FATTY ACID SIGNATURE ANALYSIS AS A POTENTIAL FORENSIC TOOL FOR MANATEES

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**Project Title:** Fatty Acid Signature Analysis as a Potential Forensic Tool for Manatees

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**Project Goals:**

In August, 2003, the Marine Mammal Commission hosted a consultation entitled *Future Directions in Marine Mammal Research.* The goals of the consultation included assessing research needs for issues of critical concern for marine mammal conservation over the next few decades. Among the ten principal topics considered by consultation participants, four dealt explicitly with health, stress, disease and trauma, and two others considered effects of habitat change and climate change on marine mammal health and well-being. It was clear that the workshop organizers and participants viewed the general issue of marine mammal health as critical. It was also apparent that scientific efforts to date have provided an incomplete picture regarding marine mammal health and the effects of various stressors. As a result, the conference proceedings report recommended that steps be take scientifically and programmatically to address deficiencies.

Identification of cause of death or effects of anthropogenic and environmental stressors for marine mammals, in general, is often difficult for a variety of reasons. For Florida manatees (*Trichechus manatus latirostris*) a comprehensive long-term program conducted by the Florida Fish and Wildlife Conservation Commission acquires and assesses cause of death of all
individuals reported dead each year. The program has received praise from wildlife pathologists, veterinarians and epidemiologists, however, significant gaps remain in scientists' understanding of disease and health in manatees (U.S. Fish and Wildlife Service 2001). Nonetheless, the extensive assessments that already occur (gross pathology, histopathology, life history analyses) for Florida manatees make the species an excellent choice for which ground-truthing of new approaches to assess health or disease can be done. In other words, lessons learned and techniques developed to facilitate understanding of manatee health, disease and mortality may be applied more generally to other marine mammals. The objectives of this proposal were to examine the fatty acid and lipid class profiles of the livers of manatees recovered for necropsy in Florida, and to assess the individual fatty acid profiles for each animal in each cause of death category. Parametric and non-parametric statistical analyses were used to identify any patterns or trends for hepatic fatty acid expression that might be indicative of a particular cause of death. Our samples included animals from four causes of death: 1) red tide exposure, 2) cold stress, 3) prolonged illness and injury, and 4) quick death. For the latter category, we assumed that the animals were generally healthy at the time of death. A total of 74 manatee livers have been made available for this study.
Introduction:

Fatty acid signature analysis (FASA) has recently been used as a tool by which to assess foraging ecology of marine mammals (see, for example, numerous papers by S. Iverson and her colleagues, and O. Grahl-Nielsen and his colleagues). Rarely for marine mammals has FASA been used to assess other questions, including effects of stress or disease (Wetzel and Reynolds 2004).

Studies of other species have, however, suggested that changes in fatty acid constituents may be associated in marine organisms (no marine mammals) with environmental change, stage of larval development, or exposure to contaminants (Grahl-Nielson and Bamung 1985). In addition, in mammals, chemical changes in perinodal adipose tissue may affect activity of lymph nodes (Mattacks et al. 2004; Pond, in press). Specifically, in humans (Siguel and Lerman 1994, 1996; de Gomez-Dumm et al. 2003), chronic stress or certain disease conditions appear to be correlated with changes in hepatic fatty acids.

Methods:

Sample acquisition:

The FWC Fish and Wildlife Research Institute’s Marine Mammal Pathobiology Laboratory (MMPL) conducts necropsies for all recovered manatee carcasses in the state. The (MMPL) sent Mote Marine Laboratory a total of 74 samples of five to ten gram size pieces of frozen liver from freshly dead manatees diagnosed as having died from four different causes. In addition, data sheets providing full information regarding each manatee sampled for the study was provided to Mote personnel.

Chemical analysis:

The chemical analyses followed the methods described by Wetzel and Reynolds (2004).
Extraction of Lipids and Derivatization to Picolinyl Esters

Approximately 1.0 g of liver was extracted employing a modified Folch extraction (Folch et al. 1957) using dichloromethane and methanol with butylated hydroxytoluene added as an antioxidant. Picolinyl ester derivatives are prepared via a modified Destaillats and Angers (2002) method. Whole lipid was added to a homogenized mixture of 3-hydroxymethylpyridine and a solution of potassium \textit{tert}-butoxide in tetrahydrofuran. The lipid mixture was then incubated at 45 °C for 30 min. The organic phase was washed with a 2.5% sodium bicarbonate solution, collected, dried over anhydrous sodium sulfate and evaporated under a stream of nitrogen. The sample was re-dissolved in hexane. Further purification of the extracts was carried out using a Florisil™ column to eliminate ancillary compounds such as sterols, fatty alcohols, hydrocarbons and residual derivatization compounds that have the potential to co-elute with fatty acids. The sample was finally derivatized and analyzed in triplicate.

