STRESS IN SPOTTED DOLPHINS (STENELLA ATTENUATA) ASSOCIATED WITH PURSE-SEINE TUNA FISHING IN THE EASTERN TROPICAL PACIFIC

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ABSTRACT

To compare evidence of chronic stress in fishery-involved and fishery-uninvolved spotted dolphin (*Stenella attenuata attenuata* and *S. a. graffmani*) targeted by the purse seiners and to study how numbers of purse-seine sets occurring in an animal's recent past and range relate to stress, we analyzed the relationship between past exposure to tuna fishing effort and stress-associated molecular changes in dolphin skin. For our analyses, we employed 424 samples collected from fishery mortalities, 202 from bow-riding biopsies, and 242 from CHESS (Chase Encirclement Stress Studies, Forney et al., 2002) biopsies. We consider the fishery mortalities and the CHESS biopsies to be fishery involved, and we assume the bow-riding biopsies to be fishery uninvolved, at least uninvolved recently. In addition, we make a relative comparison of the experience of individual dolphins with purse-seine sets occurring prior to sampling by estimating the fishing effort occurring in an animal's recent past and range by summing the number of sets, within 30, 60, 90, 120, 150, 180, 240, or 300 nautical mile radii centered on the sampling site, occurring 10, 20, 30, 40, 50, 60, or 70 days prior to sampling. Without knowledge of numbers of sets associated with each sample, a reader scored whether the sample's protein expression pattern was normal or altered based on experience with assumed healthy or clinically diseased cetaceans.

High frequencies of altered expression patterns characterize the fishery-involved samples relative to the fishery-uninvolved samples; however, potential biases regarding sampling location on the animal's body (jaw, back, or dorsal fin skin) confound the comparisons among the three sample sets. Within the fishery mortalities, more sets, occurring prior to the sampling event, increases the likelihood that a sample will have an altered expression pattern. Paradoxically, within the bow-riding biopsies, more sets decrease the likelihood that a sample will have an altered expression pattern. (CHESS results were inconclusive). We suggest an explanation for the difference in stress response between the animals that are chased and encircled (the fishery mortalities) and the animals that voluntarily approach the vessel (the bow-riding biopsies). We suggest, in the former case, fishing sets in the recent past cause stress; in the latter, fishing sets in the recent past alter behavior.

INTRODUCTION

In the eastern tropical Pacific (ETP), millions of dolphins are chased and encircled each year during purse seining for yellowfin tuna (for a general description of fishing practices, see National Research Council, 1992; Perkins and Edwards, 1999; Donahue et al., 2000). Today, for every 1,000 dolphins chased and encircled, approximately one is reported killed.[1] But even for the 99.9% released alive, few would argue that the experience is stress-free. And if the stress is substantial, there is abundant evidence from other mammals to infer negative reproductive consequences on individuals and, by extension, on populations (for general review, see Sapolsky, 1998; Curry, 1999; for specific recent examples, see Creel, 2001; Gallinelli et al., 2001; Mann, 2001). Thus, mortality rate estimated by the number of animals observed dead in the nets could fail to gauge the full impact on the three depleted dolphin populations targeted by the fishery.

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[1] For fishery mortality estimates, see Annual Reports of the Inter-American Tropical Tuna Commission, La Jolla, California.

Establishing the quantitative relationship between population stress levels and population growth rate is an intractable problem. Abundances of the impacted populations are not precipitously declining, so if stress is affecting population growth, the effect is likely subtle and distributed widely among individuals, and thus hard to detect. Or, if substantial, the effect is likely limited to a few individuals, and thus hard to sample. Equally intractable is experimental manipulation of stress in captive populations to quantify reproductive effects. This is a problem because the International Dolphin Conservation Protection Act of 1997 (IDCPA) mandates determining whether intentional chase and encirclement have a significant adverse impact (interpreted as impeding recovery from depleted status) and studies of stress are specifically mentioned. However, there is a middle ground. Although demonstrating a causal link between stress and population growth rates is virtually impossible, a more modest approach, but one that is still relevant to the goal of the Act, can be formulated.

We ask whether there is simply a relationship between fishing activity and stress on an individual. A finding that stress and effort co-vary suggests a simple cause-and-effect, i.e., fishing on stress. It is recognized that other mechanisms explaining a co-varying relationship that do not implicate the fishery can be put forth. Because correlation does not imply causality, choosing among competing explanations of a co-varying relationship can only be done by application of Occam's razor. As a result, demonstrating a relationship between stress and fishing will only add plausibility to the hypothesis that fishery practices could contribute to the depression of dolphin population growth rates. Inability to demonstrate such a relationship between fishing effort and stress (or even the simple existence of physiological stress in the population itself) will weaken but not allow rejection of the hypothesis.

Conventional determination of stress generally requires restraint to allow invasive sampling of blood or requires an autopsy (see various reports of the CHESS cruise). To take advantage of available samples and to allow sampling of wild cetaceans in the field, a new methodology for measuring stress had to be developed. Four requirements had to be met: (1) The method had to work with historical skin samples preserved in a variety of chemicals (formalin, alcohol, DMSO). Our laboratory has abundant samples of skin tissue attached to jaw sections that were collected from purse-seine fishery mortalities and used to estimate age from the attached teeth. Furthermore, skin is available via projectile biopsy from wild, unrestrained animals. The use of skin made available to us tissue samples from a wide variety of cetacean species, from animals that were apparently healthy to ones suffering from disease and likely stressed as a result. (2) The method had to be oriented towards chronic stress rather than acute stress associated with sampling, and (3) the method had to be inexpensive, rapid, and practical for a large number of samples. Based on these four constraints, the method developed by Southern et al. (2002) identifies differences in expression profiles of multiple stress-responsive proteins (SRP) in cetacean skin.

The degree of "experience" an individual animal has had with purse-seine fishing was estimated in two ways: (1) We compared stress in animals known to be involved with fishing activities and those assumed naive, and (2) we correlated stress with relative effort occurring in an animal's recent past and range by summing the number of sets, within 30, 60, 90, 120, 150, 180, 240, or 300 nautical mile radii centered on the sampling site, occurring 10, 20, 30, 40, 50, 60, or 70 days prior to sampling. Because movements of an animal prior to capture are unknowable, we

realize that a summation of effort within these temporal and spatial windows is at best an imprecise proxy for the actual number of purse-seine sets experienced by the animal.

We score whether an animal is "stressed" by an investigator's decision of whether the skin section had a "normal" or "altered" pattern of stress-responsive protein expression. The linkage of the pattern of protein expression to stress was based on (1) proteins known in humans and animals to be up-regulated in response to stressful challenges and (2) from experience of SRP expression profiles of cetaceans assumed stressed due to disease, injury or other abnormal physiological conditions, or nominally healthy (Southern et al., 2002).

