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Metabolic changes in Atlantic salmon exposed to *Aeromonas salmonicida* detected by ^{1}H-nuclear magnetic resonance spectroscopy of plasma

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ABSTRACT: ^{1}H-NMR (nuclear magnetic resonance)-based chemometric methods have been applied for the first time to investigate changes in the plasma metabolite profiles of Atlantic salmon *Salmo salar* as a result of exposure to *Aeromonas salmonicida* subsp. *salmonicida*, a Gram-negative bacterium that is the etiological agent of furunculosis. Plasma samples were obtained from salmon that survived 21 d post exposure to *A. salmonicida*, and from a control group maintained under similar conditions. 1D ^{1}H-NMR spectra were acquired and principal components analysis (PCA) was used to assess differences between the spectral profiles of plasma from salmon that survived an *A. salmonicida* challenge, and non-infected controls. PCA enables simultaneous comparison of spectra, presenting a simplified overview of the relationship between spectral data, where spectra cluster based on metabolite profile similarities and differences; information regarding the metabolite variations can therefore be readily deciphered. The major metabolite changes responsible for the spectral differences were related to modification in the lipoprotein profile and choline-based residues, with minor changes in carbohydrates, glycerol, trimethylamine-N-oxide and betaine. These changes indicated that exposure to *A. salmonicida* induced a characteristic biochemical response which could be used to determine the health status of salmon. This study suggests that with further development this metabolite profiling technique may be a useful tool for diagnosis of disease states in salmon and could provide a better understanding of the host-pathogen relationship which at present is poorly understood for *A. salmonicida* and Atlantic salmon.

KEY WORDS: *Aeromonas salmonicida* · Atlantic salmon · ^{1}H-nuclear magnetic resonance spectroscopy · Principal components analysis · *Salmo salar*

INTRODUCTION

The pathophysiological status of an organism is often reflected in the metabolite composition of biofluids such as plasma. Unlike traditional methods for determining plasma metabolite changes associated with infection or disease, metabolite profiling by ^{1}H-nuclear magnetic resonance (NMR) spectroscopy in principle can provide a comprehensive overview of all ^{1}H containing metabolites in free solution in a uniformly concentration-dependent manner, without a need for pre-selection of measurement parameters or extensive sample preparation (Nicholson & Wilson 1989, Nicholson et al. 1995, Fan 1996). Furthermore, this technique is non-destructive and reproducible. These properties, shared by no other spectroscopic method, make ^{1}H-NMR of biofluids a practical tool for generating consistent metabolite profiles for investigative research.

^{1}H-NMR spectra obtained with plasma from healthy and diseased individuals may be statistically and quantitatively compared using chemometric methods such as principal components analysis (PCA) (Eriksson et al. 1999). PCA is a means to analyze simultaneously

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large numbers of complex spectra to extract meaningful information relating to the biochemical response to the pathophysiological stimuli or disease processes under investigation. This approach to metabolite profiling, termed 'metabonomics', is defined by Nicholson et al. (1999) as, 'the quantitative measurement of time-related multiparametric metabolic responses of a living systems to pathophysiological stimuli or genetic modification'. It is being applied increasingly to investigate the metabolic effects of diseases (Brindle et al. 2003), drugs and toxins (Nicholson et al. 1999, Robosky et al. 2002, Watkins et al. 2002), particularly in mammalian systems. NMR based-metabolite profiling of tissues has also been applied in examining the effects of environmental stresses on shellfish (Viant et al. 2003).

Aeromonas salmonicida subsp. *salmonicida* is a Gram-negative bacterium that is the etiological agent of an infectious disease in salmonids called furunculosis. Furunculosis may manifest itself either as an acute septicaemia, chronic focal myodermonecrosis or as a clinically inapparent 'covert' infection. Covert infections (reviewed by Hiney et al. 1997) may be detected by clinical microbiology but the detection rate is low. The most effective method to determine covert infection is by a stress-test involving the elevation of water temperature after administration of cortisol. This process is both destructive (as it leads to the death of tested fish), and lengthy (typically 10 to 14 d for a result) (Hiney et al. 1997). We have applied a metabonomic approach to investigate differences in ^{1}H-NMR spectra of plasma from Atlantic salmon *Salmo salar*, that have survived an *A. salmonicida* infection, and a non-infected control group. Based on previous stress tests with similar fish (see below; A. Dacanay unpubl. data), up to ca. 90% of the former group may be covertly infected.

