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REPLY COMMENT

Reply to Comment on Grahl-Nielsen et al. (2003): sampling, data treatment and predictions in investigations on fatty acids in marine mammals

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Knowledge of predator-prey relationships for marine mammals is a prerequisite for understanding the ecology of these top marine predators and for appropriate management of marine resources. Sara Iverson's research group at Dalhousie University and her research partners elsewhere have dedicated considerable resources to attempt to develop a method for determining the diet of marine mammals via fatty acid (FA) signature analysis, FASA (see references in Thiemann et al. 2004a, this volume). The most outspoken skepticism regarding the usefulness of FASA for this specific purpose has perhaps been put forward by our group based at the University of Bergen and the Norwegian Polar Institute. With much at stake, it is understandable that Iverson and co-workers are sensitive to criticism implied by our data and our interpretation of it. Discourse on our disparate views has occurred on several occasions including Smith et al. (1997), Thiemann et al. (2004b) and now in the Comment by Thiemann et al. (2004a) to our paper on polar bears (Grahl-Nielsen et al. 2003).

The criticism of Grahl-Nielsen et al. (2003) by Thiemann et al. (2004a) focuses on 3 main issues; they contend that (1) we use an inappropriate tissue sampling protocol, (2) our statistical approach is invalid and (3) our conclusions are unwarranted because we fail to deal with expected metabolism of fatty acids in mammalian predators. The contents of Thiemann et al. (2004a) are closely tied to 2 publications from Iverson's research group, published after Grahl-Nielsen et al. (2003), Thiemann et al. (2004b) and Iverson et al. (2004), so this reply addresses these publications briefly, in addition to the subject matter raised directly in Thiemann et al. (2004a).

Sampling protocol. Thiemann et al. (2004a) state that a problem exists in Grahl-Nielsen et al. (2003), because we performed non-representative sampling from predator and prey lipid tissues. However, appropriate sub-sampling of the blubber column (or adipose layer) depends upon the question being asked. Our first question was whether the adipose tissue of polar bears is uniform through the whole tissue layer; sampling potentially distinct zones seemed to us to be a logical approach. Based on previous studies on other marine mammals, we suspected that there would be differences in composition with depth, although we thought that polar bears may be somewhat more homogeneous than seals and whales in this regard, because the adipose tissue of polar bears does not perform all of the structural and physiological roles that the blubber layer of these other groups does (see Pond et al. 1992). Hence, we took sub-samples from 3 parts of the adipose layer of the bears—innermost, middle and outermost—to explore potential differences. We did not suggest that these analyses represented the entire blubber column composition, as implied in Thiemann et al. (2004a).

Our second question was how the prey influenced the composition of the bear's adipose tissue. Thiemann et al. (2004b) give somewhat mixed messages about what an appropriate protocol might be for looking at the influence of diet via FA analyses. They suggest that the inner half of the blubber column of cetaceans

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should be analyzed, while in seals either full-depth or inner-half blubber FA composition must be accurately determined. But, they also state that the innermost blubber responds most rapidly to a shift in diet. Even in species where there is significant stratification, distinct layers do not exist. There is a continuous although not necessarily linear gradient throughout the blubber column (Olsen & Grahl-Nielsen 2003). We chose to be conservative in our study, focusing on the region where diet is most likely to have its strongest impact—in the innermost part of the adipose layer, to study the potential influence of prey on polar bear FA composition.

The seal samples used in our study had been prepared and analyzed, before the polar bear samples were available, for comparative work on FA composition among seal species. The sub-samples of the blubber columns of the various seal species did include the whole blubber layer (the entire core), and this was appropriate for using these samples to represent polar bear prey, because the total composition of the FA from these animals was of interest with respect to what the bears consumed (which is usually the whole blubber layer). Because these samples were from animals shot by hunters, it was possible to use more tissue and have many replicates. We performed replicates on the seals in order to have a greater appreciation of variability. There was no attempt to 'grossly inflate' our sample sizes or misrepresent our data. Our multiple sampling of individuals was very clearly indicated in the text, figures and tables, and the number of animals and samples (or sub-samples) was carefully reported throughout the manuscript. In hindsight, homogenized whole blubber cores would have been the best approach for this study with respect to the seal samples.

Thiemann et al. (2004a) also raised a question regarding the size of the sub-samples (approximately 20 mg) used in our study. Tissue samples taken from living animals will of necessity be small, and sub-samples will of course be smaller yet. If small samples were not functional, FA methods could only be applied to harvested species. However, we are confident that the size of the samples used in our polar bear study was adequate. We have explored this question in the laboratory. Multiple replicates of small size compare well with each other and with larger samples of the same tissues, as long as the depth from which the material was taken remains consistent.

