SYGM9925	frozen, formalin	Mystic	7/20/99	89		
		YES			M	J	SYGM9926			7/20/99	90		
	human	YES	Psoriasis, acute lesion biopsy.		F	A	98-1453	formalin	SWFSC	1998	91		
		YES		F	A	99-1712	1999			92			
		YES	HTLV-I associated chronic ATLL leukemia with skin lesions, skin tumor biopsy.	M	A	4538	1999			93			

Table 2b. Reference specimens classified as stressed. DMSO, formalin, frozen, SWFSC: see Table 2a. SPAWARS: US Navy Marine Mammal Program, 49620 Beluga Rd. San Diego, CA, USA. Mystic: Mystic Aquarium, Mystic, CT, USA

Table 3. Antibodies for immunohistochemical visualization of stress response proteins (SRP).

SRP	Antibody			Optimized dilutions	
	Description	Concentration	Provider	Single antibody	Antibody cocktail for spotted dolphins
β–endorphin	Rabbit antiserum to sythetic based on rat β–endorphin	Whole Serum	E1520, Sigma	1:2000	1:75,000
Caspase 8	Rabbit antiserum to human caspase-8	Whole Serum	AAP-118, Stressgen	1:4000	1:270,000
Cyclin D1	Mouse monoclonal IgG to human cyclin D1	1.0 mg/ml	KAM-CC200, Stressgen	1:600	1:36,000
Cytochrome P 450 IIE1	Rabbit antiserum to rat liver CYP450E	Whole Serum	MFO-100, Stressgen	1:2000	1:150,000
Cytokeratin	Rabbit antiserum to human skin keratin, purified	Whole Serum	YMPS33, Accurate	1: 3000	not included
Cytoplasmic cytochrome c, 1D3 antigen	Mouse monoclonal IgG1 to bovine extramitochondrial cytochrome c ^a	0.775 mg/ml	Gift, Dr. R Jemmerson, U. of Minnesota	1:1500	1:27,000
Epidermal growth factor receptor	Rabbit polyclonal IgG to human epidermal growth factor receptor, purified	Whole Serum	PU335-UP, Biogenex	1:400	1:18,000
Ferritin	Rabbit polyclonal IgG to human liver ferritin, purified	2.3mg/ml	A0133, Dako	1:2000	1:18,000
Glucocorticoid receptor	Rabbit polyclonal IgG to mouse glucocorticoid receptor alpha, purified	0.2mg/ml	SC-1004, Santa Cruz Biotechnology	1:1500	1:36,000
Glucose regulated protein 58	Rabbit antiserum to rat liver Grp58	Whole Serum	SPA-580, Stressgen	1:1000	1:60,000
Glucose regulated protein 75	Mouse monoclonal IgG to human Grp75	Ascites	SPA-825, Stressgen	1:800	1:36,000
Glutathione S-Transferase π	Rabbit polyclonal IgG to human glutathione S-Transferase ^a , purified	1.03mg/ml	A3600, Dako	1:400	1:27,000
Heat shock protein 25	Rabbit antiserum to mouse heat shock protein 25	Whole Serum	SPA-801, Stressgen	1:2000	1:60,000
Heat shock protein 40	Rabbit antiserum to human heat shock protein 40	Whole Serum	SPA-400, Stressgen	1:6000	1:240,000
Heat shock protein 60	Rabbit antiserum to <i>Synechococcus sp.</i> heat shock protein 60	Whole Serum	SPA-804, Stressgen	1:1000	1:75,000
Heat shock protein 90	Mouse monoclonal IgG to <i>Achlya ambisexualis</i> heat shock protein 90	1.0 mg/ml	SPA-830 , Stressgen	1:600	1:24,000
Heat shock transcription factor HSF-1	Rabbit antiserum to human HSF1	Whole Serum	SPA-901, Stressgen	1:2000	1:120,000
Heme oxygenase-1	Rabbit antiserum to rat heme oxygenase-1	Whole Serum	SPA-895, Stressgen	1:1500	1:48,000
Interleukin IL-1β	Rabbit polyclonal IgG to human IL-1β, purified ^a	0.2mg/ml	SC-7884, Santa Cruz Biotechnology	1:500	1:27,000