MATERIALS AND METHODS

Fish culture and plasma sampling. Animal protocols were conducted under Canadian Council on Animal Care guidelines and were approved by all relevant Local Animal Care Committees. Atlantic salmon (Sackville River stock) were obtained from a Nova Scotia hatchery certified under the Canadian Fish Health Protection Regulations as free from common pathogens of salmon including *Aeromonas salmonicida*. In the course of this study, 100 fish (average weight 67.3 g ± 11.3 SD) were challenged by immersion with 10^6 colony-forming units (cfu) per ml of a virulent strain of *A. salmonicida*. The strain used was *A. salmonicida* subsp. *salmonicida* strain A449, originally isolated from a natural epizootic in brown trout *Salmo trutta* in Eure, France. This challenge caused 91% mortality in the exposed group over a 17 d period, whereas there was no mortality in the saline-exposed control group (n = 40) (Fig. 1). After 4 consecutive days with no further mortality in the exposed group (21 d after infection), the 9 surviving fish were anaesthetised using 100 mg l^{-1} tricaine methanesulphonate (TMS, Syndel labs) and blood was drawn from the caudal vein of each surviving fish into heparinised blood collection tubes (Vacutainer™, Becton-Dickson). Similar samples were obtained by the same procedure from the control group. Cells were removed by centrifugation at 3000 × *g* and the resulting plasma was stored at –80°C until spectral acquisition. Plasma samples were thawed and a 200 µl aliquot of each sample was mixed with 300 ml of D_2O for analysis in a Wilmad 535 pp NMR tube.

Subsequently, a second study was undertaken using Atlantic salmon from the same cohort to investigate the effects of anorexia in a treatment group (n = 25) by withdrawal of food from this group for a period of 15 d. In parallel, a control population (n = 30) was fed a standard ration (Signature salmon ration, Shur-Gain) at 1% body weight per d for comparison. Plasma samples were obtained from both groups as described.

NMR spectroscopy. 1D ^{1}H-NMR spectra were acquired at 4°C on a Bruker Avance-DRX 500 spectrometer operating at 500.13 MHz using a 5 mm

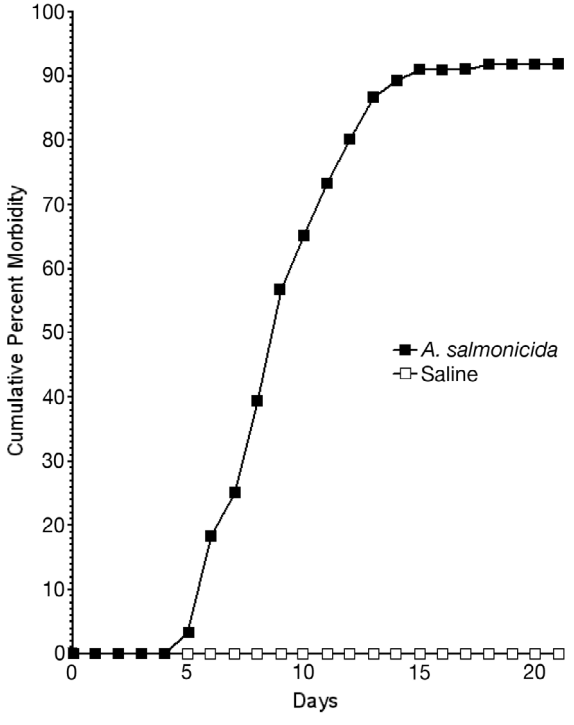

Fig. 1. *Salmo salar*. Cumulative percent morbidity of salmon vs. number of days after treatment by exposure to 100 cfu ml^{-1} *Aeromonas salmonicida* strain A449 (■) and saline (□)

Bruker triple-axis gradient, triple-band inverse (TBI) probe. Three types of 1D ^{1}H-NMR spectra were acquired for each sample: 2 were single-pulse spectra with solvent suppression using a presaturation sequence (PS) or a WATERGATE pulse sequence (WG) (Piotto et al. 1992), and the third was a T_2-edited Carr-Purcell-Meiboom-Gill (CPMG) sequence with presaturation (Bulsing et al. 1981, Evans 1995, Lindon et al. 1999). For all spectra, a total of 128 transients were acquired into 32 768 data points using a spectral width of 12 ppm, and 90° pulses (6.4 µs) were used for the WG and PS experiments. The acquisition time used for the PS and CPMG spectra was 2.73 s, and for the WG experiment 2.62 s. The delay between successive acquisition periods was 5 s for PS and 2 s for WG and CPMG.

In addition to the above ^{1}H-NMR spectra, 2D total correlation spectroscopy (TOCSY), correlation (COSY), ^{1}H-^{13}C heteronuclear single quantum correlation (HSQC) and ^{1}H J-resolved spectra (Palmer et al. 1991, Kay et al. 1992, Schleucher et al. 1994, Nicholson et al. 1995, Claridge 1999) were acquired to aid metabolite identification. These experiments provide information on molecular structure based on spin-spin coupling of nuclei via covalent bonds, and produce patterns that are highly characteristic of particular compounds or compound classes. TOCSY spectra indicate all members of ^{1}H spin systems, i.e. nuclei for which there are a continuous series of couplings. COSY spectra show which pairs of ^{1}H nuclei are mutually coupled, owing to separation by few covalent bonds (usually 2 to 3). ^{1}H-^{13}C HSQC spectra denote the ^{13}C nuclei to which ^{1}H are directly bonded. J-resolved spectra simplify ^{1}H spectra by displaying the complex coupled multiplets in the second dimension, leaving a single peak for each individual nucleus in the first dimension.

