Zoo Biology 00: 1–9 (2012)

COMMENTARY

Metabolomics Has Great Potential for Clinical and Nutritional Care and Research with Exotic Animals

Alistair D. M. Dove*

Georgia Aquarium Research Center, Atlanta, Georgia

This essay explores the potential of metabolomics for exotic animal research in a zoological setting. Metabolomics is a suite of analytical tools aimed at gaining a holistic understanding of animal metabolism without prior knowledge of the compounds to be measured. These metabolic fingerprints can be used to define normal metabolism for an unstudied species, to characterize the metabolic deviation of diseased animals from the normal state over time, to identify biomarker compounds that best capture such deviations, and to measure the metabolic impact of clinical and nutritional interventions. Two approaches, nuclear magnetic resonance (NMR) and mass spectrometry (MS) provide large amounts of complimentary pure and applied biological data. Metabolomic methods hold great potential for researchers, clinicians, and nutritionists studying exotic and aquatic animals because they can produce a huge data return on research effort, and because they do not require much a priori knowledge of the animals' metabolism, which is so often then case in zoological settings. Zoo Biol. 00:1–9, 2012. © 2012 Wiley Periodicals, Inc.

Keywords: mass spectrometry; metabolism; metabolomics; nutrition; nuclear magnetic resonance; NAG 2011; veterinary

INTRODUCTION

Adding the suffix "-omics" to a root word has become a popular trend in biology to describe a holistic or all-inclusive, discovery-based approach to a given aspect of biochemistry or physiology. The trend began when the study of genes (genetics) scaled

*Correspondence to: Alistair D. M. Dove, Georgia Aquarium Research Center, Georgia Aquarium, 225 Baker Street, Atlanta, GA 30313.
E-mail: adove@georgiaaquarium.org

Received 03 February 2012; Revised 18 March 2012; Accepted 13 April 2012

DOI 10.1002/ZOO.21024
Published online in Wiley Online Library (wileyonlinelibrary.com).

up and gave rise to studies of entire genomes (genomics). Studies of how all this genetic information is then transcribed into mRNA gave rise to "transcriptomics" and studies of how all the RNA information is expressed as proteins gave rise to "proteomics." Similarly, "secretomics" is the study of all the molecules produced by secretory epithelia and "metabolomics" is the study of all metabolites in a biofluid sample.

The emergence of all of these new -omic fields has been facilitated by advances in high-throughput lab techniques and the bioinformatic software and computing power needed to handle the large amounts of data that they generate. For example, gene sequencing methods have advanced rapidly in recent years, from dye-termination methods, through massively parallel pyrosequencing [Huse et al., 2007], to the current generation of ion torrent approaches that can produce more high quality genome data based on less sample and at lower cost than ever before. On the computing side, increases in high-volume (> terabytes) data storage and cloud-based web applications have made it possible for large and distributed teams to work on complex -omic projects while preserving the integrity of information and making the data widely available to researchers and end users.

All of the -omic approaches have potential applications in exotic and aquatic animal biology in both natural and zoological settings, but one of the most useful approaches is that of using metabolomics to characterize the end products of animal metabolism. Metabolomic approaches have the potential to identify and sometimes quantify all the smaller (i.e., nonprotein) compounds in a biofluid sample simultaneously, without a priori knowledge of what these may be [Viant et al., 2003]. These compounds can include fatty acids, sugars, amino acids and other amines, nonpeptide hormones, coenzymes, signaling molecules and vitamins, even medications and other xenobiotics. By collecting snapshots of all these compounds at the same time, metabolomics can help to define normal homeostasis in exotic species, to prospect for useful biomarker compounds, to identify metabolically deviant individuals, to characterize the nature of their metabolic perturbations, and to track their progression relative to clinical and/or nutritional interventions. The rest of this essay will therefore consider practical considerations for veterinarians, nutritionists, and researchers contemplating a metabolomic approach to questions regarding the biology of exotic and aquatic animals, and provide a portal to the literature for interested researchers.

