A description of the origins, design and performance of the TRAITS / SGP Atlantic salmon (*Salmo salar* L.) cDNA microarray

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Abstract

The origins, design, fabrication and performance of an Atlantic salmon microarray is described. The microarray comprises 16,950 Atlantic salmon derived cDNA features, printed in duplicate and mostly sourced from pre-existing EST collections (SALGENE and SGP) but also supplemented with cDNAs from suppression-subtractive hybridisation (SSH) libraries and candidate genes involved in immune response, protein catabolism, lipid metabolism and the parr-smolt transformation. A preliminary analysis of a dietary lipid experiment identified a number of genes known to be involved in lipid metabolism. Significant fold-change differences (as low as 1.5×) were apparent from the microarray analysis and were confirmed by qRT-PCR. The study also highlighted the potential for obtaining artifactual expression patterns as a result of cross-hybridisation of similar transcripts. Examination of the robustness and sensitivity of the experimental design employed, demonstrated the importance of biological replicates over technical (dye flip) replicates for the studied system. The TRAITS / SGP microarray has been proven, in a number of studies, to be a powerful tool for the study of key traits of Atlantic salmon biology. It is now available for use by researchers in the wider scientific community.

Key Words: Atlantic salmon; DNA microarray; gene expression; lipid metabolism; immune response; smoltification.

INTRODUCTION

The Atlantic salmon (*Salmo salar* L.) is an important farmed fish species throughout its native range (western Europe and east coast North America). Over the past 10 years a substantial Atlantic salmon mariculture industry has also been established in Chile. In indigenous areas the wild species also underpins valuable sectors of the rural economy founded on sport and ecotourism. Its biology is unusual in that, as an anadromous species, it adapts to very different environments in terms of temperature and salinity at different stages in its life-cycle. Atlantic salmon is a high value food source providing quality protein and oils, and together with other oily fish it is the most important source of essential "omega 3" fatty acids in the human diet. Data compiled from FAO UN database (Fishstat Plus) indicates that farmed Atlantic salmon production in the world exceeds 1.2 million tonnes per annum.

Currently, the four most important constraints on commercial production of this species are a) supply of dioxin-free highly unsaturated oils for the salmon diet, b) protein growth efficiency, c) infectious disease, and d) a long and complex lifecycle. In 2002, research groups from three UK universities (Aberdeen, Cardiff and Stirling), specialising in different aspects of Atlantic salmon biology, formed a partnership to develop and exploit transcriptomics resources to explore the molecular basis of the biology underlying these constraints. The goal of TRAITS (TRanscriptome Analysis of Important Traits of Salmon, www.abdn.sfirc/salmon) was to bolster the sustainability of Atlantic salmon farming through identification of genes and metabolic pathways influencing traits that are important in terms of a) efficiency and sustainability of farm production, b) welfare of farmed stocks and c) quality and nutritional value of salmon products for the consumer. This goal was to be achieved through selection of a set of key "indicator" genes associated with the traits of interest, in order to form the basis of a prototype DNA chip for monitoring salmon health and performance. The underlying strategy (Fig 1.) was to design a primary cDNA microarray based on extant EST collections together with novel ESTs derived from genes shown to respond, in expression terms, to relevant laboratory / field "challenges".

RNA samples derived from these and other challenges would be interrogated by the cDNA array to identify candidate responder genes. A second more focused oligonucleotide array, comprising mainly responder genes, would then be fabricated and initially validated by interrogation of the same samples that were hybridised to the cDNA array.

The development of the TRAITS cDNA microarray was initially reliant upon a *c.* 11K EST collection from the EC funded SALGENE project ("Construction of a genetic body map for Atlantic salmon"; FAIR CT98 4314), in which Stirling had been a partner. However, prior to project start-up a formal collaboration with the Norwegian Salmon Genome Project (SGP, www.salmongenome.no) was developed that allowed access to a more extensive physical EST resource (Hagen-Larsen *et al*., 2005; Adzhubei *et al*., 2007).

Several cDNA microarray platforms have been established for salmonid fish with varying numbers of features; Koskinen *et al*., 2004, (1380 features), Rise *et al*., 2004 (3700 features), Ewart *et al*., 2005 (4104 features), Jordal *et al.*, 2005 (79 features) von Schalburg *et al*., 2005 (16008 features). A number of different biological processes have been examined by microarray in salmonid fish including immune responses to bacterial infections (Ewart *et al*., 2005; MacKenzie *et al*., 2006; Martin *et al*., 2006; Rise et al., 2004; von Schalburg *et al*., 2005), viral infections (Purcell *et al*., 2006), fungal infections (Roberge *et al*., 2007), and to physiological states such as nutrition (Jordal *et al.,* 2005), mobilisation of energy reserves (Salem *et al*., 2006) and stress (Cairns *et al*., 2007). In the above studies varying complexities of experimental design were employed, using different numbers of replicates and often only genes showing two-fold or higher differences in expression were selected for further scrutiny / characterisation.

It is widely accepted that the supply of fish meal and oils for the diets of farmed fish is not sustainable (Sargent & Tacon, 1999). One consequence of this has been an increased emphasis on the

development of diets based on vegetable oil, which are suitable for aquaculture. A key aspect of salmonid lipid metabolism that is being extensively investigated is this regard is the conversion of C_{18} polyunsaturated fatty acids (PUFAs), found in vegetable oils to the C_{20} and C_{22} highly unsaturated fatty acids (HUFAs), eicosapentaenoate (EPA) and docosahexaenoate (DHA), that are the specific omega-3 fatty acids responsible for the health-promoting properties of fish and fish oil. These components are critical to the maintainance of nutritional quality of farmed fish. Two key enzymes involved in this pathway, Δ5 and Δ6 fatty acyl desaturase (FAD), have been characterised in depth (Hastings *et al.*, 2004; Zheng *et al.*, 2005*a*). Both these genes have been shown, by quantitative real-time PCR analysis (qRT-PCR), to exhibit diet-dependent differential gene expression (Zheng *et al.*, 2004, 2005*a, b*), though detected fold-change differences are minimal (1.3 – 2.0).