The assignments of peaks corresponding to trimethylamine-N-oxide (TMAO) and betaine were confirmed by supplementation of samples with the authentic compounds.

Data processing. All free induction decays were multiplied by an exponential function equivalent to a 0.3 Hz line-broadening factor prior to Fourier transformation. These spectra were manually corrected for phase and baseline using XWIN-NMR 2.6 (Bruker Analytik) and were then referred to the centre of the lactate doublet at 1.34 ppm.

The spectra over the range 0.5 to 10.0 ppm, excluding the region of the residual water resonance (4.5 to 6.0 ppm), were reduced using AMIX 3.0.1 software (Bruker Analytik) to 244 regions ('bins') each 0.04 ppm wide, and the signal intensity in each region was integrated. Prior to principal component analysis (PCA), each integral region was normalized by dividing by the sum of all integral regions for each spectrum. PCA was performed with Simca-P 10.0 software (Umetrics) using mean-centered scaling, where the average value of each variable is calculated and subtracted from the data.

RESULTS AND DISCUSSION

PCA of spectral data

The 3 types of 1D-NMR experiments (PS, WG and CPMG) emphasize different spectral features. PS and WG spectra are uniform in response to compounds with a wide range of molecular weight (MW), although in PS spectra signals from protons in exchange with water are reduced in intensity. For plasma, both PS and WG spectra are generally dominated by broad signals from macromolecules, particularly phospholipids. CPMG experiments exploit differences in spin-spin relaxation time (T_2) to reduce signals from macromolecules (short T_2) and emphasize lower MW compounds having longer T_2 (Bulsing et al. 1981, Evans 1995, Lindon et al. 1999). Hence, a combined analysis of these spectral data gives an overall picture of plasma biochemistry.

Although some differences between spectra of plasma from control and *Aeromonas salmonicida*-exposed fish were evident from visual examination, statistical analysis was necessary to obtain a reliable comparison. The method used here, PCA, reduces the dimensionality of a data set via 2D or 3D mapping procedures, and facilitates the comparison of a large number of complex objects (spectra in this case). Linear combinations (principal components, PCs) of the original variables are calculated with appropriate weighting coefficients, such that each PC is orthogonal (uncorrelated) to all other PCs. The first PC (PC1) describes the largest part of the variance of the data set, with subsequent PCs containing successively smaller amounts of variance not described in previous PCs (Aries et al. 1991, Eriksson et al. 1999). 'Score' plots in 2 or 3 PC dimensions (e.g. PC1 vs. PC2, Fig. 2A–C) highlight the maximal variation within the data set. Each point in the scores plot corresponds to an individual sample (spectrum). Samples that have similar metabolite profiles tend to map closely forming a cluster; hence for 2 groups of spectra that are characteristically different in metabolite composition, 2 clusters will be evident in the score plot. Furthermore, the degree of similarity or difference will determine the distance or overlap between the groups, and the PC in which this variation is described. Information on how the original variables are linearly combined to form the new variables (PC scores) is presented in the

PC 'loading' plots (e.g. Fig. 2D–F). The loadings describe the contribution of individual variables ('binned' chemical shift ranges) in terms of magnitude (large or small correlation) and direction (positive or negative correlation) to form the scores. Such ranges correspond to the chemical shifts of metabolites whose concentrations change in response to a perturbation of the system (e.g. survivors of infection compared to controls), and permit the compounds to be identified.

Comparison of spectra of plasma from exposed and control fish

The analysis of the score plots of PC1 vs. PC2 for each spectral data set (PS, WG and CPMG) demonstrated clustering of samples based on exposure to *Aeromonas salmonicida.* For PS and WG data, this was described in the first PC (PC1) and for CPMG data in a higher PC (PC2) (Fig. 2A–C). This indicated that the difference between the profiles of the 2 groups was greatest for PS and WG data, with intra-sample variation dominating PC1 in the case of CPMG data. Analysis of the corresponding loading plots (Fig. 2D–F) allowed for the identification of the spectral regions that were contributing to the observed clustering. The significance of the clustering was determined by cross validation methods. PC models based on each class (i.e. control [class 1] or survivor of infection [class 2]) consisting of 40% to 60% of the original data were used to predict the class of the whole original data set. Validation procedures indicated that for a 2-component PCA model, the class of each spectrum could on average be predicted accurately in >60% of the cases, with a significance level of >70% using a 95% confidence limit. Unfortunately, the sample size was low in this study owing to the 91% mortality rate. It is possible that by using a larger data set these values could be improved, as a model that is more representative of the different population could be created, and a more accurate