Interleukin IL-6	Rabbit polyclonal IgG to human IL-6, purified	0.2mg/ml	SC-7920, Santa Cruz Biotechnology	1:800	1:27,000
Interleukin IL-8	Rabbit polyclonal IgG to human IL-8, purified	0.2mg/ml	SC-7922, Santa Cruz Biotechnology	1:600	1:27,000
Interleukin IL-10	Rabbit polyclonal IgG to human IL-10, purified	0.2mg/ml	SC-7888, Santa Cruz Biotechnology	1:300	1:18,000
Interleukin IL-12	Rabbit polyclonal IgG to human IL-12, purified	0.2mg/ml	SC-7926, Santa Cruz Biotechnology	1:300	1:27,000
Laminin	Rabbit antiserum to mouse laminin	Whole Serum	PU078-UP, Biogenex	1:25	1:1,500
Leptin receptor, ObR	Mouse monoclonal IgG to mouse leptin receptor	0.2mg/ml	SC-8391, Santa Cruz Biotechnology	1:5	1:600
Metallothionein, MT	Mouse monoclonal IgG to horse self-polymerized MT-1 and MT-2	0.1mg/ml	M0639, Dako	1:4000	1:36,000
MAP kinase Mekk-1	Rabbit polyclonal IgG to synthetic peptide based on mouse Mekk-1, purified	1.0 mg/ml	KAP-SA001E, Stressgen	1:500	1:36,000
MAP kinase Mek-1	Rabbit polyclonal IgG to synthetic peptide based on rat Mek-1, purified	1.0 mg/ml	KAP-MA010C, Stressgen	1:500	1:36,000
NADPH-cytochrome P 450 reductase	Rabbit antiserum to rat liver NADPH-cytochrome P 450 reductase	Whole Serum	OSA-300, Stressgen	1:1000	1:48,000
Nitric oxide synthase II, inducible	Rabbit polyclonal to mouse iNOS, purified	0.5mg/ml	KAP-NO001C, Stressgen	1:300	1:18,000
Prostaglandin H synthase Cox-2	Rabbit polyclonal IgG to human Cox-2, purified	0.2mg/ml	SC-7951, Santa Cruz Biotechnology	1:300	1:18,000
Proto-oncogene c-fos	Rabbit polyclonal IgG to synthetic peptide based on human c-fos, purified	10-20mg/ml	PU348-UP, Biogenex	1:300	1:18,000
Proto-oncogene c-jun	Rabbit polyclonal IgG to synthetic peptide based on human c-jun, purified	10mg/ml	PU349-UP, Biogenex	1:25	1:1,500
Serotonin receptor	Rabbit polyclonal IgG to human serotonin receptor, purified	0.2mg/ml	SC-10802, Santa Cruz Biotechnology	1:400	1:18,000
Serotonin	Rabbit antiserum to serotonin	Whole Serum	PU068-UP, Biogenex	1:150	1:9,000
Substance P	Rabbit antiserum to synthetic substance P	Whole Serum	PU069-UP, Biogenex	1:300	1:27,000
Superoxide dismutase Cu/Zn	Rabbit polyclonal IgG to human SOD Cu/Zn, purified	1.52mg/ml	SOD100, Stressgen	1:3000	1:120,000
Superoxide dismutase Mn	Rabbit polyclonal IgG to human SOD Mn, purified	1.0 mg/ml	SOD110, Stressgen	1:10000	1:75,000
Transforming growth factorβ-1,2,3	Rabbit polyclonal to human Transforming growth factorβ-3, purified	0.2mg/ml	SC-7892, Santa Cruz Biotechnology	1:500	1:24,000
Tumor suppressor p53	Mouse monoclonal IgG to human p53	0.5mg/ml	KAM-CC002, Stressgen	1:800	1:36,000
Vasoactive intestinal peptide	Rabbit antiserum to synthetic vasoactive intestinal peptide	Whole Serum	PU044P-UP, Biogenex	1:1000	1:120,000

^a Biochemistry 38: 3599 (1999); Accurate, Westbury, NY, USA; BioGenex, San Ramon, CA, USA; Dako, Carpinteria, CA, USA; Santa Cruz Biotechnology, Santa Cruz, CA, USA; Sigma, St. Louis, MO, USA; StressGen, Victoria, BC, Canada.