It must be recognized that sub-sampling a fat layer or blubber column is a subjective task, no matter how carefully it is approached. In living animals some of the innermost lipids are quite fluid and some are undoubtedly lost in sampling. Also, the columns extracted from living animals are vertically stretched to varying degrees, so even if biopsy width is standardized, samples from the same depth in different columns may represent somewhat different proportions of the blubber column as it stood in the animal. The FA composition of the blubber also differs in different areas of the bodies of marine mammals (Käkelä & Hyvärinen 1996, Olsen & Grahl-Nielsen 2003), although this variation is smaller than the vertical differences in the blubber column. The actual gradient is also dependent on the state of the animal—for example, whether it is in a fattening or fasting period. We have found that the inner layer of fasting seals has a FA composition that is similar to the composition in the middle layer of seals in a fattening period. All of these issues introduce some imprecision to FA analyses, especially when they are based on small samples taken from living animals.

Statistical treatment of the data. Thiemann et al. (2004a) are correct in being critical of how we approached the exploration of individual FAs among species via the use of *t*-tests. These tests are robust, but larger sample sizes do of course have greater potential to identify differences between groups. We should have at least used a blocked method that accounted for the source of the tissue sub-samples at the level of the individual. After receiving their Comment, we repeated these analyses using a single averaged value for each animal when comparing the seals' blubber with the polar bears' adipose tissue for each FA by means of Student's *t*-test. The results were not substantially different from those reported in our paper. We found that 3 of the 28 FAs were not different at the 99% level for the ringed seals, 8 for the harp seals, and 8 for the bearded seals. Thus, the majority of the FA did occur at significantly different ($p < 0.01$) proportions when sample sizes reflected the number of individual animals analyzed, so our conclusions remain unaltered. We should of course also have reported exactly what statistical test was producing our results at each stage, and we regret that this was not clear in the manuscript.

Thiemann et al. (2004a) maintain that our arguments 'rely almost exclusively on multiple univariate comparisons', and they characterize our use of PCA as an 'invalid approach to the analysis of the data' (p. 298), suggesting that PCA cannot be used when the number of variables exceeds the number of samples. In Grahl-Nielsen et al. (2003) we use multivariate PCA both qualitatively (Fig. 2) and quantitatively (Fig. 4) in the comparisons between the bear adipose tissue and the various seal species, so our arguments are supported by multivariate as well as univariate statistics. Thiemann et al. (2004a) infer that we use an inflated dataset to obtain more samples than variables for our PCA. This is not the case. In the qualitative analysis, it is clearly stated (Grahl-Nielsen et al. 2003, legend to

Fig. 2) that the seal samples 'are projected onto the PC plot without being included in the principal component analysis' (p. 279), and in the quantitative analysis the RSDs of the seal samples are measured from the space-filling PC model for the samples of the inner adipose tissue of the polar bears.

The contention in Thiemann et al. (2004a) that PCA needs more samples than variables, also advocated on an earlier occasion (Smith at al. 1997), is fundamentally wrong. As S. Wold, one of the founders of chemometrics, states:

> The classical methods of multivariate analysis, namely multiple regression, linear discriminant analysis and analysis of variance were all developed around 1930. At that time measurements were expensive and therefore one made few on each object. Typically one compensated this by having many objects. Hence the data tables were long (n large) and lean (p small). Consequently, the data analytical methods at the time were developed for the situation with n >> p. After a while everybody was taught that one has to have many more objects than variables, and this is today almost a dogma in statistics and data analysis. The typical situation in 1983 in chemistry, biology, geology and medicine is, however, different. With the modern instruments and separation methods many measurements are made on each object. Each measurement is cheap and once one has an object in hand, one might as well measure many variables. The objects are now usually much more expensive than the variables (think of one brain operation compared with one GC peak). Hence, the data matrices in chemistry are today often short (n small) and fat (p large). The classical methods of statistics break down and chemists have to use other methods. Fortunately, the projection methods such as PCA give good results even when the number of variables far exceeds the number of objects. In fact, the object scores are better estimated the larger the number of relevant variables (for a given number of objects). This because the *t*-values are linear combinations of all the variables and thus have the character of weighted averages. And averages are more precise the larger the number of relevant elements on which they are based'
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> (Wold et al. 1984, p. 49–53).

A vast number of publications where analytical chemical results with more variables than samples are treated by PCA have been published since the introduction of chemometrics in the early 1970s (see Kowalski & Bender 1972).