DISCUSSION

Practical Approaches to Metabolomics

One of the first questions to be addressed when considering a metabolomic approach is which biofluid sample should be used. Each fluid in an animal is likely to reflect metabolism relevant to the origins of that fluid, and therefore each can be expected to produce different results. Blood is the obvious choice because of its ubiquity in clinical assessments and the fact that it can reflect so well the systemic metabolism of the whole animal. Blood contains a substantial cellular fraction and large amounts of protein, however, so it cannot be used without a significant amount of sample preparation because these fractions will interfere with the analytical methods to be applied. The first step is to separate serum from the remainder of the blood constituents. In the case of animals with nucleated erythrocytes, this must be done promptly, lest the ongoing metabolism of the blood cells alter the biochemical

make-up of the serum after the blood sample has been drawn from the animal. Serum is still relatively high in protein, so it must then be deproteinized using one of several protocols, most of which involve chemical coagulation or precipitation followed by ultrafiltration or centrifugation. Further processing may then be required depending on the specific approach to be applied (see below), or the samples can be frozen at -80°C for future analysis.

Blood is not the only fluid that can be used for metabolomic studies. Urine, cerebrospinal fluid, and even saliva can be collected in relatively noninvasive fashion, and urine in particular may be more useful for cases of suspected excretory disease or studies of drug/toxin metabolism or clearance rates. Milk can also be used in a similar fashion. In cases where animals are euthanized for ethical reasons or as part of an experimental protocol, it is also possible to prepare cell-free filtrates from homogenized organ samples, to determine tissue-specific metabolic profiles. This more radical approach is probably most useful in cases where a particular target organ is suspected of involvement and requires a more mechanistic assessment than that afforded by a standard histopathological evaluation.

Once a biofluid has been settled upon, the next decision to be made is which metabolomic technique to use, because there are several available and each has its advantages and disadvantages. One of the most popular is proton nuclear magnetic resonance spectroscopy (^{1}H NMR). This method detects the hydrogen atoms in organic molecules based on their response to radio frequency bombardment when contained within a powerful electromagnetic field. The specifics of NMR are beyond the scope of this paper, so interested readers should consult [Viant et al., 2003; Solanky et al., 2005a; Coen et al., 2008] for further information. ^{1}H NMR provides structural information about the molecules in a mixture because the signal obtained from the protons is modulated by the proximity and geometry of other hydrogen atoms attached to the same molecule. Thus every molecule has a unique NMR spectrum that can be decoded manually or by computer software, in order to reveal the most likely underlying compound. ^{1}H NMR is also a quantitative technique. The major drawback of using ^{1}H NMR is that biofluid mixtures are complex and the spectra associated with each constituent compound are overlain on the same axis (called chemical shift and measured in ppm), such that disentangling the sample spectrum is at least computationally difficult and at worst practically impossible. It is possible to resolve spectra on a second axis (2D or JRES NMR); this is a more advanced and time-consuming analytical technique [Schroeder et al., 2007] but one worth pursuing if time and resources allow. Due to this confounding of ^{1}H spectra in complex mixtures, NMR is not the best approach for identifying specific biomarker compounds unless those compounds happen to occupy unique parts of the chemical shift spectrum where they stand alone, allowing for unequivocal identification. Despite the relative insensitivity of NMR as a technique for identifying specific biomarkers in complex mixtures, the overall spectrum of the mixture is still sensitive to holistic disturbance and the method is very good for defining population normals and deviations therefrom [Viant et al., 2003, 2005]. This is done by using multivariate statistics, typically principal components analysis (PCA), to collapse the metabolic spectrum of each sample into a single point in multidimensional space and then looking for independent variables that explain groupings of samples or deviations therefrom. This is shown in Figures 1 and 2 and discussed in more detail in the section below on metabolic trajectories.

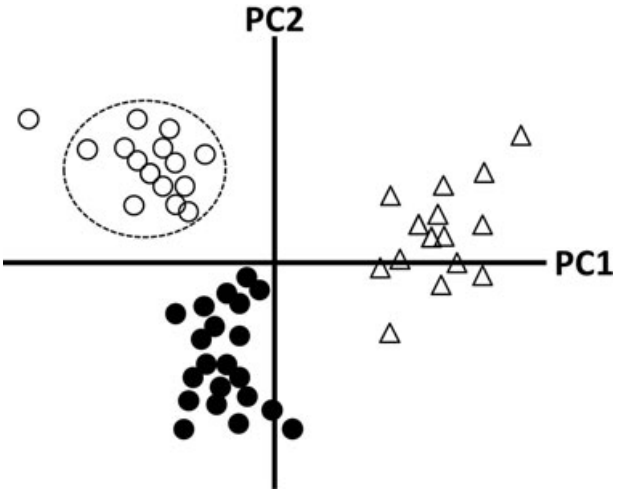

Fig. 1. Discriminating three populations based on metabolic fingerprinting of NMR spectral data using principal components analysis (PCA). Multidimensional metabolic fingerprints are collapsed onto the first two PC axes, which capture the majority of the variance in the data. The dashed ellipse represents a Normal Metabolic Operating Range (NMOR *sensu* Viant) for the open circle population: a region encompassing 95% of normal individuals.