APPENDIX - REVIEW RESPONSE

S. Southern, Ph.D.
April 25, 2002

Five reviewers (selected by an independent organization) examined this document (CIE-S08, January 24, 02) and submitted independent reports. The current version (version April 26, 02) attempts to address the concerns the reviewers directed to the new method for molecular analysis of stress. These concerns focused on specific details in two categories: methodological validation of SRP profiling, and validation of the interpretation of SRP profiles. The current version has been edited to acknowledge this, to explain several technical steps more clearly and to discuss the interpretative limitations. In addition, the reviewers' comments, and my responses are summarized below.

Methodological validation of SRP profiling

Comments

R. Ortiz

- 1/ General methodological validation of SRP profiling.
- 2/ Validation of the cross-reactivity of anti-SRP antibodies with dolphin antigens.

D. Martineau

- 1/ Validity of reference samples: different preservation, different species, different body sites. Suggested improvement: biopsies before and after stress from the same animal/species.
- 2/ The authors decided to measure gene expression at the protein level instead of the RNA level. To justify the choice they rationalize that the poor quality of dolphin skin RNA would not allow interpretable results.
- 3/ The effect of sample preservation. *Luckily* the reference specimens gave consistent results despite the different methods used for their preservation.
- 4/ The use of antibody cocktail.
- 5/ Optimization of the antibody concentrations.
- 6/ The cross-reactivity of anti-SRP antibodies with dolphin antigens.

G.D. Bossart

- 1/ The effects of skin fixation-associated variables on the accuracy and validity of the immunohistochemical staining.
- 2/ General applicability of the staining technique for all 40 SRP antibodies, in particular antigen retrieval and reactivity with formalin-fixed tissues.
- 3/ General applicability of a standard image analysis protocol for measuring the immunohistochemical signal of SRP with different subcellular localization.
- 4/ The use of antibody cocktail: potential effects of cross-binding among the individual antibodies?
- 5/ To validate the technical parameters of SRP profiling, it was suggested to develop a laboratory animal model permitting experimental manipulation of critical variables.

S. DeGuisse

- 1/ Using the reference study to both define and validate the SRP stress markers.
- 2/ Validation of the cross-reactivity of anti-SRP antibodies with dolphin antigens.
- 3/ The use of antibody cocktail.
- 4/ Reproducibility of the subjective classification of SRP expression in the spotted dolphins.

Dr. J. Mann

- 1/ Classification of SRP profiles in the large sample of spotted dolphins into normal/alterd, rather than using a continuous scale.

Responses

Definition of the 40 stress response proteins (SRP) as stress markers.

Analysis of proteins rather than mRNA, was chosen not only because we had to analyze preserved skin which did not have suitable RNA. Proteins, not RNA messages, are directly involved in the molecular pathways of the stress response process, and therefore proteins are more suitable proxy molecules for measuring physiological stress responses than mRNAs.

The reference study was not used to define the SRP markers. Rather, the particular 40 SRP proteins were identified based on a review of medical literature. The SRPs were defined in separate, published studies, as proteins with known and well conserved functions in physiological stress responses of humans and other mammals (and other species, in many cases), as summarized in Table 3. Furthermore, all the SRPs were shown to be expressed in mammalian epidermal keratinocytes in previously published studies.

Validity of the reference specimen set.

The reference set of specimens was assembled with a goal to identify among the candidate SRP those that could discriminate between healthy and diseased cetaceans. In order to design a SRP panel constituting a broadly applicable generic stress marker, the discrimination would optimally be independent on the type of the stress, and the type of mammalian skin sample (species, gender, age, skin sampling site, and the initial preservation by freezing, formalin or DMSO). Therefore the reference specimens were chosen from several cetacean species and humans, both genders, different ages. In most cases, the precise skin sampling locations were not known, and were supposed to represent random sites on the dorsum and the head. We chose individuals exposed to a variety of physiological and pathological conditions in order to allow the identification of common, generic differences in the SRP expression pattern between the normal and stressed groups.

