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Metabolomics of aquatic organisms: the new 'omics' on the block

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ABSTRACT: Environmental metabolomics can be defined as the application of metabolomics to characterise the metabolism of free-living organisms obtained from the natural environment and of organisms reared under laboratory conditions, where those conditions serve to mimic scenarios encountered in the natural environment. This approach has considerable potential for characterising the responses of organisms to natural and anthropogenic stressors. The current essay introduces environmental metabolomics, discusses the challenges of measuring metabolites, and then highlights the dynamic nature of the metabolome that can be exploited to provide a holistic view of an organism's health. Dealing with metabolic variability is a considerable challenge in environmental metabolomics. Here, I propose the concept of a normal metabolic operating range (NMOR), defined as the region in metabolic space in which 95% of individuals from a population reside, with stress identified as a deviation from the NMOR. Furthermore, I emphasise the importance of genotypic and phenotypic anchoring (e.g. knowing species, gender, age) to facilitate interpretation of multivariate metabolomics data.

KEY WORDS: Metabolomics · Environmental metabolomics · Environment · Phenotypic anchoring · Normal metabolic operating range · Fish · Invertebrate · Stress

INTRODUCTION

Metabolomics is the study of the endogenous low molecular weight metabolites within a cell, tissue or biofluid (termed the metabolome). 'Environmental metabolomics' is the application of metabolomics to characterise the metabolic responses of an organism to both natural and anthropogenic stressors that can occur in its environment. Although it is the newest 'omic' approach, and is therefore considerably less developed and utilised in marine environmental research than genomics, transcriptomics and proteomics, it potentially affords several benefits for assessing organism function and health at the molecular level. For example, metabolomics shares many of the advantages of the other omics approaches in that it enables a rapid, unbiased and simultaneous measurement of many tens, hundreds or even a thousand endpoints (i.e. metabolites), and therefore differs substantially from traditional biochemical methods that typically detect only 1 or a few metabolites. As a result, metabolomics is a particularly powerful approach for discovering biomarker profiles of toxicant exposure and disease, and for identifying the metabolic pathways involved in such processes. Metabolomics now offers us a systems-based approach for studying individuals in the marine environment. Other advantages that are specific to metabolomics include the high degree of functionality of metabolic measurements that can be directly related to an organism's phenotype, and the flexibility with which it can be applied to any organism irrespective of the knowledge of the genome for that species.

To date there have been only 10 publications that have applied metabolomics to aquatic species, which can be grouped into the study of biological stress (i.e. disease), chemical stress (i.e. toxicity), temperature stress (Viant et al. 2003a) and fish embryogenesis (Viant 2003). The toxicity studies include exposure of embryos of medaka *Oryzias latipes* (Viant et al. 2005, Viant et al. 2006a) and chinook salmon *Oncorhynchus tshawytscha* (Viant et al. 2006b) with the goal of evalu-

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ating metabolomics as a high-throughput screening tool for chemical risk assessment. Samuelsson et al. (2006) have utilised metabolomics to study the effects of ethinylestradiol, an endocrine disruptor, in juvenile rainbow trout *Oncorhynchus mykiss*. The effects of diseases on the metabolome have been studied in fish and an invertebrate, including a bacterial infection in Atlantic salmon *Salmo salar* (Solanky et al. 2005), liver cancer in dab *Limanda limanda* (Stentiford et al. 2005), and withering syndrome in red abalone *Haliotis rufescens* (Viant et al. 2003b, Rosenblum et al. 2005). Unexplored applications of metabolomics that could benefit from this rapid, unbiased systems-based approach include its use in environmental monitoring and in the aquaculture industry to optimise husbandry and productivity.

An important characteristic that in many situations sets the metabolome apart from the genome, transcriptome and proteome is the degree to which it varies under normal and stressful conditions. The metabolome is often the first to respond to anthropogenic stressors (e.g. pollutant exposure) and natural daily events (e.g. feeding), and in some cases no changes in the transcriptome and proteome occur. Furthermore, these metabolic changes can occur directly (e.g. oxidative stress associated with antioxidant depletion) or indirectly (e.g. by redistribution of energy reserves away from growth and reproduction towards cellular defence and repair). As discussed later, this large variation in the metabolome has a number of major ramifications for the applicability of metabolomics in environmental studies. First, however, it is important to address and clarify some issues relating to the measurement of metabolites.