Mass spectrometry (MS) is another suite of analytical methods that is useful in metabolomic studies. MS determines the mass to charge ratio of each constituent compound in the biofluid mixture, a ratio which is unique to compounds with a given molecular formula and arrangement of ionizable moieties. MS is not as good as NMR at providing spectra to capture subtle variations in overall metabolism determined statistically, but it is very useful for providing lists of candidate compounds in a sample, which is key when looking for specific biomarker compounds [Viant, 2007; Zhou et al., 2010]. On the other hand, MS does not provide any structural data to help identify which exact compound it is that matches that particular molecular formula. For example, MS may tell you that a compound with the formula $C_6H_{12}O_6$ is present in the sample, but it may not be able to tell you whether it represents glucose, fructose, or mannose, which all have the same formula but different structures. Two other important features of MS methods are that they cannot detect nonionizable compounds and that they are generally not quantitative; they can only say whether a compound is present in a sample, not in what amount.

For both NMR and MS methods, the processing and analysis of the resulting data can be a major challenge and should not be underestimated [Fiehn and Weckwerth, 2003; Kind and Fiehn, 2007; Coen et al., 2008; Prince and Pohnert, 2009]. Researchers, nutritionists, or clinicians interested in using metabolomic approaches should seek out collaboration with an experienced bioinformatician in order to assure data quality and expedite the analytical process. Thankfully, the profusion of -omic approaches in biology has all but assured the availability of willing collaborators at most major universities, whether they are in traditional biology/biochemistry departments, in medical research units, or even chemistry or physics departments.

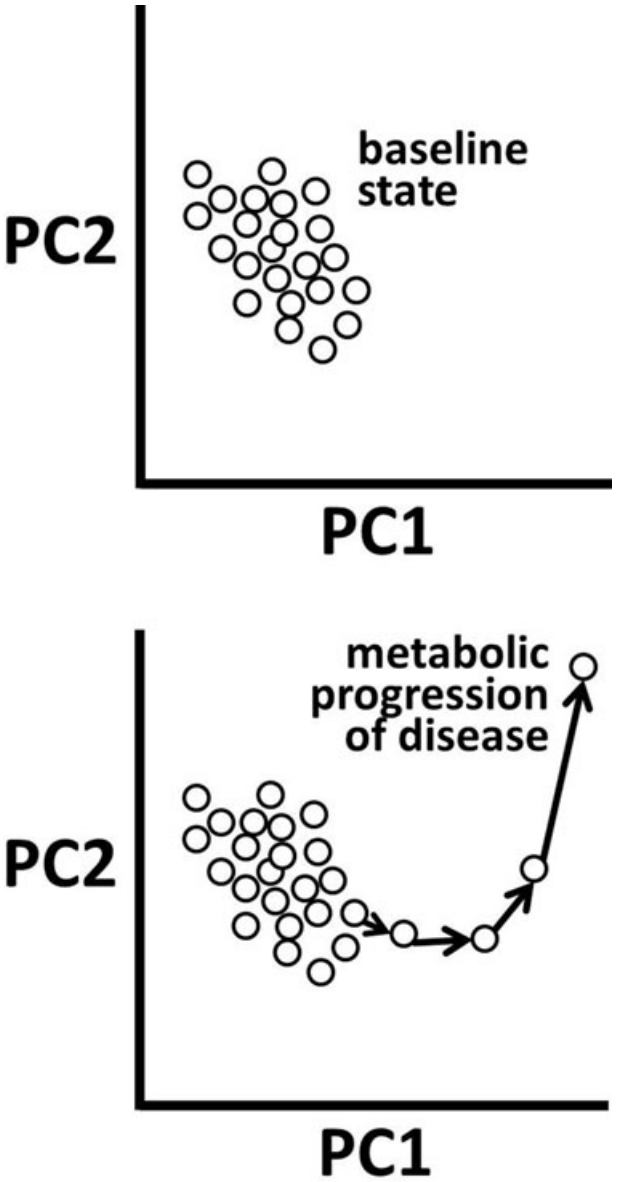

Fig. 2. Metabolic trajectory using PCA of ^{1}H NMR metabolomics data. (A) A cloud of data points in principal components space representing the normal or baseline metabolic condition of that animal. Each point is a snapshot of the whole metabolism extracted from a spectrum of binned ^{1}H NMR data of a single serum sample. (B) As disease progresses, the metabolism departs from the baseline condition on one or more PC axis. Examination of loading scores will reveal which parts of the spectrum (and therefore which metabolites) are driving the trajectory.