Here we describe a set of analyses examining the relationship between fishing effort and stress in two subpopulations of the pantropical spotted dolphin (*S. a. attenuata* and *S. a. graffmani*, Perrin, 2001). The former is composed of two stocks or management units, the northeastern and the western-southern, although in the following analyses, only the northeastern offshore one was available to us. *S. a. graffmani* is managed as only one stock, the coastal one. We test whether there is a relationship between the likelihood of exhibiting a normal or an altered pattern of stress responsive protein expression and whether an animal is fishery-involved (chased and encircled in the course of fishing operations) or fishery-uninvolved (biopsied while bow riding). We also test whether the samples showing a normal versus an altered pattern were drawn from the same population of accumulated sets. Three samples sets were employed: (1) offshore stock individuals accidentally killed in purse-seine operations and sampled ten years ago by observers on purse-seiners, (2) offshore and coastal individuals sampled in 1998-99 via projectile biopsy from animals that approached research vessels to bow ride, and (3) offshore individuals biopsied after chase and encirclement during the CHESS cruise of 2001. Because determination of stress was based on a judgment of whether a skin section looked normal or altered, experimenter bias was controlled by scoring each sample without prior knowledge of the effort level associated with the sample.

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.

MATERIALS AND METHODS

Sample Sets

Fishery mortalities. Chasing and encircling dolphin schools in order to capture yellowfin tuna in the ETP has been going on since 1959 and systematic collecting of mortality and biological data from the U.S. fleet by on-board observers employed by the National Marine Fisheries Service since 1972. In the late 70's, increasing participation by other countries prompted the Inter-American Tropical Tuna Commission to start a program of their own. Comprehensive details about the fishing practices such as set location, chase and set duration, time of capture, school size and species composition, and numbers and biological observations about incidental mortalities were recorded in standardized logs by the vessel captain and the observer, when the vessel carried one. Whenever time and circumstances permitted, the observers collected biological samples to be returned for laboratory analyses in addition to simply enumerating observations in log books. Over the years, a variety of samples were collected from the incidental kills. Those samples included blubber, female

reproductive tracts, fetuses, male reproductive tracts, stomachs, parasites, spleens, lymph nodes, teeth, etc. The teeth were used for estimating age by counting tooth layers and collected by using shears to cut out a section of mandible, which was initially preserved in formalin and later, back at the laboratory, in 70% isopropyl alcohol (Jefferson et al., 1994). External skin adhering to the jaw sections was used for our analysis of stress in this sample set.

It is important to realize that because these samples were from incidental mortalities, there is no expectation that they are necessarily a random sample of the school or the population. Furthermore, sampling instructions to the observers regarding which tissues to collect over the years differed depending on interests and needs of investigators on land. We limited our sample set to those jaws taken from the 1988 through 1991, when observer coverage of the fleet was high. After 1992, biological samples were no longer collected for us.

Figure 1A shows the distribution of collection sites for the 443 samples sectioned for immunohistochemical analysis. Of those, 424 yielded usable data. All were from the northeastern offshore stock, and sampling was distributed reasonably homogeneously throughout the range of stock (Dizon et al., 1994). There were 178 males, 245 females, and 1 with unrecorded sex. (Generally, the observers preferentially collected biological samples from females). In spotted dolphins, age is roughly reflected by color pattern (Perrin, 1969). About half of the individuals showed an adult color pattern, while the balance was distributed approximately evenly among juvenile or sub-adult patterns. Only 11 of the samples were judged to be neonates. For some of the females, information regarding whether they were lactating and whether a fetus was present and its sex and size was determined. Samples were collected from Jan 27, 1988 through Oct 27, 1991.

Bow-Riding Biopsies. Dolphins voluntarily approach vessels to wave ride on the bow wake from minutes to hours in what seems to be a playful and effortless behavior, and this behavior can be exploited to collect skin samples via crossbow biopsy. A crossbow bolt is tipped with a 0.7cm biopsy punch equipped with internal barbs to retain the sample. A flange padded with a section of vacuum hose limits penetration, and the bolt is tethered via a light line to retrieve it after sampling. Reactions are minimal, and in many cases taking a sample does not interrupt the bow-riding behavior. For these stress studies, samples were preserved by freezing in liquid nitrogen.

Figure 1B shows the distribution of 202 bow-riding biopsies sectioned for immunohistochemical analysis (and the CHESS biopsies). Of the bow riders, 108 were from the northeastern offshore stock, 65 were from the coastal stock, and 29 could not be identified to stock. Note that these samples, in contrast to fishery mortalities, concentrate within approximately a few hundred nautical miles from the coastline. As distance from the coastline increases, dolphins become increasingly reluctant to bow ride, and although cruise tracks cover the same areas from where the fishery mortalities were collected, no bow riding was observed far offshore. Figure 2 shows that sightings of spotted dolphin schools during the research cruises of 1998 and 1999 were made throughout the ETP, but that bow riding only occurred and consequently biopsies acquired up to about 300nm from the coast. There is a clear cline in boat-evasive behavior ranging from infrequent near shore through areas where some schools are evasive and others not, or some members of the school are evasive, to far offshore where all schools are evasive (Mesnick et al., 2002).

From the bow-riding biopsies, sex was determined molecularly (Rosenberg and Mesnick, 2001), and 89 of the samples were female, 91 male, and 22 were undetermined because insufficient

tissue was left after the SRP expression pattern analyses. No data are available on size, age, or reproductive condition. Samples were collected on the 1998 and 1999 ETP research cruises conducted by the SWFSC (Aug 6 to Dec 1 and Aug 9 to Dec 4, respectively).

CHESS Biopsies. A study involving repeated chasing and capturing of dolphins designed to collect data on physiological indicators of stress was mandated by the IDCPA (Forney et al., 2002). Two vessels were employed: the NOAA Ship McARTHUR and a chartered purse seiner for a two-month cruise, Aug 8 through October 9, 2001. During the cruise, an extensive set of samples and data were collected from the dolphins chased and encircled by the seiner. Primarily northeastern offshore spotted dolphins were targeted by the cruise although a few eastern spinner dolphins (*Stenella longirostris orientalis*) were also sampled although not analyzed for this report. The area of operations and sampling positions of the spotted dolphins are plotted on Figure 1B.

Once the dolphins were encircled and the net completely pursed, skin samples were collected in three ways (Forney et al., 2002): (1) Some animals were chosen for fairly extensive sampling consisting of tagging, bleeding, etc. Our sample was the core from the dorsal fin taken out when a telemetry package or tag was applied to the dorsal fin. The tagging enabled subsequent capture and identification. These animals on recapture were re-sampled from the dorsal surface of the body, posterior to the dorsal fin. The samples taken the first time an animal was captured are called the "tagging/bleeding" samples; some were recaptured a second and, in one case, a third time. (2) Some animals were simply singled out by a swimmer, grabbed by hand, and brought over to a raft to just have an identification applied through a hole cored in the dorsal fin. These are called the "drive-thru" [2] animals, and our sample was again the dorsal fin core. Some of these were recaptured a second time. And (3) a limited number of animals were sampled with a biopsy tool mounted on a pole. In these cases our sample was not from the dorsal fin, it was, like the recaptures, from the dorsal surface posterior to the dorsal fin. This sampling procedure was used only on the first two sets because the fishing captain felt that sampling this way disturbed the school and interfered with the backdown procedure. These are called the "pole-spear" biopsies. The biopsies were stored at -80˚C or in liquid nitrogen and kept frozen until transfer to the SWFSC.

Two hundred and eighty skin biopsies from northeastern offshore spotted dolphins were collected and returned to the SWFSC for subsequent analyses. Of those, 260 were of adequate size and thickness to be processed. After sectioning and staining, 3 were discarded due to poor section quality. Of the 257 skin samples available for statistical analyses, 15 were from recaptured animals.