MEASUREMENT OF THE METABOLOME

As stated above, metabolomics is the newest of the omic approaches and is still very much under development. This is particularly true for the methods used to measure metabolite levels. Metabolites, unlike genes, transcripts and proteins, are a highly physically and chemically diverse group of chemicals. Some, like glycine, are present at high concentration, have a low molecular mass and are extremely polar. Others, such as testosterone, have the opposite characteristics. Several thousand additional metabolites have intermediate or even more extreme properties. So, unlike the measurement of genes, transcripts and to some extent proteins (which are all polymers of nucleotide bases or amino acids), no one bioanalytical technique is capable of detecting all metabolites.

The 2 most widely used methods in metabolomics are ^{1}H nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry. A comparison of these techniques is beyond the scope of this commentary, so readers are referred to articles by Dunn & Ellis (2005), Pelczer (2005), and Villas-Boas et al. (2005). It is important to note, however, that both techniques have considerable value in metabolomics and that neither method has yet been fully developed for this application. Perhaps the most striking statistic that illustrates this point is that of the estimated several thousand metabolites in the cellular metabolome; current NMR methods are believed to detect only about 100 metabolites (less than 10%) and mass spectrometry up to approximately 1000 metabolites. It is therefore important for those engaged in environmental metabolomics research to stay acquainted with technological advances in this rapidly developing field, and to implement them as they occur. The 2 areas that are in most urgent need of development include methods to extend the coverage of the metabolome and an improved ability to identify and quantify metabolites unambiguously. Before leaving this topic, it is important to recognise a definitive advantage associated with measuring metabolites that stems from the conservation of metabolites across species. Any technical advances in the measurement of metabolites in one species will in general be applicable to all other species, and no *a priori* genomic knowledge is required. Exploitation of this fact is discussed below.

THE DYNAMIC METABOLOME

One of the most significant advantages of metabolomics is the close dynamic relationship that exists between the metabolites that are measured and the physiological status of the whole organism. For example, metabolomics includes the measurement of ATP and glycogen, which can vary as a function of the energetic status of an organism (Wasser et al. 1996). Metabolomic methods can also detect molecules like glutathione and ascorbate, which can change as a function of cellular redox status (Kristal et al. 1998). Steroids are another class of molecules that are of considerable interest, and so measurement of oestradiol and ketotestosterone could be used to help inform on the reproductive status of an organism (Noaksson et al. 2004). Taken together, as metabolomic technologies are developed to the point where many hundreds of metabolites are measured simultaneously, the exciting potential to rapidly assess many aspects of an organism's current energetic, oxidative and perhaps even reproductive status may be realised. Measurement of the genome and transcriptome are less able to provide this information, because genes and transcripts are not guaranteed to manifest themselves as functional

changes at the organismal level. Conversely, metabolomics is not useful for assessing population structure and genealogy of marine organisms, for which genomics is vital. Although the proteome can provide a window into functional organismal changes, the measurement of protein levels is still not able to provide such a direct link to physiology (such as energetic status) as can be achieved via the metabolome. The attempt to prove a causal relationship between metabolic biomarker profiles and an individual's Darwinian fitness (reproductive health, growth and survival) is, therefore, an important area of current and future research.

The ability of the metabolome to change so readily is not only a considerable strength but also creates a major challenge. Measurement of the metabolomes of several individuals from a free-living population will necessarily include considerable metabolic 'noise', i.e. the metabolite concentrations will be highly variable among individuals owing to differences in the individual's local environment, their genetics, and possibly the time since they last ate! This biological noise will tend to mask the metabolic differences between healthy and stressed animals, as well as the more subtle differences among closely related stressful states. Coping with this biological noise is in my view the greatest challenge in environmental metabolomics. There are, fortunately, a number of approaches that will help address this problem, including the simultaneous measurement of multiple metabolites, supervised methods of multivariate analysis, and knowledge of the organism under investigation, which are all addressed below.