It would have been optimal to have reference specimens from individuals biopsied at multiple standardized sites before and after stress, stored/fixed in a standard way. However, the scope of the study precluded obtaining such specimens. Instead, the study had to rely on gifts of differently fixed specimens obtained by opportunistic sampling.

Preliminary results in humans, elephants and birds, with standardized specimens of skin and other tissues confirmed that the SRP panel can discriminate between healthy and diseased individuals (chronic infections, cancer).

The effect of specimen preservation and fixation.

The anti-SRP antibodies chosen for SRP profiling were known to react optimally with formalin-fixed tissues. To assure consistent immunohistochemical reactivity with the antibodies, all specimens were chemically standardized before histological processing. Specimens initially stored in DMSO, formalin or frozen were transferred to fresh 10% normal buffered formalin, and fixed for at least 24 hrs before embedding in the paraffin blocks.

Complex “banded” patterns of the altered SRP expression in spotted dolphins: processing artefacts?

The hypothesis that the “banded” patterns of SRP expression, observed in the large sample of the spotted dolphins, are artefacts cannot be accepted at this time because of the following empirical observations. (1) Non-random distribution of the banded patterns: The normal, constitutive patterns of the SRP expression were not affected. Only the altered SRP profiles were more complex: ie “discontinuously altered/banded” in addition to “continuously altered”. (2) The same four types of the “banded” SRP profiles were observed in two very different groups of specimens, regarding processing/preservation: formalin-preserved jaws, and frozen biopsies post-fixed in formalin. (3) The banded profiles were consistently observed with different markers, and at distant sections. (4) Significant correlation between the frequency of the banded patterns and the history of the exposure to the fishing operations both in the jaw section specimens, and in the biopsy specimens.

Specific detection of cetacean antigens using cross-reactive anti-SRP antibodies.

The anti-SRP antibodies used in our study (Table 2) were well-characterized, commercially available reagents identified based on a review of technical literature. We selected such antibodies that were

designed to recognize highly conserved SRP epitopes, as documented by actually observed, published specific cross-reactivity against homologous SRP antigens in different tissues of humans and mice (and other species, in many cases). When possible, we have chosen rabbit polyclonal antibodies that recognized multiple epitopes, rather than monoclonal antibodies, to further increase the probability of specific cross-reactivity with cetacean proteins. The antibody design and the empirically validated pan-mammalian cross-reactivity of these antibodies strongly suggested that a homologous protein would be recognized in other mammalian species, including cetaceans.

To eliminate non-specific cross-reactivity in our experiments, the concentrations of the individual antibodies were optimized by multi-step titrations using skin specimens from the 8 reference species. To control for non-specific staining we used negative control antibodies: commercial non-immune rabbit sera and epitope-matched mouse IgGs.

Detailed molecular identification of the cross-reactive antigens was not carried out for several reasons. (1) Within a scope of this study, knowledge of the precise molecular identity of the cetacean antigens was not required. It was sufficient to establish empirically that a cross-reacting cetacean antigen was useful for discriminating between healthy and diseased individuals. (2) Precise molecular characterization of the cross-reactive cetacean antigens was not technically possible with the majority of the reference specimens because formalin and DMSO fixed tissues were not suitable for W. blotting and cDNA analysis. (3) The limited scope of this study precluded the development and use of cloned cultured cetacean cells as an alternative route to characterize the SRP antigens in cetaceans.

Optimization of antibody concentrations and the use of control antibodies.

The concentrations of all the individual antibodies were optimized using multi-step titrations on positive cetacean and human tissues (Table 2).

To control for non-specific staining we used negative control antibodies (commercial non-immune rabbit sera and epitope-matched mouse IgGs).

To assess tissue preservation, the expression of keratin (ubiquitous keratinocyte protein) was visualized in all specimens, and was determined to be consistent. In following experiments, visualization of keratin and SOD Mn, an easily detectable SRP, were used as positive control staining.