Applications of Metabolomics Methods

Whichever method or combination of methods is used, metabolomics can also differ markedly in its applications to the clinical and nutritional sciences. I will

consider three applications here: population studies, metabolic trajectories, and nutritional studies.

Population Studies

When dealing with exotic species, about which so little is usually known, learning about the presence or concentration of a given compound in blood or other biofluids of an individual animal is of pretty limited use. Is that compound supposed to be there or is it a xenobiotic? Is its concentration normal or has this animal lost homeostasis? There is really no way to tell, so one of the best applications of metabolomics in basic biology is to establish population values for each compound (either presence/absence, concentration, or both) and/or for the metabolic profile as a whole. Regarding the whole metabolism, a leading researcher in this field, Viant [2007] has termed this the Normal Metabolic Operating Range or NMOR, that region of PCA space that encompasses 95% of normal animals (Fig. 1). It is against these population measures that deviations can then be discriminated by marking data points according to various independent variables, which can help identify unhealthy animals, provide hints as to functions, or reveal useful biomarker compounds [Viant et al., 2003; Hines et al., 2007; Viant, 2007]. It is important to recognize that the NMOR is context specific. In other words, what represents NMOR for a group of bongos, for example, living in a zoo and feeding on a consistent nutritionist-formulated diet will likely not be the same as the NMOR for wild bongos feeding on a seasonally variable diet. In this way, deviations must be measured against NMOR for that context.

The laboratory effort required to do these sorts of population studies is typically not taxing; sample processing is relatively straightforward and a good NMR lab can create a large number of ^{1}H spectra in a relatively short period of time. The hardest part of population level studies of a metabolomic nature is data processing and analysis. In our MS studies [Dove et al., submitted] of the metabolomics of whale shark serum, we identified between 80 and 600 putative compounds per individual serum sample, and in concomitant ^{1}H NMR experiments, we created thousands of rows of data from just 60 serum samples. The spectral data are continuous, so before they can be analyzed statistically using PCA, they need to be grouped into discrete bins. For a larger population level study, this combination could lead to unmanageably large data sets and demand a lot of effort to ensure good data quality and robust biological interpretation of the results.

Identifying the normal constituents of metabolism in an exotic animal species can reveal new aspects of biology and identify compounds with potential as biomarkers of health [Vanravenzwaay et al., 2007; Collings, 2008]. By their very nature, these discoveries are largely serendipitous, since the analyses are done without a priori knowledge (i.e., they are what statisticians call "unsupervised"). In our NMR and MS studies of whale shark serum [Dove et al., submitted], we found the compound homarine (N-methyl picolinic acid) and noted that it varied in seemingly meaningful ways with whale shark health. The functions of homarine in whale sharks remain, however, unknown, because our study also represented the first time that this compound had been recorded from an elasmobranch. It seems reasonable to assume that the source of homarine in whale shark serum is dietary because this species is a planktivore and is fed large amounts of krill in the aquarium setting; homarine is most well known as a haemolymph metabolite of crustaceans [Gasteiger et al., 1955; Gasteiger et al., 1960;

Dall, 1971]. In one of the few studies of this compound in fish, however, Ito et al. [1994] found little correlation between the concentration of cyclic betaines in serum of several species and their concentration in each species' diet.

Focal Animal Metabolic Trajectories—a Clinical Application

Once a population average has been established for any analyte or for the metabolism in general, the possibility exists for recognizing outlying data. In population studies, these outliers would represent individual animals that were deviant from the "normal" condition and therefore worthy of further investigation [Viant et al., 2003]. But another approach is to look at the same animal through time, an approach that has been termed "metabolic trajectory" [Viant et al., 2005]. Consider a hypothetical animal in a zoological collection that has had routine blood samples taken throughout its history. Over time, a normal metabolic profile of that individual would develop and be expressed as a cloud of data points in PCA space (Fig. 2A). If that animal were to become sick, however, the metabolism would deviate from the normal profile and this would be reflected as outlier data points moving away from the cloud that represents the "normal" or baseline state (Fig. 2B). By literally connecting the dots in the time series, an investigator can track the loss of homeostasis and the progression into clinically significant disease, perhaps even to death. Importantly, it is then possible, using discriminant functions, to identify which parts of the ^{1}H spectrum are driving the deviation, which can in turn shed light on the nature of the disease or possibly reveal compounds that may be worth further investigation as biomarkers. For example, if the metabolic trajectory of an animal was driven by parts of the spectrum most commonly occupied by amino acids, then a problem of protein metabolism may be indicated. In its simplest form, a metabolic trajectory could be driven by a toxicant measured directly, or by metabolites of the toxicant, and for this reason care must be taken with these sorts of analyses, because the NMR spectrum will also capture medications or supplements (and their metabolites) given to the animal during the course of veterinary/nutritional intervention [Rosenblum et al., 2006].