Procedures

Estimating Stress. Physiological stress was identified using high throughput SRP profiling. See Southern et al. (2002), for detailed methodology. Briefly, SRP expression was visualized by immunohistochemical staining evaluated by computerized image analysis and visual inspection, and then classified as "normal" or "altered" without knowledge of the associated fishing effort.

Estimating Fishing Effort. Spotted and other dolphins that have been targeted by the fishery, at least in the recent past, are less likely to approach boats but rather run and hence have to be

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[2] These animals were called "raft-side" samples in Forney et al. (2002).

chased and herded (Mesnick et al., 2002).   On the basis of this, we subdivided our sample sets first by whether the sample was obtained during fishing operations (fishery mortalities and CHESS biopsies) and clearly "fishery-involved" or whether the sample was obtained during bow riding and assumed relatively "fishery-uninvolved."

Another approach was based on estimating the relative experience that a dolphin has had with purse seining in the ETP by accumulating the number of sets that occurred prior to sampling within a circumscribed area and time. A Visual Basic 6 computer program was written at the SWFSC and provided to the IATTC for application to their data sets. On a sample-by-sample basis, the program steps through extractions of the observer and skipper data bases, which contained a time-ordered array of sets made by the U.S. and international yellowfin tuna purse- seine fleet. Our program summed sets occurring within 30, 60, 90, 120, 150, 180, 240, and 300 nautical miles centered on the sampling location and occurring 10, 20, 30, 40, 50, 60, and 70 days prior to sampling. The summation of effort within these temporal and spatial windows is at best a proxy for the actual number of purse-seine sets experienced by the animal. However, as a relative measure for making effort comparisons among samples and within sample groups (fishery mortalities, bow-riding biopsies, CHESS biopsies), it is likely unbiased.

The files used by the IATTC contained information from calendar year 1972 to 2000 that involved sets on dolphin schools that had at least one dolphin species present. The data are taken from two sources: the IATTC Observer database for fishing trips that had an observer aboard, and the IATTC Log Book database for unobserved trips. A single set was taken from the Observer database if present, and, if not, from the Log Book database. A single set was never taken from both. Data taken from the Observer database included all intentional and accidental dolphin sets. An intentional one was defined as a set made with the intention of capturing dolphins; an accidental one was one in which the target of the set was not dolphins (e.g., on logs or free-swimming schools), but dolphins were nevertheless captured. Sets involving only whales were not included.

The limits of the spatial windows were based on the practice of the IATTC and others of using 1˚ and 5˚ squares to display fishery and sample data in the ETP and the temporal windows, on the average 70-day lifetime of an epithelial cell from differentiation in the germinal layers at the base of epithelium to shedding at the skin surface (Geraci et al., 1986). Thus for each sample, an 8 x 7 array was produced and provided to us by the IATTC, which depending on a complex and unknowable set of movements by the individual and the fleet, some elements of which would presumably be a proxy for the individual animal's experience with fishing. In the final analyses, a choice of a single space / time window was not required, and it was possible to draw conclusions simply from the patterns of sets among the 56 space / time windows.

Statistics. Our analytical approach was extremely simple. Because of the classificatory scale of the stress data and the non-normal distribution of the set data, the following non-parametric statistical procedures were used: Pearson two-way cross tabulations and Mann-Whitney U tests, (Siegel, 1956). These tests were implemented in SYSTAT. In addition, a permutation-based, pair-wise χ^2 test, which circumvents the problems of low frequency cells, was used (1000 permutations; Roff and Bentzen, 1989). Effects of potential explanatory variables when data were available (sets [effort], sex, reproduction condition, age, length, stock, vessel evasiveness, and sampling location) were tested in a univariate manner. We used $\alpha \leq 0.05$ as our level of significance.

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RESULTS

Fishery Mortalities

Among the fishery mortalities, there were many more individuals scored as having an altered expression pattern compared to a normal one (354 versus 70). The geographic distribution of the normal and altered patterns seemed random among the samples (Figure 3A).

There was no significant difference in the distribution of normal and altered patterns between males versus females; the ratios were near parity. Surprisingly, there was also no significant difference between lactating versus non-lactating females and between resting or early term pregnant versus lactating or late-term pregnant. One would expect lactation or carrying a late-term fetus would be stressful or predispose the animal to be stressed by other causes. Early- and late-term pregnancy was based on the observer's ability to observe a fetus. In instances when a fetus was not observed (and the female was not lactating), we assumed the dolphin was either resting or in an early stage of pregnancy. One might also expect that younger animals would also be stress-susceptible, but we also observed no significant difference basing age on length or on color pattern.

There were significant differences in the number of associated purse-seine sets between individuals scored as having a normal pattern versus ones having an altered pattern. Table 1A compiles the median sets and whether the *P* values of the Mann-Whitney U test were significant for each space / time window used to accumulate the number of sets prior to the set where the individual was killed and a jaw section collected. The test examines whether two independent groups (the samples showing a normal versus an altered pattern) were drawn from the same population of accumulated sets. For the fishery mortalities, average and median number of sets in the smallest window (30 nautical mile radius, 10 days) were 5.4 and 4, respectively; average and median number of sets in the largest (300 nautical mile radius, 70 days) were 325.1 and 274. In all 56 windows, the average rank of the number of accumulated sets experienced by individuals with normal patterns was smaller than the average rank of sets experienced by individuals with altered patterns. From the composition of accumulated sets among the space / time windows, we conclude that more sets, occurring prior to the sampling event, increases the likelihood that a sample will have an altered expression pattern.

Bow-riding Biopsies

Among the bow-riding biopsies, there were many more individuals scored as having a normal expression pattern compared to an altered one (127 versus 75). In a two-way comparison, if the frequencies of normal versus altered expression patterns are compared between fishery mortalities (70 versus 354) and bow-riding biopsies (127 versus 75), the difference is highly significant (two-way cross tabulation $\chi^2 = 136.2$, $P < 0.001$).

The geographic distribution of the normal and altered patterns as with the fishery mortalities seemed random among the samples (Figure 3B). There were no significant differences in the distribution of normal and altered patterns between males versus females or between the 108 northeastern offshore individuals, the 65 coastal individuals, or the 29 that could not be identified to stock. No information was available on reproductive condition or age although generally only adult animals bow ride.

Again as with the fishery mortalities, there were significant differences in the number of associated purse-seine sets between individuals scored as having a normal pattern versus ones having an altered pattern. Table 1B compiles the median sets and whether the *P* values of the Mann-Whitney U test were significant for each space / time window used to accumulate the number of sets prior to the date and location where the individual was biopsied. In order to compare the data with the fishery mortality and CHESS biopsy data set, only samples from the offshore stock were tabulated. (For the coastal stock animals, no significant relationship pattern for any space / time window was observed). Relative to the fishery mortalities, somewhat larger windows were necessary to observe a significant relationship. The average and median number of sets in the smallest window (30 nautical mile radius, 10 days) were 0.5 and 0, respectively; average and median number of sets in the largest (300 nautical mile radius, 70 days) were 134.3 and 124. For the coastal animals, the values were 0.1 and 0, 116.4 and 72, respectively. For the offshore spotted dolphins, the values were 0.6 and 0, 146.6 and 128, respectively. The overall number of sets experienced by the fishery mortalities compared to bow-riding biopsies (offshore stock) was about 3½ times greater. The coastal stock animals experience about half the number of sets that the offshore stock ones do.