MEASUREMENT OF MULTIPLE METABOLITES, NORMAL OPERATING RANGES AND MULTIVARIATE MODELS

Biological variability has long been the thorn in the side of the environmental biomarker research community. Numerous studies have reported that seemingly well established biomarkers for pollutant exposure such as metallothionein, heat shock proteins and antioxidant defence mechanisms exhibit seasonal variability (Sheehan & Power 1999, Geffard et al. 2001, Lacorn et al. 2001). This has limited the application of biomarkers as a tool for ecological monitoring. Metabolomics, as with all the omics, brings an interesting new dimension to biomarker research, that being the simultaneous measurement of potentially 100s or 1000s of metabolites. It might be construed that this exacerbates the problem because the total amount of variability captured in these multiple measurements will be significantly greater than the variability in any one metabolite. However, the advantage of the simultaneous measurement of multiple parameters stems from the fact that there will be a subset of metabolites within all those measured that can each (partially) discriminate between healthy and stressed organisms. The integrated profile of this subset of metabolic biomarkers will be able to discriminate between the healthy and stressed groups more robustly than any one biomarker alone. In effect, the biomarker profile becomes stabilised by the inclusion of many relevant variables, even if each of these variables is noisy (Eriksson et al. 2001). The challenge is to determine which subset of the hundreds of metabolites is able to provide this discrimination, which I address below.

Aside from the advantage of providing subsets of biomarkers that are potentially specific to a defined stressor, the simultaneous measurement of multiple metabolites enables us to obtain a more holistic view of the metabolic status of individuals in a population. That is, multiple measurements allow us to determine the 'normal operating range' (NOR) of an aquatic organism, a concept discussed by Kersting (1984). The metabolic status of an organism is necessarily a multivariate property in which the concentrations of each of the few thousand metabolites that define the metabolome are represented along unique axes in multidimensional metabolic space. The normal metabolic operating range (NMOR) can be defined as the region in that space in which 95% of the individuals from a population reside. Stress can then be defined as a deviation from the NMOR, with different stressors inducing different metabolic responses and therefore moving away from the NMOR in unique directions. Since we are unable to visualise high dimensional space, we use dimensionality reduction tools such as principal components analysis (PCA; Eriksson et al. 2001) to project multi-dimensional space down to just a few dimensions. This is illustrated in Fig. 1, which shows the PCA scores plot for a cohort of 15 marine mussels *Mytilus galloprovincialis* from Port Quin, Cornwall, UK. These mussels had been submerged for at least 2 h, were actively respiring, and were then dissected and the adductor muscles rapidly frozen. Metabolites were extracted from the muscle, analysed by NMR spectroscopy, and the resulting spectra were subject to PCA. In the PCA scores plot (Fig. 1), the ellipse — drawn at 2 SD from the mean metabolic status of the adductor muscles — represents the NMOR. The NMOR concept provides a useful and visibly meaningful approach for summarising high dimensional metabolomics data.

In conjunction with multivariate analyses, the concept of NMOR can be extended to help visualise the effect of stressors. When comparing stressed and unstressed animals, some of the metabolites with the

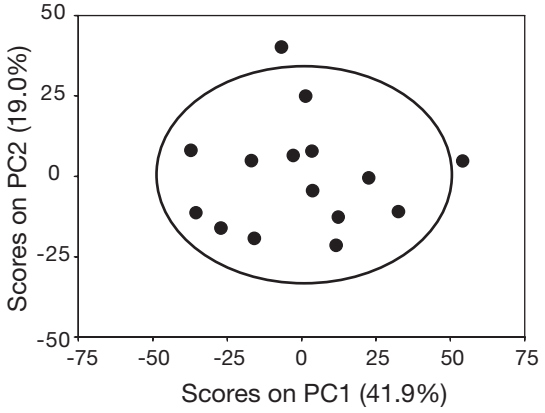

Fig. 1. *Mytilus galloprovincialis*. PCA scores plot from analysis of the metabolic fingerprints of adductor muscle from 15 mussels collected from Port Quin, Cornwall. Each data point corresponds to an entire NMR metabolic fingerprint comprising ca. 100 metabolites, and PCA axes are linear combinations of the most variable metabolites. If 2 data points are closely spaced, then this indicates that metabolomes of those samples are similar. The ellipse (±2 SD) defines the normal metabolic operating range (NMOR) for 95% of individuals within the population; % variance accounted for by each PC is shown