Metabolism of fatty acids. Despite the tone of Thiemann et al. (2004a) and their identification of real, although somewhat minor concerns, it was heartening for us that they acknowledged that the most important part of this discussion is in fact understanding FA metabolism in the predator. After having advocated during their initial years in this field of research (both verbally at a variety of meetings, as well as in print; e.g. Iverson et al. 1995) that diet FAs are deposited without modification in the predator's blubber, it is refreshing that Iverson's group now acknowledges that 'predator FA profiles will be influenced by biosynthesis of certain FAs and by reduced deposition of other FAs' (Thiemann et al. 2004a, p. 299). We have always advocated this position, and—although Thiemann et al. (2004b) accuse us of ignoring this issue—most of the discussion in Grahl-Nielsen et al. (2003) is dedicated precisely to the changes that occur in the FAs when metabolized by the predator.

Experiments carried out by Iverson's group demonstrate nicely that there is some influence on seal blubber FA composition that can be traced to their prey. We have reported similar results for other species (e.g. Andersen et al. 2004). Thiemann et al. (2004a) are correct in stating that there is no evidence in our investigation for the conclusion that the composition of the adipose tissue of polar bears is independent of the diet—and there is no such conclusion in our publication. What we did conclude was that 'it will not be simple to deduce' relative effects of various prey species (Grahl-Nielsen et al. 2003, p. 281).

Our opinions diverge with respect to the 'precision potential' in deducing the diet of predators using FAs. Iverson and her group appear to be firm in their belief that a given prey species influences predator species' blubber composition in a very consistent, predictable way. They have created correction factors for the incorporation of individual FAs by a predator, established by feeding captive animals with the same diet over a long enough time for the blubber FA composition to be expected to be stable (Iverson et al. 2004). However, the correction factor, in addition to varying by prey species, also varies according to the predator. They have found different correction factors for harp seals fed herring than for grey seals fed the same herring. This clearly shows that there will be a species effect on the FA composition of various predators, even when they have identical diets. It therefore seems unreasonable to suggest that it is appropriate to apply calibration factors obtained from specific predator/prey experiments to identify other prey species consumed by other predators.

We remain open-minded regarding the potential of the QFASA method. Nevertheless, initial reflection suggests that if the method has any potential to work, correction factors for all possible prey species versus all possible predators would have to be established as a minimum. This can only be done via controlled experiments carried out with captive animals. It is obvious that this is an impossible task for many marine mammal species. Additionally, to apply accurate correction factors for analysis of a specific predator, does one not need to know the diet composition in advance? Assuming that this is somehow accounted for in QFASA—the composition of the predator's blubber may still be affected by a host of

variables, such as age, activity level, condition, health status, feeding/fasting state etc. This is in addition to all the possible variances in the prey. Energy density of prey varies with its chemical composition and especially its lipid content. The lipid content in a given fish varies with age (younger fish tend to store less lipids than older fish; see Paul et al. 1998), reproductive status (spawning versus non-spawning, see Dygert 1990), geographical area, season (seasonal variation in the energy content of mature herring is 3.9 to 13.0 kJ g^{-1}, a variation of over 300% mainly in lipid content; see Mårtensson et al. 1996). The digestive efficiency of marine mammals also varies with the lipid content of their food, with higher digestibility values for more lipid-rich prey (Mårtensson et al. 1996, Rosen & Trites 2000). In addition, lipid digestibility in pinnipeds varies considerably, depending on total lipid intake (harbour seals fed herring reduced lipid digestibility from 90 to 50% when lipid intake exceeded 60 g kg^{-1} d^{-1}, Trumble et al. 2003). To further complicate the picture, most marine mammals feed on more than one type of organism, even during a single meal, and digestion of mixed diets becomes even more complicated. Even if the QFASA method could conclude accurately, e.g. that 20% of the fatty acids in the blubber of a predator were derived from herring, it is still far from quantifying its diet. Another set of correction factors to attempt to estimate what this corresponds to in terms of quantity of herring ingested would be required. And once again, one would require substantial knowledge in advance regarding what had been ingested in order to apply the correct correction factors. A quantitative model, such as QFASA, will produce a result if the program and input parameters dictate the production of an end product. But, how closely this will reflect the real diet of marine mammals is currently, in our opinion, very uncertain. Nobody would be more pleased than us to have a method that allowed us to non-lethally and accurately identify the diets of marine mammals. But we remain skeptical regarding a simplistic solution to this very complex task.

We freely acknowledge that the FA of prey organisms to a degree affects the FA composition of the adipose tissue/blubber of the predator. However, we also believe that there are clear species-specific influences on the composition of these tissues. We remain firm in our belief that '…the differences in the FA composition of the different dietary items would have to be very large in order to leave a pattern that would survive the metabolic rearrangements in the tissues of the predator' and that it 'would not be simple to deduce' direct influences specific prey species on the FA composition of the adipose tissue of polar bears (Grahl-Nielsen et al. 2003, p. 281).

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