Nutritional Applications

Metabolomics has found its greatest applications in toxicology, where the focus of research is on the impacts of exogenous compounds (toxicants) on the biology and health of animals [Viant et al., 2003; Robertson, 2005; Viant et al., 2006; Chen et al., 2007; Coen et al., 2008]. At their heart, though, nutritional studies are similar in nature, except that the exogenous compounds are not toxicants but the macro and micronutrient components of food [Gibney et al., 2005; Solanky et al., 2005a; Kullgren et al., 2010]. Nutritional metabolomics suffers from the same challenges as other metabolomic studies, the biggest of which are always the questions "Which compounds are truly endogenous" and "How do we tease out nutritional processes from the myriad other nonnutritional metabolic processes taking place in complex physiological systems?" [Gibney et al., 2005]. In animal nutritional studies in particular, there can be an additional dimension to these questions that derives from the presence of obligate microbial floras that may assist the animal in digestive processes, but may also obscure the origins of given metabolites and further complicate the biochemical snapshot [Gibney et al., 2005].

These questions can best be answered by controlling as many aspects of the system as possible. In other words, rather than using metabolomics to assess a previously unsampled population, it is better to apply metabolomic methods to gathering response data for a very controlled dietary study wherein the impacts of a specific dietary intervention are to be measured [Solanky et al., 2005a]. This is still a very young approach, having been first applied to human nutritional studies in 2003 [Solanky, 2003] and rarely since in animals (but successfully for Atlantic salmon, see [Solanky et al., 2005b] and [Kullgren et al., 2010]). Nonetheless, the potential research returns are great: new insight into animal responses to nutritional intervention and large libraries of metabolic data that can be mined in the future to answer similar questions as yet unimagined.

CONCLUSIONS

1. Holistic techniques of the sort that qualify for "-omic" status are no longer the realm of large, federally funded multiinstitutional research programs; they are continually being refined as tools that can be applied routinely in biological research.
2. Metabolomics tools are a terrific development for nutritional and clinical studies of exotic species, where the baseline understanding of metabolism is typically deficient relative to laboratory model species, domestic animals, or humans.
3. By identifying qualified collaborators with access to metabolomic tools and skills, researchers, clinicians, and nutritionists now have opportunities to make quantum leaps in scientific understanding of the metabolism of exotic species in zoological care.
4. This knowledge can then be leveraged for better understanding, management, and conservation of these same species in their natural setting.

ACKNOWLEDGMENTS

I appreciate the invitation of Mike Maslanka and Ann Ward to present the substance of this essay at the 2011 Nutrition Advisory Group conference in Kansas City, MO, in November 2011.

REFERENCES

Chen C, Krausz KW, Idle JR, Gonzalez FJ. 2007. Identification of novel toxicity-associated metabolites by metabolomics and mass isotopomer analysis of acetaminophen metabolism in wild-type and cyp2e1-null mice. J Biol Chem 283:4543–4559.

Coen M, Holmes E, Lindon JC, Nicholson JK. 2008. NMR-based metabolic profiling and metabonomic approaches to problems in molecular toxicology. Chem Res Toxicol 21:9–27.

Collings F. 2008. Novel technologies for the discovery and quantitation of biomarkers of toxicity. Toxicology 245:167–174.

Dall W. 1971. Role of homarine in decapod Crustacea. Comp Biochem Physiol 39:31–44.

Fiehn O, Weckwerth W. 2003. Deciphering metabolic networks. Eur J Biochem 270:579–588.

Gasteiger EL, Haake PC, Gergen JA. 1960. An investigation of the distribution and function of homarine (N-methyl picolinic acid). Ann NY Acad Sci 90:622–636.