In all 56 windows, the average rank of the number of accumulated sets experienced by individuals with normal patterns was larger than the average rank of sets experienced by individuals with altered patterns (Southern et al., 2002). In contrast to the fishery mortalities, fishing effort paradoxically seems associated with a higher frequency of normal patterns. From the composition of accumulated sets among the space / time windows, we conclude that fewer sets, occurring prior to the sampling event, increases the likelihood that a sample will have an altered expression pattern.

Banding Patterns

Southern et al. (2002) noted that a "discontinuous level of staining intensity" characterized a certain fraction of the expression patterns scored as altered. In the fishery mortalities with altered patterns, 258 were uniform, 54, banded; in the bow-riding biopsies with altered patterns (all stocks), 63 were uniform, 13, banded. When the patterns of accumulated sets are examined in both the fishery mortalities and the bow-riding biopsies sample sets, more effort was associated with animals exhibiting a uniform pattern than exhibiting a banded pattern (Table 1D – E). Because the uniform pattern is associated with more sets in all significant space / time windows for, we speculate that the banded pattern represents an intermediate level of stress relative to the uniform pattern perhaps caused by a lower frequency of chase and encirclement events.

CHESS Biopsies

The CHESS biopsies were processed together with a sub-set of the bow-riding biopsies in order to compare fishery-involved and fishery uninvolved animals in the present-day fishery. The earlier comparison of bow-riding biopsies and fishery mortalities is suspect considering the decade-long separation in sampling dates, the differences in preservation, and the fact that the fishery samples came from killed animals. In contrast, the bow-riding sub-set and CHESS biopsies are nearly contemporaneous, were collected from live animals, were preserved the same way, and were

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processed together. Furthermore, they were evaluated without reader knowledge of their source. In this manner, potential reader bias was eliminated.

When the subset of the bow-riding biopsies (101 normal versus 62 altered) that were processed together with the non-recaptured CHESS biopsies (34 normal versus 208 altered) are compared (two-way cross tabulation χ^2 = 100.6, $P < 0.001$), the results are very similar to those obtained when the fishery mortalities were compared with the full set of bow-riding biopsies. Fishery-involved animals have higher frequencies of altered patterns than fishery-uninvolved ones. And in addition, the presumably stressful effects of drowning in the fishing nets does not seem to produce a detectable signal because the frequency of normal expression patterns are actually slightly higher in the fishery mortalities than in the CHESS biopsies (Table 3A).

In the CHESS biopsies, animals with normal versus altered patterns seemed distributed randomly in the study area and among schools processed (Figure 3C).

In contrast with the fishery mortalities and the bow-riding biopsies of the offshore population, in the CHESS biopsies, the pattern of significant Mann-Whitney U tests for each space / time window was hard to interpret. For the CHESS biopsies, some space / time windows with significant Mann-Whitney U tests had the average rank of sets larger for the specimens with altered patterns and in other windows the average rank was smaller for the altered pattern (Table 1C). The average and median number of sets in the smallest window (30 nautical mile radius, 10 days) were 2.5 and 2, respectively; average and median number of sets in the largest (300 nautical mile radius, 70 days) were 437.6 and 421. We suspect the lack of a predictable pattern was due to the fact that only 27 sets were made in a limited area, but on balance, there were more space / time windows where the animals with altered patterns show lower average rank values.

In all preceding CHESS analyses, samples that came from recaptured animals were not used although they were scored, along with the non-recaptured CHESS biopsies and the sub-set of bow-riding biopsies, without the knowledge that they were from recaptured animals. In total, we have analyzable tissue from 15 recapture events (1 animal was recaptured twice; Table 2A). For 3 of the 15, skin was not collected when the animal was first handled. Of the 22 paired samples plus 1 second capture, 10 were scored altered and 1 normal at the first capture, but on subsequent captures, only 7 were scored altered and 4 plus 1 second capture were scored normal. Three animals on their second recapture exhibited a normal pattern in place of the altered one they had exhibited on their first sampling; one animal exhibited an altered pattern on the first and second sampling but a normal pattern on the third sampling (Table 2B). The number of specimens demonstrating a normal pattern is significantly higher among recaptures than among animals sampled just once either from a mortality event or from a first-time CHESS biopsy (Table 2C - E). (In this case, all 15 recaptures are included). Thus, normal patterns seem to occur in higher frequencies among the recaptures than among the fishery mortalities and CHESS biopsies of animals captured only once, and four animals in a span of 1 to 5 days demonstrated a seeming ability to recover from an altered expression pattern.

This suggestion of higher frequencies of normal patterns in subsequently captured animals, and the puzzling ability to recover even when confronted with the continuing stress of chase and recapture, may be due to sampling position on the animal's body. In the 54 tagged/bled and the 166 drive-thru animals, the sample came from the dorsal fin, but when recaptured, the sample came from the back. In the 22 pole-spear animals, the sample came from the back. When the frequencies of normal and altered patterns are compared for those sample sets as well as the fishery mortality and bow-riding biopsies, a strong pattern is evident. When considering the frequency of altered to

normal, the drive-thru and the tagging/bleeding specimens strongly resemble the fishery mortalities and the pole-spear and recapture biopsies resemble the bow-riding biopsies (Table 3A - B). The former three sets of samples were collected from dorsal fin cores and show a dearth of normal expression patterns (0.09, 0.13, and 0.17, respectively). The latter three were collected from skin punches in the back and show a clear preponderance of normal expression patterns (0.55, 0.40 [Table 2C], and 0.63, respectively).

DISCUSSION

If we assume that animals that bow ride are inexperienced or have little recent experience with purse-seine fishing, a rough grouping of the samples into fishery-involved and fishery-uninvolved animals can be made. Samples that are collected from animals in a purse seine after chase and encirclement are obviously involved. When the frequencies of normal and altered expression patterns were examined, the differences among the fishery mortalities, the bow-riding biopsies, and the CHESS biopsies sample sets were significant. The odds of observing a normal expression pattern in fishery-mortality samples were about 1 in 6. With 1st captured CHESS-biopsy samples, the odds were about 1 in 7. With bow-riding biopsies, the odds were about 2 in 3. However, before drawing the obvious conclusion that a relationship between fishing and stress exists, the following must be considered.

Our initial assumption was that our stress test was free of sampling bias in that it measured the stress experienced by the animal over the two months or so prior to the sampling event. We assumed that the transitory stress accompanying collecting the sample was too brief to be detectable in our test, and we assumed that sampling location on the body was unimportant. Data from other cetacean species suggest that the first assertion is reasonable (Southern et al., 2002). The authors cite instances where 21 cetaceans subject to transient trauma or capture (accidental drowning, aboriginal hunts lasting up to 30 minutes, and netting and tissue sampling as part of a long-term ecological study) show no increased incidence of altered patterns. The authors also cite instances where samples collected close together on the body responded the same. However, we may have been overly sanguine, in not suspecting a potential bias in sampling skin as different as jaw, dorsum, and dorsal fin.