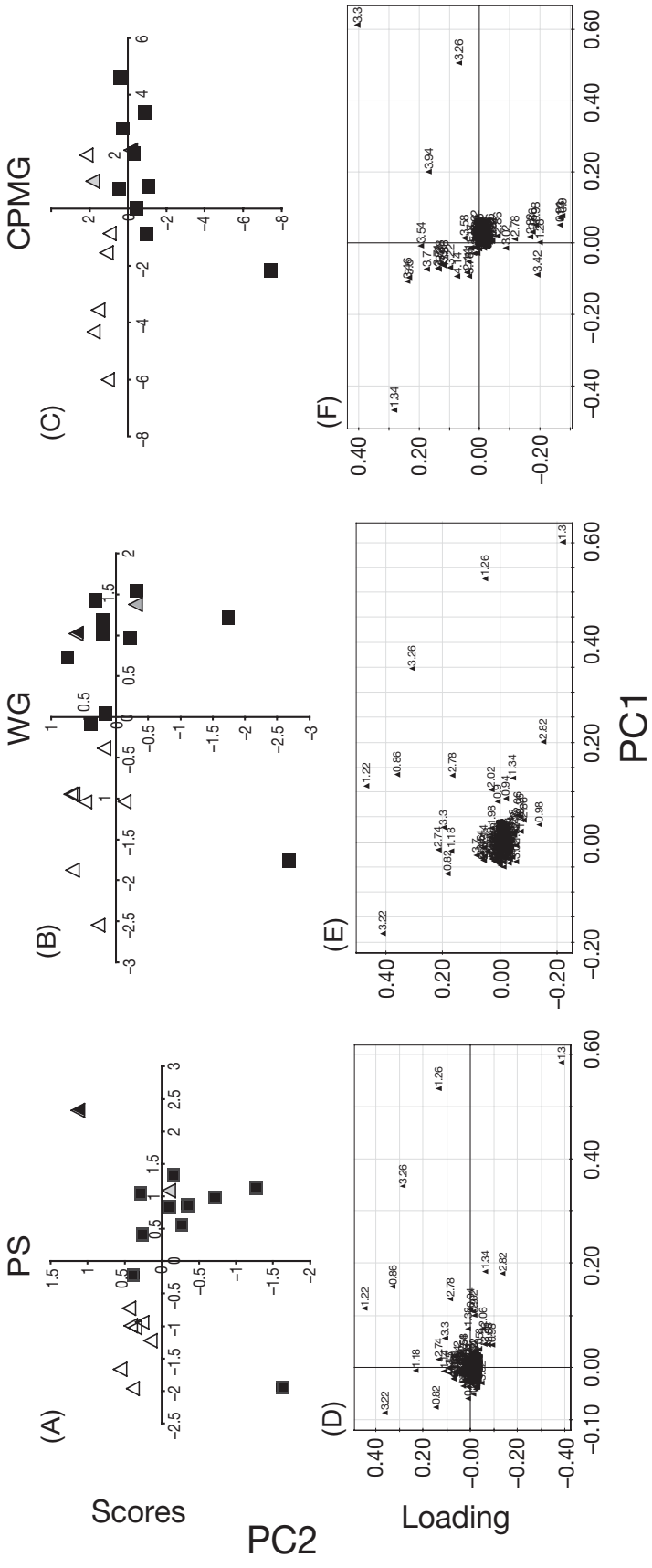

Fig. 2. Principal component score plots (PC1 vs. PC2) derived from centre scaled data of (A) PS (presaturation pulse sequence), (B) WG (WATERGATE pulse sequence) and (C) CPMG (Carr-Purcell-Meiboom-Gill pulse sequence) ^{1}H-NMR spectra of plasma from control salmon (■), and from salmon that survived 21 d after exposure to *Aeromonas salmonicida* (Δ). Corresponding loading plots are shown below each score plot: (D) PS, (E) WG and (F) CPMG. The 2 survivors (22%) that fall within the control space (▲ & ▲) are highlighted in each score plot (A–C)

method of cross validation applied. It is noteworthy that in the scores plots for the 3 data sets (PS, WG and CPMG) the same 2 individual survivors of *A. salmonicida* exposure (22% [2/9]) clustered with the control group (Fig. 2A–C), indicating that the metabolite profiles of these individuals were not affected by exposure to *A. salmonicida*.

Similar studies with this strain of salmon and *Aeromonas salmonicida* have shown that of salmon that survive an initial epizootic, up to 90% may be covertly infected, the remaining 10% are either uninfected or somehow immune (A. Dacanay unpubl. data). Although the infection status of the animals in this study was not determined by stress-test (e.g. Specker et al. 1994) or clinical microbiology (Schotts 1994), our data suggest that 2 (22%) of the *A. salmonicida*-challenged animals may represent such uninfected individuals.