\textit{GC-MS analysis}

All samples were analyzed by a Thermo Finnigan DS Q quadrapole GCMS, equipped with a 30-M x 0.25 id x 0.25 μm film thickness, DB5 fused silica capillary column with helium as the carrier gas. The mass spectrometer was scanned from mass 50-500 in 0.5 s at an ionization potential of 70 eV. The program rate for the picolinyl ester analysis begins at 80 °C for 2 min, increased from 80 °C to 185 °C at 7 °C•min\textsuperscript{-1}, was held for 5 min, then from 185 °C to 260 °C at 5 °C•min\textsuperscript{-1}, held for 5 min, and from 260 °C to 325 °C at 5 °C•min\textsuperscript{-1}, and held for 10 min.

\textit{Lipid class analysis}

A subset of the liver samples were to be analyzed for lipid class using an Iatroscan, an analytical instrument that permits assessments of lipid class composition (e.g., triglycerides, wax
esters, phospholipids, free fatty acids, etc.). Examination of lipid class would complement and supplement the analyses of the individual fatty acids and may help demonstrate diagnostic changes associated with stress or disease.

**Statistical analysis**

Statistics were computed to compare each of the hepatic fatty acids between the red tide (RT) manatees versus the manatees which died from other causes. There were 35 FA variables, 8 of which were excluded from the analysis because they were detected in less than 10% of the samples (i.e. detected in 3 or less samples). Because many of the fatty acid variables were not normally distributed, a nonparametric test (Mann-Whitney) was employed. Because there were multiple variables, a sequential Bonferroni correction was applied to control experiment-wise error.

In order to visualize differences between RT and non-RT samples, a Principal Components Analysis (PCA) was used to transform the large number of FA variables into a smaller number of uncorrelated variables. The correlation matrix was used to compute principal components because in many cases the values of FA variables varied by several orders of magnitude, and Analysis of Variance (ANOVA) was conducted to compare PC1 scores between samples from manatees with differing causes of death.

**Results:**

Assessments of the manatee liver samples indicate that:

- The fatty acid profiles of the manatees assigned to the brevetoxin category are distinctive;

- Some fatty acids appear uniquely in animals suffering from prolonged injury or exposure to cold stress;
• Some animals assigned to other mortality categories demonstrate the “brevetoxin fatty acid profile”. On subsequent examination of the necropsy reports, we found that those moribund animals were recovered around the time of heavy red tide blooms.

There were approximately 39 different fatty acids found in the manatee liver lipids. The numbers of fatty acids found in each sample were generally higher in the red tide exposed animals than in the other three causes of death samples (Figure 1). This suggests a) the animals are converting endogenous fatty acids into a variety of other fatty acids not typically found in most other causes of death, b) that the fatty acids are exogenous in nature, perhaps coming from the fatty acids associated with inhaled or ingested red tide cells, or c) some other source or mechanism resulted from the exposure to red tide.

Figure 2 represents the total suite of fatty acids found in four liver lipid samples. These samples were from two red tide exposed animals and two quick death animals. While there are generic fatty acids found in all four samples in similar ratios, the red tide exposed animals also expressed a large number of fatty acids that were characteristic of that cause of death profile only.

We looked at the number of indicator fatty acids in each of the samples. The FA were scored from -100% to 100% by their occurrence in the four cause-of-death classes. This was calculated as the percent presence in each class added to the negative percent presence in the other classes, e.g. a FA present in every RT sample and absent in all others gets a +100% for RT. A FA present in every sample gets 0%, i.e. no bias towards any class. The only FA that showed strong correlations to a class were from the red tide animals. Ten were over 50% and these are plotted as (possible) indicators of red tide (Figure 3); many of the hepatic fatty acids (14:0, 14:1n5, 15:0, 16:0, 16:1n7, 16:1n9, 17:0, 17:1n8, 20:0) differed significantly between
manatees that died from red tide and manatees which died from other causes. However, there were four other manatees which also seemed to have a large number of these indicator fatty acids. All except one of the samples, a cold stressed animal, died around the time and areas of significant red tide blooms.