The poly-SRP antibody cocktail.

The goal of the mandated study was a high throughput molecular analysis of stress in a large number of specimens. To compress the staining procedure, and still retain the broad functional potential of multiple stress markers, we implemented a cocktail of all the individual antibodies. The use of cocktail antibodies requires a careful design and empirical validation of specific reactivity. Cocktail antibodies are used routinely in medical diagnostics, for example for rapid identification of HIV-1, 2 positive blood in the VA Hospital in San Diego (Abbott Diagnostics HIV-1,2 kit: see a letter from the director of the VA Blood bank). Based on the experience from previous studies (eg. SO Southern, J. Human Virol 1998, 1:328), highly compatible primary antibodies (see below) were used to design the poly-SRP cocktails. The optimal dilutions of antibodies for the cocktail use were established empirically, starting with the optimized concentrations of the individual antibodies. At first we designed a generic cocktail, applicable to different mammalian species. Such cocktail contained all antibodies at 40-fold lower concentrations than the optimized single antibody concentrations. This cocktail for used to analyze all reference specimens. The empirical results of this analysis showed that this cocktail revealed 45 altered SRP profiles (perturbation index>14%), and thus provided 88% identification of the stressed individuals. Then we designed a cocktail specifically tailored for highly specific staining of the spotted dolphin specimens (Table 2). This cocktail contained antibodies whose concentrations were optimized separately, and were 30-60-fold more diluted than the optimal single concentrations (Table 2). The use of this tailored cocktail antibody allowed a rapid and inexpensive screening of SRP profiles in over 1000 specimens, within 5 months. While analysis of the individual SRP markers would provide much more accurate results, it would require a 40-fold larger time, personnel and money budget, which was not available. The empirical results of this reference study, and other studies demonstrate the utility of well designed cocktail antibodies, while the precise reaction mechanism of their immunoreactivity remains to be elucidated.

General applicability of a standard immunohistochemical staining procedure for all SRP antigens.

We selected such mouse or rabbit commercial antibodies that were known to recognize SRP antigens under common reaction conditions, and whose binding could be visualized using a common detection procedure.

- 1/ Reactive with formalin-fixed, paraffin-embedded tissues (tested empirically with cetaceans).
 - 2/ Requiring or not affected by citrate antigen retrieval.
 - 3/ Requiring pretreatment with goat serum to block non-specific binding.
 - 4/ Specific immunochemical binding at 4°C, in 16 hrs.
 - 5/ Detection of the primary antibody using either of two reagents, reacting under the same conditions (mouse or rabbit links).
 - 6/ Detection of the secondary antibody using a common reagent (AP-label).
 - 7/ Visualization of the antibody-link-label sandwich using a common reagent (a red pigment Fuchsin).
- The compatible properties of the anti-SRP antibodies provided basis for the use of a standard staining procedure with all the individual antibodies, and with the poly-SRP antibody cocktail.

General applicability of the image analysis-based quantification for all SRP antigens.

The image analysis measures the density of the immunohistochemical signal in a selected area. In our study, we selected the entire epidermis as the area of interest, and quantified signal deposition within all subcellular and extracellular compartments within that area.

Classification of SRP profiles into normal and altered rather than using a continuous expression level scale.

Based on the results of the reference study, we planned to use a continuous scale based on SRP levels measured by the computerized image analysis. However, when we stained the large sample of the spotted dolphins, we discovered that while the normal SRP profiles resembled the reference specimens, the altered SRP profiles were much more complex, and contained about 4 types of expression patterns with discontinuous layers of higher and lower staining (banded patterns). We had no means to objectively assess these complex patterns (development of new image analysis program for a complex pattern recognition would take over one year). Therefore we had to use the simple normal/altered classification.

Reproducibility of the normal/altered classification in the spotted dolphins.

As described in the report, a high reproducibility of the normal/altered SRP profile classification was demonstrated by a blinded repeated analysis of 200 specimens (137/158=87% duplicated classification, Spearman rank correlation test $r_s=0.999$). This level of reproducibility is sufficient for a population survey.

Validation of SRP techniques using cultured cetacean cells or a laboratory animal model.