The puzzling finding that four recaptured animals reverted from an altered expression pattern to a normal one on subsequent capture one to five days later even though stress was presumably unremitting may be explained by a bias for normal skin patterns in the skin of the back relative to the dorsal fin (Table 2C). This reversion could be dismissed as reader error, but the chance of drawing six normal and nine altered pattern recaptured samples out of the population represented by the drive-thru and tagging/bleeding samples is only 1 out of 500 (permutation $\chi^2 = 12.0$, $P = 0.002$). There is some evidence that frequently repeated stressors can cause decreased molecular responsiveness to stress potentially creating non-linear dynamics in the profile development (Southern et al., 2002).

The other puzzling finding that a majority of the pole-spear biopsy samples exhibited a normal pattern similarly may be explained by this bias for normal skin patterns in the skin of the back relative to the dorsal fin (Table 3A). Here, the chances of drawing 12 normal and 10 altered pattern pole-spear biopsies out of the same population of 1st captured CHESS is even smaller, at least less than 1 out of 1000 (permutation $\chi^2 = 32.9$, $P < 0.001$).

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Although the evidence for bias caused by skin sampling location is based on a small number of samples, i.e., 22 CHESS pole-spear biopsies and 15 recaptures, it is consistent in both. And, it is only empirical evidence we have relating to the problem. There are likely other mechanisms that could be put forth to explain the differences in expression patterns among the sample sets (see Southern et al., 2002). However, such postulated mechanisms are unlikely to be sufficiently compelling to allow a conclusion of a fishery effect based on comparisons of frequency among the three sample sets: fishery mortalities, bow-riding biopsies, and CHESS biopsies.

Estimating fishing experience of an individual by accumulating the number of sets within a time and area relative to the geographical sampling location and date circumvents potential biases of sampling site on the dolphin's body. It allowed us to examine the effect of fishing *within* samples collected in a more-or-less identical manner (the fishery mortalities' jaw tissue and the bow-riding biopsies' back tissue). From these data, we conclude that for the fishery mortalities, more sets occurring prior to the sampling event *increases* the likelihood that a sample will have an altered expression pattern. For bow-riding biopsies, more sets *decrease* the likelihood that a sample will have an altered expression pattern. For the CHESS biopsies, we are unsure about the relationships between sets and expression patterns. The problem may be due to the CHESS biopsy sample being represented by relatively few sets or the potential bias of sampling from the dorsal fins.

The inverse and significant relationship of effort and frequencies of normal or altered expression patterns in bow-riding animals suggests an effect of purse-seine fishing just like the direct one observed with the fishery mortalities. It could be argued that as one moves inshore, the fraction of fishery-naive dolphins increases and bow-riding behavior increases. Behavioral analyses of Mesnick et al. (2002) support this. With relatively low or no fishing effort, animals exhibiting both altered and normal patterns bow ride. We observed that with the coastal stock animals. We assume whatever causes the stress, which produced the altered pattern, was not fishing activity or, at least, not recent fishing activity. With increasing but still relatively low fishing effort, we suggest that those "pre-stressed" animals are less likely to approach the boat for a bow ride. Thus, in areas of little or no fishing effort, the pattern frequencies approach that expected in fishery-naive populations, but in areas of intermediate fishing effort, the bow-riding biopsy sample is biased towards animals with normal expression patterns.

The observation that there is a relationship between past fishing events and frequencies of altered and normal expression patterns argues that we are observing a chronic effect independent of the proximate effects of sampling. In addition, there is the strong relationship between number of sets and whether an animal exhibits a banded or a uniform pattern (see Southern et al., 2002). Because the number of sets are accumulated over periods of 10 days or longer, the expression or banding pattern responses we observe seem to reflect more than simply the exertions and possible novel stressors of the single set when the sample was taken. These results must reflect response to past sets that is observable when the animal is re-set on and sampled or killed.

In summary, we have identified SRP profiles indicating stress in dolphins associated with fishing activities; however, the precise relationships between perturbed profiles and long-term health of individuals remains unclear. Therefore, we cannot clearly identify whether purse-seine fishing activities today are having a negative impact on dolphin populations. It is important to realize that although our test was based on comparisons of animals that were apparently healthy and ones that had a range of health problems, we have little idea about the dynamics of the stress response in skin or the degree of short- or long-term stress that is necessary to convert a normal pattern to an altered

one. The SRP profile classification scale (Southern et al., 2002) was nominal, i.e., normal or altered, and we presumably cannot discern the differences between an animal that has been inconsequentially or massively stressed. Another important caveat when considering these results is that because of the questions of sampling bias in the CHESS biopsies, our evidence of a relationship between fishing and stress is limited to samples that are over a decade old and from killed dolphins rather than biopsies of chased and encircled ones. Today, mortality rates are insignificant demographically compared to what they were then, and fishing practices are much improved presumably minimizing the stressful effects of chase and recapture.

If the opportunity arises to repeat the CHESS experiments, even on a much simpler scale not requiring repeated chase and encirclement, the question of bias caused by skin sampling location could be readily addressed allowing meaningful comparisons between the levels of stress in fishery-involved and fishery-uninvolved animals. A new collection of biopsies from the back of encircled spotted dolphins during normal fishing operations would allow a meaningful comparison with the bow-riding biopsies. In addition, we are exploring potential situations where we could obtain biopsies simultaneously from the dorsal fin and the back.

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Table 1. Median value of sets accumulated in each space / time window stratified by sample set and by expression pattern. Bolded values are those for which the Mann-Whitney U test was significant at the 0.05 level. Sample sets were tested in separate tests. The test examines whether two independent groups (the samples showing a normal versus an altered pattern or uniform versus a banded pattern) were drawn from the same population of accumulated sets. Relationship of sets to normal versus altered expression patterns in fishery mortalities (A), bow-riding biopsies, offshore stock only (B). CHESS biopsies, 1st captures only (C). Relationship of sets to uniform versus banded patterns in fishery mortalities (D), bow-riding biopsies, all stocks (E).

Sample Set	Expression Pattern	Radius (nm)	Days						
			10	**20**	**30**	**40**	**50**	**60**	**70**
A. Fishery Mortalities	Normal (n=70)	**30**	4	4	4	4	**5**	**5**	**6**
		60	7	8	16	16	**16**	17	19
		90	**16**	**16**	22	**22**	23	27	31
		120	**22**	**25**	32	**33**	**35**	**37**	**43**
		150	**27**	**33**	**43**	**44**	**47**	**48**	**61**
		180	**30**	**41**	**54**	**57**	**64**	**64**	**77**
		240	**35**	**53**	**79**	**83**	**85**	**90**	**165**
		300	**39**	**58**	**107**	**107**	**120**	**136**	**245**
	Altered (n=354)	**30**	4	4	5	6	**6**	**6**	7
		60	**15**	**16**	16	18	**19**	**19**	22
		90	**20**	**24**	29	**37**	**37**	**38**	**38**
		120	**28**	**29**	41	**61**	**61**	**62**	**64**
		150	**36**	**44**	**57**	**85**	**97**	**97**	**101**
		180	**43**	**56**	**76**	**106**	**122**	**132**	**140**
		240	**51**	**82**	**119**	**152**	**183**	**198**	**224**
		300	**66**	**104**	**146**	**195**	**235**	**284**	**303**
			10	**20**	**30**	**40**	**50**	**60**	**70**
B. Bow-riding Biopsies	Normal (n=68)	**30**	0	0	**0**	**0**	**0**	1	2
		60	0	0	0	0	1	**2**	5
		90	0	0	2	3	**4**	**7**	**9**
		120	1	3	6	6	14	**15**	**15**
		150	2	5	11	14	**20**	**24**	**29**
		180	3	7	23	**29**	**32**	**37**	**43**
		240	12	**25**	**41**	**61**	**68**	**80**	**93**
		300	28	**64**	**96**	**111**	**124**	**137**	**161**
	Altered (n=40)	**30**	0	0	**0**	**0**	**0**	**0**	**0**
		60	0	0	0	0	**0**	**0**	**0**
		90	0	0	1	1	**1**	**2**	**3**
		120	1	1	2	2	2	**4**	**6**
		150	1	2	4	4	**6**	**8**	**10**
		180	1	3	6	**8**	**14**	**14**	**16**
		240	8	**15**	**22**	**24**	**34**	**37**	**43**
		300	23	**39**	**47**	**63**	**71**	**80**	**98**