Principal Components Analysis (PCA) was used to transform the large number of FA variables into a smaller number of uncorrelated variables. The correlation matrix was used to compute principal components because in many cases the values of FA variables varied by several orders of magnitude. By graphing the first two principal components (PCs) and labeling the samples by cause of death (Figure 4), it can be seen that the first PC almost completely separates the samples from manatees which died during red-tide events from manatees which died from other causes. The PC1 scores were > +2.0 for manatees which died during a red-tide event, while the majority (all but the four “non-conformers” mentioned earlier) of the samples from other causes of death had PC1 scores near or below zero. This first PC accounted for the largest proportion (33%) of the variance in the data.

The loadings of the FA variables for PC1 are given in Table 1. The most significant positive loadings (> 0.80) were for 14:0, 15:0, 16:1n7, 16:1n9 and 17:1n8. Loadings for 14:1n5, 16:0, 17:0 and 20:0 were slightly less significant, but still greater than 0.60. Only 18:0 showed a substantial negative loading (< -0.74) on PC1. These results are consistent with the MW test results which indicated significant differences between red tide and non-red tide samples for the same fatty acids. The exception is 18:0, which was not found to differ significantly between red-tide and non red-tide samples by the MW test.

An Analysis of Variance (ANOVA) was conducted to compare PC1 scores between samples from manatees with differing causes of death. As expected, the ANOVA showed that
there were significant differences (p<0.0001). A follow-up Tukey’s honestly significant differences (HSD) test was conducted for pair-wise comparisons between groups. PC1 scores for the RT samples differed significantly from all other groups (p=0.0002, p=0.002, p=0.0002 for RT vs. CS, RT vs. LT, RT vs. QD, respectively) but did not differ significantly between the other three groups (Figure 5). This suggests that RT samples could be discriminated from non-RT samples very well by a function of the hepatic fatty acids: 14:0, 14:1n5, 15:0, 16:0, 16:1n7, 16:1n9, 17:0, 17:1n8, and 20:0.

Discussion:

Identifying fatty acid alterations in animals, particularly humans, has been well documented as a clinical diagnostic marker of certain stressors, including disease and exposure to toxicants. Although our sample size is small at this point, our results indicate clearly that manatees are likely to respond in similar ways to certain stressors, including exposure to biotoxins from red tides.

Conservation Benefits:

Management decisions are made regarding Florida manatees based on the “best available scientific information” including locations and causes of death. Understanding what kills manatees allows managers to focus on mitigation measures that have the potential to reduce specific mortality causes. Because cause of death for manatees can only be determined in about 50% of cases, the development of forensic tools that may allow better determination should be of great value to managers.

Our results demonstrate that hepatic fatty acid profiles are a useful forensic tool to help to determine when deceased manatees were exposed to brevetoxin. In addition, our results suggest that this approach may be useful in assessing when manatees die from other causes as well.
Finally, our results demonstrate that some proximal causes of death determined at necropsy may have been influenced by an animal’s prior exposure to brevetoxin.

**Future Directions:**

Our research will progress in the near term in three ways:

- Expanding the number of manatee liver samples analyzed;
- Assessing hepatic fatty acid responses in other marine mammals exposed to harmful algal blooms; and
- Attempting to determine whether analysis of fatty acids in blood (which can be obtained from living manatees) may provide the same sorts of insights as analysis of fatty acids and lipid profiles in liver.
Literature Cited:


List of Tables:

Table 1. PC1 loadings and results from Mann-Whitney test to compare fatty acids between red-tide and non red-tide samples.

List of Figures:

Figure 1. Number of fatty acids per category of death.

Figure 2. Individual fatty acids profiles of red tide and non red tide associated deaths in manatee liver.

Figure 3. Number of indicator fatty acids for each animal in each category of death.

Figure 4. PC1 versus PC2. Red squares represent RT samples, blue circles represent samples from manatees that died from other causes.

Figure 5. Group means for PC1 score. RT samples differed significantly from all other groups (Tukey’s HSD pairwise, all p<0.01), but none of the other groups differed significantly from each other.
Table 1. PC1 loadings and results from Mann-Whitney test to compare fatty acids between red-tide (red) and non red-tide (black) samples.

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<th>FA</th>
<th>MW p-value</th>
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