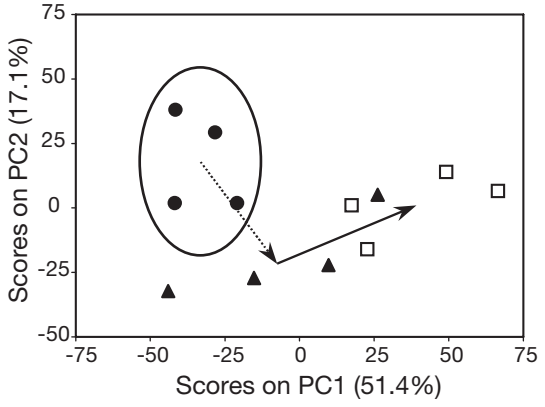

Fig. 2. *Mytilus galloprovincialis*. PCA scores plot from analysis of the NMR metabolic fingerprints of adductor muscle from mussels exposed to 0 (●), 250 (▲) and 1000 (□) ppb copper for 48 h. The NMOR of the control group is shown by the ellipse (±2 SD). Copper-induced stress forces the average metabolic status of the mussels away from the NMOR, as indicated by the dotted (low dose) and solid (high dose) arrow

most variable concentrations will be completely unrelated to the stress, and these will tend to mask those induced by the stressor. The application of unsupervised methods of analysis[1] such as PCA will only identify the most variable metabolites, irrespective of whether they are related to the stressor. Occasionally, when the induced stress is large relative to the biological noise, PCA will detect the differences between the metabolic phenotypes of the 2 groups. An example is given in Fig. 2, which shows the effect of a 48 h exposure of mussels to 0, 250 and 1000 ppb copper. Despite the small number of samples in this pilot study, a dose-dependent deviation from the NMOR is clearly visible. For the more typical scenario in which the induced stress is small relative to the biological noise, a statistically more powerful approach is required for determining specific biomarkers of stress. Supervised methods of analysis[2] can be used to search specifically for those metabolites that discriminate the stressed and unstressed groups (assuming sample sizes are sufficiently large). It is highly likely that supervised methods will be essential for characterising the effects of stressors in free-living aquatic organisms. Supervised methods for robust classification of metabolic phenotype and for biomarker discovery include partial least-squares discriminant analysis (Eriksson et al. 2001) and genetic algorithms (Jarvis & Goodacre 2005). Results from these analyses must be accompanied by appropriate parameters such as cross-validation misclassification rates, sensitivity and specificity, which can assess the quality of the multivariate model. This and the earlier discussions highlight a considerable challenge in environmental metabolomics, namely that knowledge in several disciplines spanning the ecology and biology of the organism through to sophisticated bioanalytical and bioinformatic techniques must be mastered, which necessitates collaboration between research groups. Furthermore, owing to the expense of establishing, and expertise required to operate, a metabolomics bioanalytical laboratory, a logical strategy is to establish centralised facilities that act as centres of excellence in environmental metabolomics.

IMPORTANCE OF GENOTYPIC AND PHENOTYPIC ANCHORING

From my laboratory's studies on field-sampled mussels it has become evident that, in order to elucidate metabolic biomarker profiles for specific stressors, we need a complete phenotypic and genotypic characterisation of these animals. For example, metabolomics studies of rodents established that urinary metabolite composition depends upon strain (Gavaghan et al. 2000), sex (Stanley et al. 2005) and age (Plumb et al. 2003). It is logical to conclude that similar genotypic

[1]Unsupervised analyses do not use class identifiers (e.g. control or diseased). They aim to detect clusters in the metabolic data that may not be trivially observable and that indicate which animals have similar metabolomes