Table 1. Continued.

Sample Set	Expression Pattern	Radius (nm)	Days						
			10	20	30	40	50	60	70
C. CHESS Biopsies	Normal	**30**	2	**4**	6	**8**	**8**	**10**	10
	(n=34)	**60**	**5**	11	17	**22**	**28**	**35**	36
		90	**7**	20	**32**	52	67	**74**	74
		120	**11**	26	**48**	79	104	**116**	116
		150	**16**	43	**66**	109	150	**168**	168
		180	**19**	54	**74**	139	201	**215**	216
		240	**32**	64	**99**	186	279	294	**315**
		300	**49**	87	**164**	273	377	394	**415**
	Altered	**30**	2	**3**	3	4	7	**8**	8
	(n=208)	**60**	**7**	11	15	**16**	18	**24**	24
		90	**11**	16	**24**	31	41	**56**	56
		120	**16**	24	**35**	49	73	98	112
		150	**21**	42	**54**	73	109	**156**	171
		180	**31**	54	**71**	104	158	**208**	225
		240	**53**	64	**98**	149	219	295	**329**
		300	**58**	87	**142**	201	287	395	**424**

Table 1. Continued.

Sample Set	Banding Pattern	Radius (nm)	Days 10	20	30	40	50	60	70
D. Fishery Mortalities (Altered only)	Uniform (n=258)	30	4	5	6	7	7	7	8
		60	16	16	17	23	23	25	28
		90	22	24	31	38	39	55	57
		120	28	32	46	67	67	77	77
		150	37	45	58	90	102	102	102
		180	43	58	81	110	129	138	142
		240	51	83	128	163	193	221	229
		300	71	106	166	212	252	291	321
	Banded (n=54)	30	4	4	4	4	4	4	5
		60	11	11	16	16	16	17	19
		90	19	21	22	22	23	28	32
		120	26	27	32	35	37	42	48
		150	31	34	43	48	51	53	63
		180	34	38	50	61	67	75	91
		240	41	62	74	103	115	134	167
		300	56	80	120	143	163	179	247
E. Bow-riding Biopsies (Altered only)	Uniform (n=62)	30	0	0	0	0	0	0	0
		60	0	0	1	1	1	1	2
		90	0	1	2	3	4	4	5
		120	1	4	5	7	9	11	12
		150	1	4	9	11	13	16	20
		180	2	7	12	14	16	26	32
		240	10	19	23	37	43	45	53
		300	23	49	61	77	95	104	117
	Banded (n=13)	30	0	0	0	0	0	0	0
		60	0	0	0	0	0	0	0
		90	0	1	2	3	3	3	4
		120	1	2	4	4	4	5	5
		150	1	4	4	4	4	5	5
		180	1	4	4	4	4	5	10
		240	6	9	9	9	9	10	21
		300	9	12	17	22	31	35	57

A.

Znum	RecNum	A/N	Dnum	Sampling	Sex	Set	Date
24926	1	A	19	t	m	2	8/20
24931		N	19	t	m	3	8/22
24937	2	A	29	t	f	6	8/28
24960		A	29	t	f	8	8/30
24964		N	29	t	f	11	9/3
24940	3	N	34	t	f	6	8/28
24948		N	34	t	f	7	8/30
25083	4	A	42	t	m	20	9/13
25113		A	42	t	m	21	9/14
24967	5	A	47	t	f	11	9/3
24968		A	47	t	f	12	9/5
25137	6	A	63	t	m	23	9/19
25142		A	63	t	m	24	9/20
25140	7	A	67	t	m	23	9/19
25143		A	67	t	m	24	9/20
25096	8	A	193	d		21	9/14
25119		A	193	t	f	22	9/15
25127	9	A	215	d		23	9/19
25145		N	215	t	m	24	9/20
25134	10	A	242	d		23	9/19
25148		N	242	t	m	24	9/20
25151	11	A	245	d		25	9/22
25174		A	245	t	m	27	9/25

B.

	1st capture	2nd capture	3rd capture
Altered (A)	10	7	0
Normal (N)	1	4	1

C.

	normal	altered	frac normal
Fishery mortalities	70	354	0.17
Bow riders	68	40	0.63
CHESS 1st capture	34	208	0.14
2nd or 3rd recapture	6	9	0.40

D. Chi Square above diagonal and permutation probability values below

P\chisq	morts	bow	1st	recap
Fishery mortalities		96.7	0.5	5.6
Bow riders	0.00		85.0	2.9
CHESS 1st capture	0.51	0.00		6.9
2nd or 3rd recapture	0.03	0.09	0.02	

Table 2. Tagged and recaptured animals. A. Data table. (Znum = specimen number. RecNum = recapture number. A/N = expression pattern judged altered or normal. Dnum = individual number. Sampling [d = drive-thru, t = tagging / bleeding]. Set = set number.) B. Frequencies of paired CHESS recaptures (animals skin sampled on 1st and subsequent recaptures) exhibiting normal and altered expression patterns. C. Comparison of frequencies of fishery mortalities, bow-riding biopsies, and CHESS biopsies (1st and subsequent recaptures) exhibiting normal and altered expression patterns. Note addition of three animals to 2nd or 3rd recapture cell. Those animals were not skin sampled on first capture. D. Pair-wise permutation chi-square tests of frequency differences (1000 permutations); chi-square values above diagonal, and probability values below. Bow-riding biopsies include only offshore stock.

A.

	normal	altered	frac norm
Fishery mortalities	70	354	0.17
Bow riders	68	40	0.63
Drive-thru	15	151	0.09
Tagging/bleeding	7	47	0.13
Pole Spear	12	10	0.55

B.