Within the constraints of the mandated study, it was not possible to implement cell culture experiments, or a laboratory animal model.

Interpretation of SRP profiles

Comments

G.D. Bossart

- 1/ The effect of the skin sampling sites.
- 2/ Interpretation of SRP profiles in the cetaceans when the information on the patho-physiological mechanisms of SRP expression is limited.
- 3/ Prognostic significance of the observed SRP profiles needs to be assessed.
- 4/ Suggesting the use of a captive experimental cetacean population to provide information on the dynamics of SRP expression in normal and diseased cetaceans, such as necessary for diagnostic and prognostic interpretation of SRP profiles.

J. Mann

- 1/ Resolving the relation between SRP profile and skin sampling site.

R. Ortiz

- 1/ The effects of sun exposure.
- 2/ The effect of the skin sampling sites.

D. Martineau

1/ The effects of solar exposure on the SRP profiles in the skin. Differences in solar exposure for different body sites (dorsum and jaws less exposed than dorsal fin).

S. DeGuisse

1/ Effects of the skin sampling sites.

2/ Interpretation of SRP profiling when the information on the dynamics of the SRP expression is limited.

3/ Interpretation of the SRP profiles in the CHES recaptures.

RESPONSES

The effect of skin sampling sites.

The reference skin specimens were obtained by opportunistic sampling of mostly wild, free-ranging cetaceans. While providing very valuable field situation specimens, this approach precluded precise choice and even knowledge of the sampling sites. Based on color pattern, shape and morphology, the reference specimens were from different sites on the dorsum and the head. Since this is a common situation in field sampling of wild cetaceans, we aimed to develop a stress-detection method which would not be dependent on the precise sampling site location. In the reference study, the type of SRP expression profile (normal or altered) was clearly and consistently related to the health of the sampled individuals rather than to the (presumably random) sampling sites. In the large sample of spotted dolphins, the sampling procedure was most consistent in the fishery mortalities (jaws skin), less in the biopsies from bow-riding animals (random sites on the dorsum). In CHES, specimens were collected in several ways. The majority of specimens were from the dorsal fin (222/242), 20 were from the dorsum. The frequencies of the normal and altered SRP profiles were significantly different in specimens from the dorsal fin and the dorsum. However, firm conclusions cannot be drawn from the differences between the dorsal and dorsal fin specimens from CHES due to the small number of the dorsal specimens and potential confounding variables related to these specimens (geographical location, previous stress exposure).

Therefore, the relationship between the skin sampling site, and the type of SRP profile needs to be investigated rigorously in future studies with a specifically designed sampling procedure.

Limited information on the patho-physiological mechanisms of SRP expression in cetaceans.

The relationship between diverse manifestations of pathology in cetaceans and SRP expression was established in the reference study of 49 killed or diseased cetaceans, including two diseased wild spotted dolphins and seven wild spotted dolphins killed in the fishing nets (Table 1). In addition, three humans with well characterized clinical diseases were included for comparison.

Limited information on the dynamics of SRP expression in cetaceans.

Within the scope of this study it was not possible to investigate the dynamics of SRP expression. In a related study we have shown that altered SRP profiles can be induced in directly stimulated skin within 30 minutes (SO Southern and V Morhenn, JAAD, submitted 2002).

Prognostic significance of SRP profiles.

In a related study, SRP profiling was applied to blinded blood and skin specimens from 3 healthy and 2 diseased semi-domesticated bottlenose dolphins (SOS, unpublished results). Altered SRP profiles were found only in the blood and the skin from the diseased dolphins. The blood specimens from the two diseased dolphins were obtained one day or one month before death, respectively. The skin specimens from the diseased dolphins were obtained during necropsy. These results suggest that SRP profiling of blood could have prognostic utility as a generic indicator of compromised health.

Interpretation of SRP profiles in the CHES recaptures.

Full interpretation of SRP profiles in CHES recaptures is not possible without a better understanding of the SRP expression dynamics during rapidly repeated stressful impacts.

The effect of sun exposure.

Within the scope of this study, it was not possible to investigate the effect of the sun exposure.