Metabolite identification and biochemical implications

Analysis of PC1 vs. PC2 loading plots (Fig. 2D–F), corresponding to the PC score plots Fig. 2A–C generated from 1D ^{1}H-NMR spectral data, enabled identification of the chemical shift ranges that contributed to the clustering of spectra from survivors of an *Aeromonas salmonicida* challenge and controls. This information was used to guide identification of biomarkers of covert infection (Table 1). Identification of the corresponding metabolites, labelled in Fig. 3, was based on previously published data (Nicholson et al. 1983, Foxall et al. 1993, Lindon et al. 1999) and ^{1}H 2D COSY, TOCSY and HSQC spectra of representative samples. The spectral profile variations (Fig. 3A,B) were related mainly to changes in the lipid profile including decreases in triglyceride (TG), low-density

Table 1. *Salmo salar*. Changes in plasma metabolite levels following exposure to *Aeromonas salmonocida* as determined from ^{1}H-NMR spectral profiles of salmon plasma. The variations are based on PC loadings analysis of Presaturation (PS) and Carr-Purcell-Meiboom-Gill pulse sequence (CPMG) spectral data accompanied by spectral interrogation. +: concentration increase in that metabolite in survivors relative to control; –: decrease in that metabolite in survivors relative to control; NA: metabolite not significant to scores for that spectral data set; NA$^{\#}$: significance of metabolite undetermined as the metabolite signal is obscured by larger lipoprotein signals. TMAO: trimethylamine-N-oxide; LDL: low-density lipoprotein; VLDL: very-low-density lipoprotein; s: singlet; d: doublet; dd: doublet of doublets; t: triplet; q: quartet; spt: septet; m: multiplet; b: broadened. $\star$: metabolites confirmed by supplementation of samples with authentic compounds. Chemical shifts (referred to centre of lactate doublet at 1.34 ppm) and multiplicities (number of peaks associated with each resonance) agree with metabolite assignments(Nicholson et al. 1995)

Chemical shift (ppm) (Multiplicity)	Metabolite	Pathway	Biological role	Change relative to control in PS	Change relative to control in CPMG	Contribution to clustering
3.27(s)	TMAO$\star$	Methylamine pathway	Molecular chaperone, and osmolyte	+	+	Major
1.34(d) 4.14(q)	Lactate	Anaerobic energy metabolism	Energetic homeostasis, indicator of stress	NA	+	Major
2.72(m) 2.00(m) 0.93(m)	Lipid	Fatty acid metabolism	Lipid transport, bile, energy storage and mobilisation	+	NA	Major
2.69(m)	Lipid					
0.84(t) 1.25(m)	LDL			+	NA	Major
3.21(s) 3.66(m)	Choline, phosphocholine	Biosynthesis of acetylcholine, and phosphotidylcholine	Cell membranes	+	+	Major
3.87(m) 3.54(s)	Glycerol, glycine	Lipid metabolism		NA	+	Minor
0.70(m) 0.87(t) 1.29(m)	VLDL			–	NA	Major
1.57(m)	VLDL					
3.27(s) 3.9(s)	Betaine$\star$	Product of choline metabolism	Osmolyte	–	–	Minor
3.2–3.9	α, β carbohydrates	Glycolysis	Energetic homeostasis	–	–	Minor
2.04(s)	Glycoprotein	Amino acid metabolism, gluconeogenesis, fatty acid metabolism, TCA cycle	Protein turnover and muscle activity	–	–	Minor
0.97(d) 0.95(d) 1.71(m) 3.69(dd)	Leucine			NA$^{\#}$	–	
2.05(m) 3.45(m) 4.12(m)	Proline					
3.34(m) 1.99(m) 2.36(m)	Proline			NA$^{\#}$	–	Minor
1.28(m) 1.00(d) 1.96(m)	Isoleucine					
0.93(t) 1.47(m)	Isoleucine			NA$^{\#}$	–	Minor
0.97(d) 1.02(d) 2.24(spt of d)	Valine			NA$^{\#}$	–	Minor
0.91(bd) 0.84(m)	Cholesterol in lipid	Fatty acid metabolism	Lipid transport, bile function, energy storage mobilisation	–	NA	Minor