[2]Supervised analyses do use class identifiers. The aim is to build a multivariate model that can predict those classifications (e.g. can discriminate between healthy and diseased animals) and discover relevant biomarkers

and phenotypic traits are important for understanding changes in the metabolome of mussels. To date, however, toxicity studies with field-sampled mussels from the UK have mostly been conducted without regard to these traits. This is particularly worrying when one considers that the UK coastline is populated by the native *Mytilus edulis* and the Mediterranean *M. galloprovincialis*, as well as a viable hybrid species (Hilbish et al. 2002). As such, we first need to assess the effects of species, sex and age on the metabolome, prior to characterising the metabolic responses of these animals to environmental stressors. That is, we need to deconvolute the overall biological noise into components with regard to major phenotypic and genotypic traits, so that these can effectively be eliminated. Of course we will never be able to characterise all noise, but to understand (and to effectively anchor to known traits) just some of it will lessen the computational challenge associated with finding biomarkers to specific stressors. Furthermore, characterising the metabolic effect of natural stressors such as hypoxia and food limitation will also be important for helping to unravel the effects of natural stressors, anthropogenic stressors and residual biological noise. Since this is true for all environmental metabolomics studies, I recommend that as metabolomics becomes more widely used in marine ecology, the baseline biochemistry of aquatic animals should be much more thoroughly characterised as a function of species, sex, age, reproductive cycle and the effects of natural stressors. For many species this will require studying the metabolic changes throughout an entire annual cycle. Such studies have the potential to add significantly to our knowledge of these organisms and their interactions with the environment.

An additional complication arises when comparing the metabolomes of organisms collected from multiple sites. It is quite plausible that comparison among these organisms will show metabolic differences that arise from, for example, differences in food availability or differences in temperature that affect the timing of the reproductive cycle. This will complicate the interpretation of the metabolomics data because these differences may mask effects resulting from anthropogenic stressors. One approach to lessen this problem builds on the concept of phenotypic anchoring, and that is to additionally anchor the metabolic measurements with multiple physical and chemical descriptors of each site. A more robust solution would be to conduct temporal studies at a series of independent sites, and to use each of those sites as its own internal control. This could then enable temporal changes in environmental quality at these sites to be assessed via changes in the metabolomes of the resident sentinel species.

TOWARDS MULTI-SPECIES ASSESSMENT OF ECOLOGICAL HEALTH

The conservation of metabolites among species (i.e. alanine is conserved in marine mammals, fish and invertebrates) provides metabolomics with a significant advantage for multi-species assessment compared with the other omics that rely on species-specific information. Metabolomics could therefore assess the metabolic status and derive NMORs for multiple species at one geographical location. Using the same argument as above—that the measurement of multiple metabolites (versus one) can provide a more robust assessment of organismal metabolic health—one can argue that characterising the health of multiple species can provide a more complete assessment of ecosystem responses to environmental stressors. This approach was previously used to analyse invertebrate community responses to an anionic surfactant in stream mesocosms, but in that case the invertebrate populations were simply counted (Wong et al. 2003). Metabolomics could afford a more sensitive window into the deterioration of organism health (prior to death) and could be used to identify which species in the community are most sensitive. Furthermore, the specific metabolic changes observed could help to inform on the nature of the stressor.

CONCLUSIONS

Metabolomics, being the new omics on the block, has benefited from some of the lessons learned from the development of the other omics. This is particularly true in terms of the recognition that sophisticated multivariate analyses and data standardisation are both essential, together with the acceptance (by some) of the significant value of unbiased discovery driven research. Unfortunately, metabolomics has also been labelled with the same shortfalls and viewed by some with the same scepticism applied to the other omics. But at this stage is this justified? Considering that only a handful of research laboratories are engaged in aquatic environmental metabolomics, and given the small amount of funding that has been available to date, it is not surprising that there are only 10 publications in the field. While we do not yet know to what extent metabolomics will impact on marine ecological studies, what is definitely true is that the approach offers considerable potential for rapid assessment of the metabolic status of marine organisms, is capable of multi-species investigations, and can provide molecular information that is closely related to whole-organism physiology and function. Ultimately, we should strive towards the integration of omics data sets because this will enable us to

exploit the advantages of each approach and will provide the most comprehensive molecular description of organisms in the aquatic environment.

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