Gasteiger L, Gergen J, Haake P. 1955. A study of the distribution of homarine (N-methyl picolinic acid). Biol Bull 109:345.

Gibney MJ, Walsh M, Brennan L, Roche HM, German B, Ommen Bv. 2005. Metabolomics in human nutrition: opportunities and challenges. Am J Clin Nutr 82:497–503.

Hines A, Oladiran GS, Bignell JP, Stentiford GD, Viant MR. 2007. Direct sampling of organisms

from the field and knowledge of their phenotype: key recommendations for environmental metabolomics. Environ Sci Technol 41:3375–3381.

Huse SM, Huber JA, Morrison HG, Sogin ML, Welch D. 2007. Accuracy and quality of massively parallel DNA pyrosequencing. Genome Biol 8:R143.

Ito Y, Suzuki T, Shirai T, Hirano T. 1994. Presence of cyclic betaines in fish. Comp Biochem Physiol B-Biochem Mol Biol 109:115–124.

Kind T, Fiehn O. 2007. Seven golden rules for heuristic filtering of molecular formulas obtained by accurate mass spectrometry. BMC Bioinformatics 8:105.

Kullgren A, Samuelsson LM, Larsson DGJ, Björnsson BT, Bergman EJ. 2010. A metabolomics approach to elucidate effects of food deprivation in juvenile rainbow trout (*Oncorhynchus mykiss*). Am J Physiol Regul Integr Comp Physiol 299:R1440–R1448.

Prince EK, Pohnert G. 2009. Searching for signals in the noise: metabolomics in chemical ecology. Anal Bioanal Chem 396:193–197.

Robertson DG. 2005. Metabonomics in toxicology: a review. Toxicol Sci 85:809–822.

Rosenblum ES, Tjeerdema RS, Viant MR. 2006. Effects of temperature on host−pathogen−drug interactions in red Abalone, Haliotis rufescens, determined by1 H NMR metabolomics. Environ Sci Technol 40:7077–7084.

Schroeder FC, Gibson DM, Churchill ACL, Sojikul P, Wursthorn EJ, Krasnoff SB, Clardy J. 2007. Differential analysis of 2D NMR spectra: new natural products from a pilot-scale fungal extract library. Angew Chem Int Ed 46:901–904.

Solanky K. 2003. Application of biofluid 1H nuclear magnetic resonance-based metabonomic techniques for the analysis of the biochemical effects of dietary isoflavones on human plasma profile. Anal Biochem 323:197–204.

Solanky K, Bailey N, Beckwithhall B, Bingham S, Davis A, Holmes E, Nicholson J, Cassidy A. 2005a. Biofluid H NMR-based metabonomic techniques in nutrition research-metabolic effects of dietary isoflavones in humans. J Nutr Biochem 16:236–244.

Solanky K, Burton IW, MacKinnon SL, Walter JA, Dacanay A. 2005b. Metabolic changes in Atlantic salmon exposed to Aeromonas salmonicida detected by 1H-nuclear magnetic resonance spectroscopy of plasma. Dis Aquat Organ 65:107–114.

Vanravenzwaay B, Cunha G, Leibold E, Looser R, Mellert W, Prokoudine A, Walk T, Wiemer J. 2007. The use of metabolomics for the discovery of new biomarkers of effect. Toxicol Lett 172:21–28.

Viant MR. 2007. Metabolomics of aquatic organisms: the new 'omics' on the block. Mar Ecol-Prog Ser 332:301–306.

Viant MR, Bundy JG, Pincetich CA, de Ropp JS, Tjeerdema RS. 2005. NMR-derived developmental metabolic trajectories: an approach for visualizing the toxic actions of trichloroethylene during embryogenesis. Metabolomics 1:149–158.

Viant MR, Pincetich CA, Tjeerdema RS. 2006. Metabolic effects of dinoseb, diazinon and esfenvalerate in eyed eggs and alevins of Chinook salmon (*Oncorhynchus tshawytscha*) determined by 1H NMR metabolomics. Aquat Toxicol 77:359–371.

Viant MR, Rosenblum ES, Tjeerdema RS. 2003. NMR-based metabolomics: a powerful approach for characterizing the effects of environmental stressors on organism health. Environ Sci Technol 37:4982–4989.

Zhou M, McDonald JF, Fernandez FM. 2010. Optimization of a direct analysis in real time/time-of-flight mass spectrometry method for rapid serum metabolomic fingerprinting. J Am Soc Mass Spectrom 21:68–75.