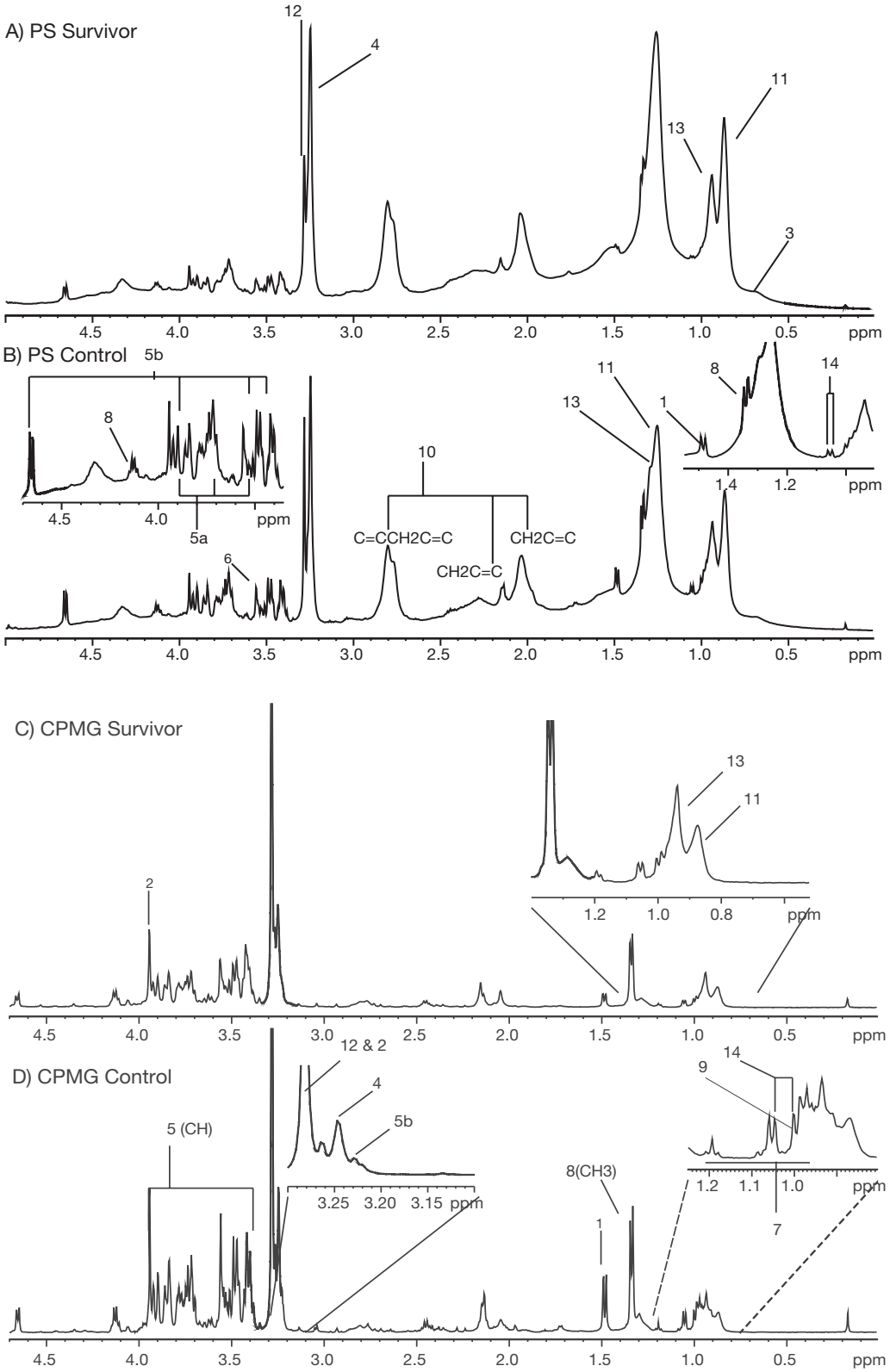

Fig. 3. Representative partial ^{1}H-NMR spectra: (A) PS (presaturation) survivor, (B) PS control, (C) CPMG survivor, (D) CPMG control. The metabolites labelled were identified by analysis of PC loadings and relate to the clustering of samples based on exposure to *Aeromonas salmonicida*. (1) Alanine, (2) betaine, (3) cholesterol, (4) choline moiety, (5a) (Carr-Purcell-Meiboom-Gill) α-glucose, (5b) β-glucose, and amino acid CH residues, (6) glycine, (7) isoleucine, (8) lactate, (9) leucine, (10) lipid, (11) low-density lipoprotein, (12) trimethylamine-N-oxide, (13) very-low-density lipoprotein, (14) valine

lipoproteins (LDL) and phosphocholine, with increases in very-low-density lipoproteins (VLDL), cholesterol and choline. Changes in the amino acid and carbohydrate levels were identified by analysis of CPMG spectral data, though these were not as significant to the clustering as changes in the larger molecules, as demonstrated by the level of separation between the 2 clusters in score plots for the PS and WG data (Fig. 2A,B) when compared with the score for CPMG spectral data (Fig. 2C). These analyses suggested that exposure to *A. salmonicida* resulted in major modifications of lipid metabolism with underlying changes in carbohydrate and amino acid metabolism that are associated with modulation or alteration in energy metabolism, protein turnover, and/or muscle activity. The changes might be due to modification of the metabolic activity of the host in response to the pathogen, the direct action of the pathogen on the host, or a combination of both factors.