P\chisq	mort	bow	drive	tag	pole
Fishery mortalities		96.7	5.4	0.4	20.2
Bow riders	0.00		90.1	3.2	0.5
Drive-thru	0.02	0.00		0.7	32.7
Tagging/bleeding	0.58	0.00	0.44		14.4
Pole Spear	0.00	0.47	0.00	0.00	

Table 3. CHESS sampling methods. A. Frequencies of specimens whose expression pattern was judged normal and altered stratified by fishery mortality and bow-riding biopsies and sampling methods within CHESS biopsies. Bow-riding biopsies include only offshore stock. See text for how sampling methods differ. B. Pair-wise permutation chi-square tests of frequency differences (1000 permutations). Chi-square values above diagonal, and probability values below.
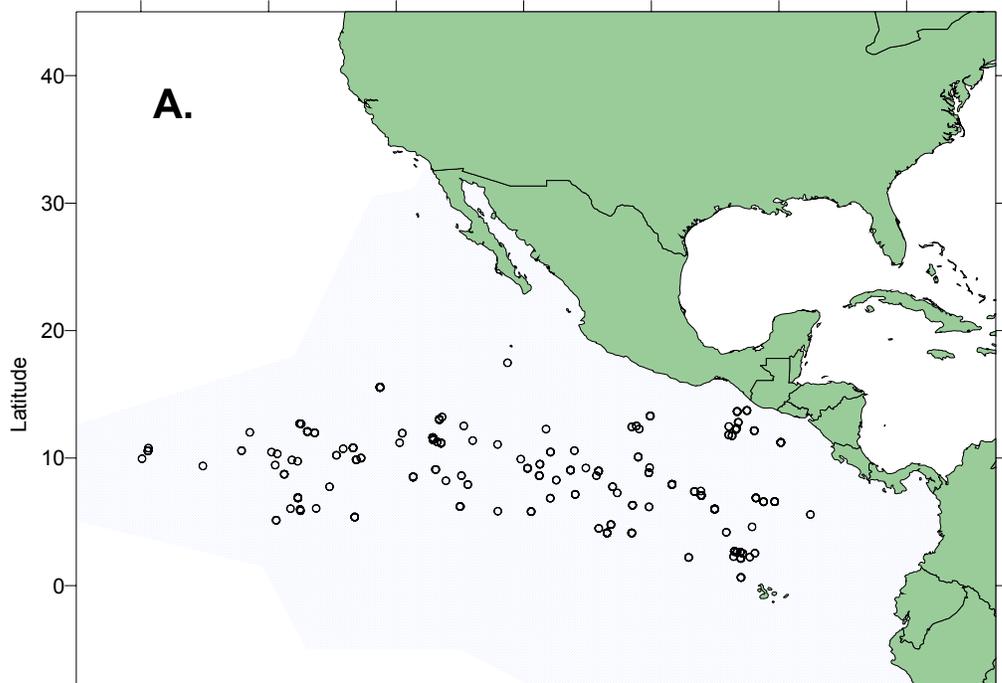
Figure 1. Spotted dolphin sampling locations. ETP boundaries stippled. A. Fishery mortalities (all offshore stock). B. Bow-riding biopsies (o = offshore; □ = ?; + = coastal stock) and CHESS biopsies (all offshore stock).