The observed metabolite changes in energy utilisation could be associated with starvation, which is linked with an increase in cortisol secretion (Bergendahl et al. 1996), an indicator of stress. Furthermore, anorexia is known to be a common clinical sign of *Aeromonas salmonicida* infection (Pirhonena et al. 2003), therefore an experiment was conducted to determine whether the observed spectral changes between challenged and control salmon could be attributed to this cause. Feed was withheld from a third group of Atlantic salmon (n = 25) for 15 d before plasma was drawn, and additional samples were also obtained from a second control group (n = 30) for comparison. PCA of the ^{1}H-NMR spectral data (PS, WG and CPMG) from plasma collected from salmon under the 3 different conditions (control, challenged and starved, Fig. 4) showed clustering of the spectra from survivors of *A. salmonicida* exposure away from that of the control and starved animals in the first PC (PC1) while effects of starvation were described in higher PCs (PC2). The scores indicated that profiles of the starved animals were closer to those of the uninfected controls than to those of the challenged animals, hence the differences between challenged and control salmon seen in Fig. 2 were characteristic of the exposure of salmon to *A. salmonicida*, and were not significantly related to the metabolic effects of starvation.

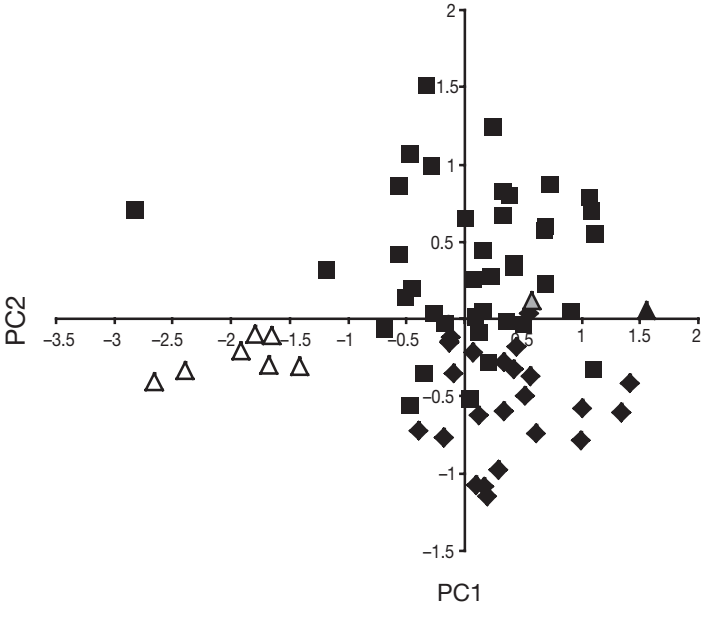

Fig. 4. Principal component score plot PC1 vs. PC2 derived from centre scaled PS (presaturation) spectral data, illustrating clustering of plasma samples based on treatment. (■) Spectra of plasma from controls; (△) spectra of plasma from salmon that survived 21 d after exposure to *Aeromonas salmonicida*; (◆) spectra of plasma from salmon that were starved for 15 d; (▲ & ▲) spectra of plasma from the 2 challenged fish that consistently fall within the control population space

CONCLUSIONS

PCA of ^{1}H-NMR spectra of plasma collected from Atlantic salmon challenged with *Aeromonas salmonicida*, and controls, showed that challenge resulted in characteristic metabolite profile changes, with 7 of the 9 survivors (78%) clustering in an area of multivariate space distinct from the controls. The remaining 2 of the challenged fish clustered with the control group rather than the infected fish, as independently determined from the analysis of 3 different types of spectra. Although the disease status of these animals was not confirmed by stress test or microbiology, based on previous challenge trial data the 7 survivors that clustered outside the control space were likely to represent a covertly infected sub-population, whilst the 2 survivors that clustered within the control space represent uninfected individuals. Furthermore, although suggestive of anorexia, the differences in spectral profiles were not due to anorexia per se.

In summary, infection with *Aeromonas salmonicida* produced a distinctive response leading to changes in the host plasma metabolite composition. The pattern of metabolite changes may be of diagnostic value for this disease, as well as for the determination of health status. By identifying fluxes in certain metabolites, metabonomics can help in understanding the specifics of the *A. salmonicida*–*Salmo salar* pathogen–host interaction, a process which remains poorly defined.

This work presents the first reported application of metabolite profiling techniques for the investigation of an infectious disease in lower vertebrates. These analyses demonstrate the potential of a metabonomic

approach in both experimental studies and routine clinical diagnosis of aquatic animal disease.

As these techniques could in principle be used in the aquaculture industry to address problems that cannot be solved by traditional techniques, we are undertaking studies focusing on some significant issues including: sex determination of immature fish from species with lengthy life-cycles aimed at early selection of females for brood-stock programs, identification of biomarkers related to health status (including covert infection) of fish prior to stocking, for monitoring individual responses to veterinary treatments, and as complementary research tools (with functional genomics or proteomics) for the analysis of host-pathogen relationships. Further horizontal studies are under way to follow individual animals through the infection process to further understand the mechanisms of *Aeromonas salmonicida* infection and to validate metabolite changes that correlate with disease outcome.