CHESS samples

Figure 2. Sightings where a biopsy was taken and sightings where a biopsy was not taken (● = bow-riding biopsies; + = sightings with no biopsy). In most cases but not all, when bow-riding animals were present, a biopsy was taken.
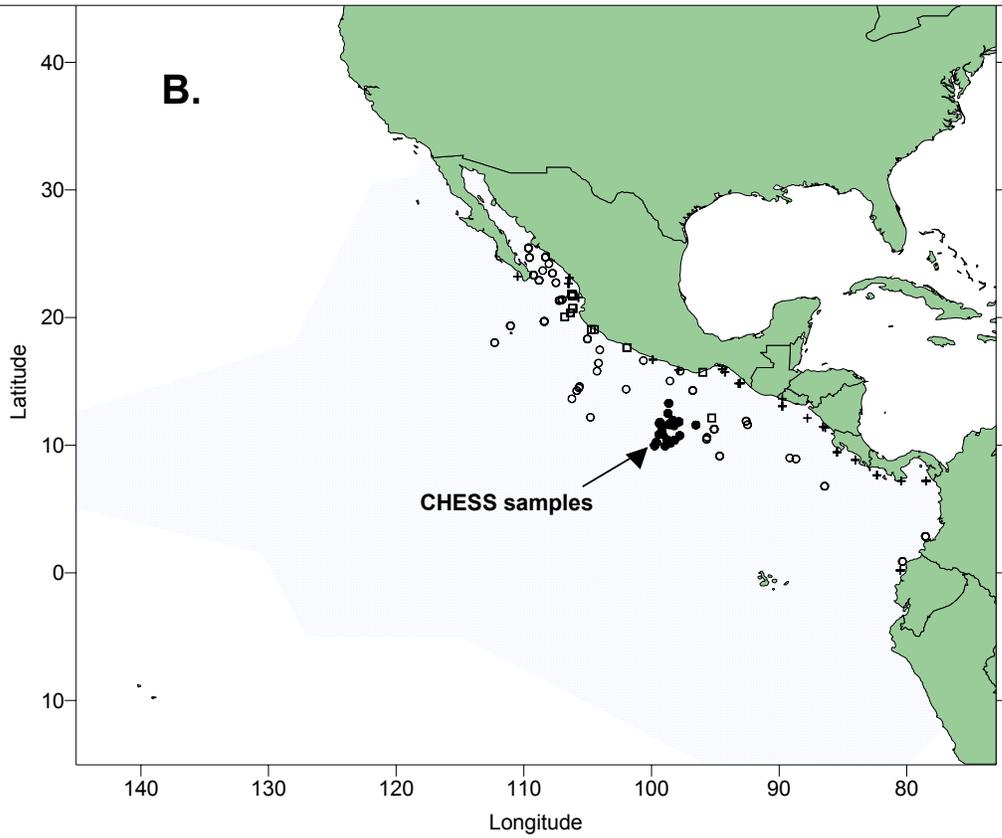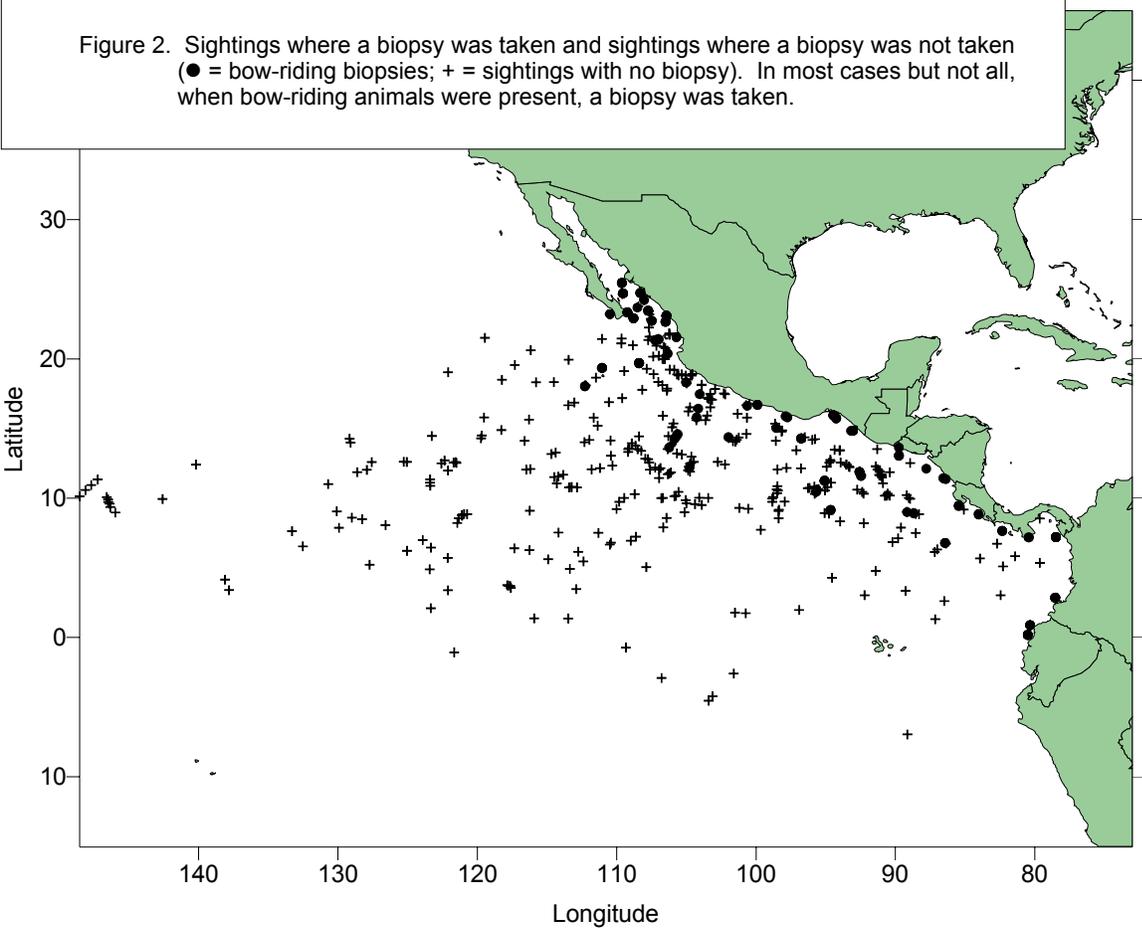
Figure 3. Distribution of samples exhibiting a normal pattern (o) and an altered one (x).
A. Fishery mortalities. B. Bow-riding biopsies. C. Chess biopsies.
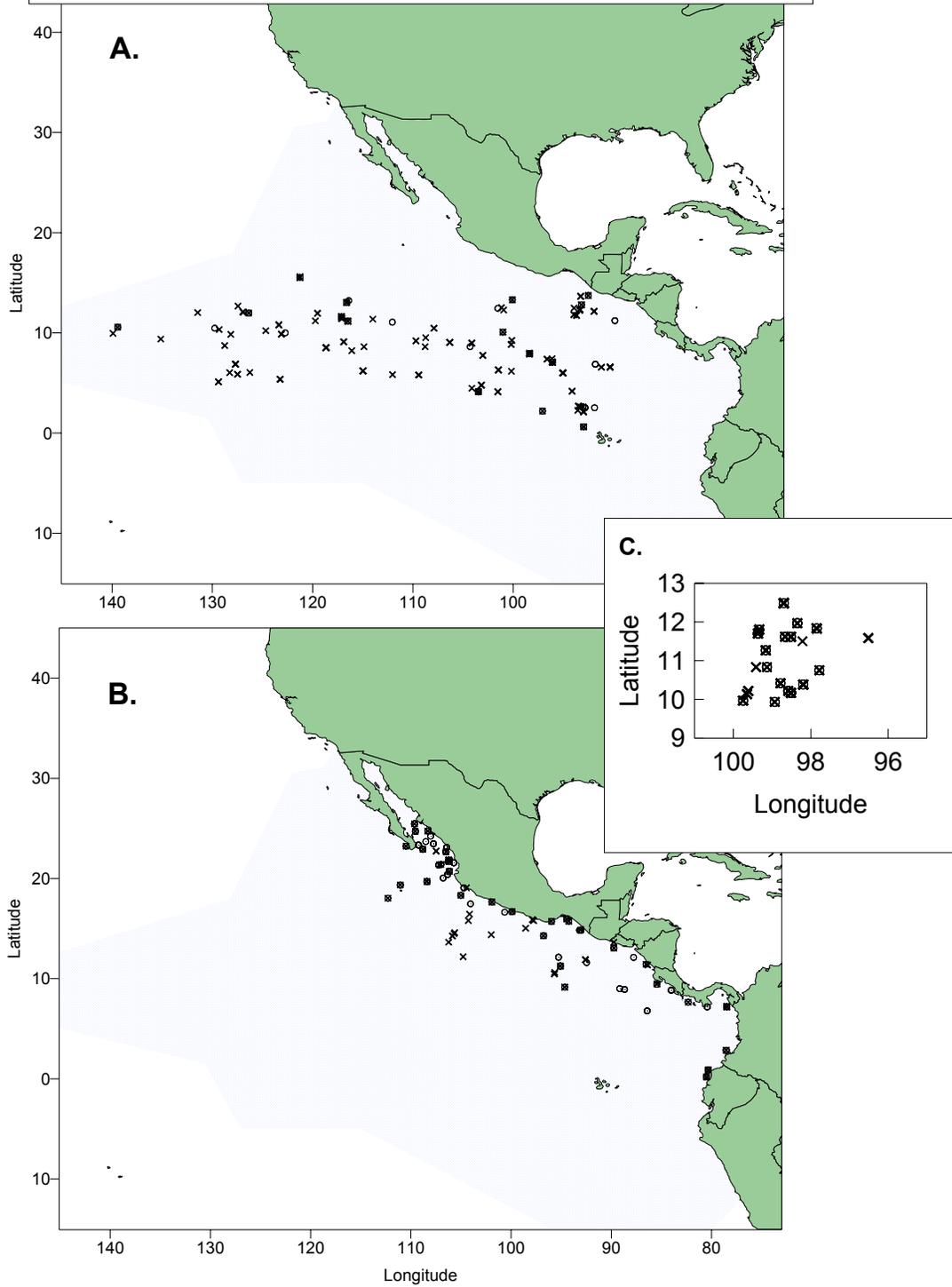
APPENDIX – Responses to Reviewer Comments

Five reviewers (selected by an independent organization) examined this document (CIE-S07, version 7) and submitted independent reports. The current version (version 8) attempts to address the concerns the reviewers specifically directed to the core subject of the study—comparing stress and effort in a sample of spotted dolphins. These concerns focused on the imprecision in the estimate of effort. The current version was edited to acknowledge this and to emphasize that effort was a relative measurement used to compare *among* samples *within* the three sample set groupings.

The methodology for estimating stress was the subject of another report, and the concerns expressed by the reviewers regarding this methodology are addressed in that report.

One reviewer suggested using a power analysis to determine whether sample size was an explanation of why the "CHESS patterns did not show a clear relationship to set exposure (Mann review)." However, I do not think a power analysis is appropriate when the null hypothesis is rejected, at least not a straightforward one power analysis. In the examination of the CHESS biopsies, the problem was that some space / time effort windows showed a significant positive relationship and others showed a significant negative one. I think the issue is the lack of consistency among the 56 sampling windows likely due to the limited number of independent sets observed, rather than a strict question of low statistical power leading to an inability to reject a null hypothesis of a lack of relationship between fishery effort and stress.