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LITERATURE CITED

Aries ER, Lidiard PD, Spragg AR (1991) Principal components analysis. Chem Br 29:821–824

Bergendahl M, Vance ML, Iranmanesh A, Thorner MO, Veldhuis JD (1996) Fasting as a metabolic stress paradigm selectively amplifies cortisol secretory burst mass and delays the time of maximal nyctohemeral cortisol concentrations in healthy men. J Clin Endocrinol Metab 81(2):692–699

Brindle JT, Nicholson JK, Schofield PM, Grainger DJ, Holmes E (2003) Application of chemometrics to ^{1}H NMR spectroscopic data to investigate a relationship between human serum metabolic profiles and hypertension. Analyst 128: 32–36

Bulsing MJ, Sanders JKM, Hall LD (1981) Spin echo methods for resolution control of lanthanide-shifted NMR spectra. J Chem Soc Chem Commun 1201–1203

Claridge TDW (1999) High-resolution NMR techniques in organic chemistry, Vol 19. Elsevier Sciences, Amsterdam

Eriksson L, Johansson E, Kettaneh N, Wold S (1999) Introduction to multi and megavariate data analysis using projection methods (PCA & PLS). Umetrics, Umea

Evans JNS (1995) Biomolecular NMR spectroscopy. Oxford University Press, Oxford

Fan TWM (1996) Metabolite profiling by one- and two-dimensional NMR analysis of complex mixtures. Prog Nucl Mag Res Sp 28:161–219

Foxall PJ, Parkinson JA, Sadler IH, Lindon JC, Nicholson JK (1993) Analysis of biological fluids using 600 MHz proton NMR spectroscopy: application of homonuclear two-dimensional J-resolved spectroscopy to urine and blood plasma for spectral simplification and assignment. J Pharm Biomed Anal 11(1):21–31

Hiney MP, Smith P, Bernoth EM (1997) Covert *A. salmonicida* infections. In: Bernoth EM, Ellis AE, Midtlyng PJ, Olivier G, Smith P (eds) Furunculosis: multidisciplinary fish disease research. Academic Press, San Diego, CA, p 53–97

Kay LE, Keifer P, Saarinen T (1992) Pure absorption gradient enhanced heteronuclear single quantum coherence spectroscopy with improved sensitivity. J Am Chem Soc 114: 10663–10665

Lindon JC, Nicholson JK, Everett JR (1999) NMR Spectroscopy of biofluids. Ann Rep NMR Spectrosc 38:1–88

Nicholson JK, Wilson ID (1989) High resolution proton NMR spectroscopy of biofluids. Prog Nucl Mag Res Sp 21: 449–501

Nicholson JK, Buckingham MJ, Sadler PJ (1983) High resolution ^{1}H n.m.r. studies of vertebrate blood and plasma. Biochem J 211(3):605–615

Nicholson JK, Foxall PJD, Spraul MR, Farrant D, Lindon JC (1995) 750 MHz ^{1}H and ^{1}H-^{13}C NMR spectroscopy of human blood plasma. Anal Chem 67(5):793–811

Nicholson JK, Lindon JC, Holmes CE (1999) Metabonomics: understanding the metabolic response of living systems to pathological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica 29: 1181–1189

Palmer AG III, Cavanagh J, Wright PE, Rance M (1991) Sensitivity improvement in proton-detected 2-dimensional heteronuclear correlation NMR spectroscopy. J Magn Reson 93:151–170

Piotto M, Saudek V, Sklenar V (1992) Gradient-tailored excitation for single-quantum NMR spectroscopy of aqueous solutions. J Biomol NMR 2(6):661–665

Pirhonena J, Schrecka CB, Renob PW, Ogut H (2003) Effect of fasting on feed intake, growth and mortality of chinook salmon, *Oncorhynchus tshawytscha*, during an induced *Aeromonas salmonicida* epizootic. Aquaculture 216(1–4): 31–38

Robosky LC, Robertson DG, Baker JD, Rane S, Reily MD (2002) In vivo toxicity screening programs using metabonomics. Comb Chem High Throughput Screen 5(8): 651–662

Schleucher J, Schwendinger M, Sattler M, Schmidt P, Schedletzky O, Glaser SJ, Sorensen OW, Griesinger C (1994) A general enhancement scheme in heteronuclear multidimensional NMR employing pulsed field gradients. J Biomol NMR 4(2):301–306

Schotts EB (1994) Chapter XI: Furunculosis. In: Thoesen JC (ed) Suggested procedures for the detection and identification of certain finfish and shellfish pathogens. 4th edn, Version 1. Fish Health Section, American Fisheries Society, Bethesda, MD

Specker JL, Portesi DM, Cornell SC, Veillette PA (1994) Methodology for implanting cortisol in Atlantic salmon and effects of chronically elevated cortisol on osmoregulatory physiology. Aquaculture 121(1–3):181–193

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(21):4982–4989

Watkins SM, German JB (2002) Metabolomics and biochemical profiling in drug discovery and development. Curr Opin Mol Ther 4(3):224–228

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