In this paper, design and fabrication details relating to the TRAITS / SGP cDNA microarray are reported. Results of a preliminary transcriptomic analysis from a dietary lipid feeding trial are used to explore both the robustness and the sensitivity of analysis that may be achieved by the use of this microarray.

MATERIALS & METHODS

cDNA RESOURCES

Archived ESTs

Two main EST collections (SALGENE *c.* 11K clones and SGP *c.* 30K clones) were available to the TRAITS consortium in 2004. In all cases, fish were sourced from farm stocks of European origin and library construction began with mRNAs from tissues being used as template for oligo(dT)-primed

reverse transcription. The SALGENE resource comprised ESTs from seven tissue-specific non-normalised libraries and two tissue-specific normalised libraries, with all cDNAs being directionally cloned into vectors. Details of tissues used, life-history stage (juvenile *i.e.* freshwater phase or adult *i.e.* marine phase) and cloning systems employed are given in Table I. Non-normalised library construction has been detailed elsewhere (Martin *et al*., 2002; Hagen-Larsen *et al*., 2005; Adzhubei *et al*., 2007). Insert size varied among libraries but ranged from 300 bp – 4 kbp. Single pass sequence data (5' end) were available for all clones. Normalised libraries were made in M.B. Soares' laboratory, University of Columbia, USA, following their standard methodology (Bonaldo *et al*., 1996). Single pass sequence data available for these clones was a mixture of both 5' and 3' end reads. SGP clones were derived from 14 tissue-specific non-normalised libraries; brain, eye, gill, head-kidney, heart, intestine, kidney, liver, white muscle, ovary, skin, spleen, swim-bladder and testis. All tissues were sampled from parr (freshwater phase). The cDNAs were directionally cloned into pBlueScript II SK(+) XR phagemid vector and transformed into XL10–Gold host cells (Hagen-Larsen et al., 2005; Adzhubei *et al*., 2007). EST data comprised single pass 5'-end sequences.

Trait-specific enriched libraries

All enrichments used a standard approach – suppression subtractive hybridisation (SSH; PCR-select cDNA subtraction kit, Clontech). Non-directional cloning was subsequently used to insert subtracted cDNA fragments into the pGEM T-Easy vector (Promega). Resultant cDNA fragments generally ranged in size between 150-700 bp.

<u>Immune response genes</u>

A bacterial challenge was undertaken using *Aeromonas salmonicida,* the bacterial pathogen responsible for furunculosis of salmon. Three tissue-specific enriched libraries (head kidney, gill, and liver) were constructed (Martin *et al*., 2006). Fish were anaesthetised with benzocaine (Sigma 20 mg L^{-1}) and injected intraperitoneally with 100 μL (10^9 CFU mL^{-1}) of a genetically attenuated strain ($aroA^-$) of *A*. *salmonicida* (Brivax II, Marsden *et al*., 1996). in phosphate buffered saline (PBS) or 100 μL of PBS as control. Brivax II is a non virulent strain, but acts in a similar manner to the intact virulent pathogen, however, after several rounds of replication the fish clears the bacteria. Intraperitoneal injection of Brivax II induces a protective immune response, with fish resistant to a later challenge with virulent strains. The two groups of fish were kept separately and RNA was pooled from 10 'challenged' fish and 10 control fish at 24 h and 48 h post injection. In each case the challenged RNA was "tester" and the control "driver". Approximately 500 clones from each library were sequenced, a mean redundancy of *c.* 33% being observed. Following BLASTx sequence homology matching, 20%, 23% and 50 % of genes had sequences homologous to immune-associated genes for head-kidney, gill and liver respectively (Martin *et al*., 2006).

Protein catabolism genes

Two tissue-specific enriched libraries (white muscle and liver) were constructed following a starvation trial. RNA was pooled from 10 fish starved for 14 days and from 10 fish fed *ad libitum*. RNA from the starved pool was used as "tester" and RNA from fed fish as "driver". For genes enriched following short term starvation, a highly heterogeneous group of genes was found, as many different biological processes were altered by this treatment including those related to protein turnover. For the library generated from liver, 92% of the sequences were found to have homologies following BLASTx searches. Key groups of genes represented in the library encoded metabolic enzymes, serum proteins and immune response genes, with other minor groupings being iron-binding proteins, globins and factors involved in transcription and translation. For the genes enriched in

muscle following starvation, 77% had BLASTx homologies, with key groups of sequences encoding metabolic enzymes, structural proteins and transcription and translation factors (10%) and minor groups including heat shock proteins.

Diet responsive genes

Atlantic salmon were fed from first-feeding on diets containing either fish oil (FO; capelin oil) or a 25% FO : 75% blended vegetable oil (VO) diet (detailed in 'feed trial' section below). Four subtracted liver cDNA libraries (two timepoints – 52 weeks (pre-smolt / freshwater) and 55 weeks (post-smolt / seawater) and two directions, FO driver and VO driver) were made. Pooled RNA from 12 (pre-smolt) and 4 (post-smolt) female fish on each diet were used. A total of 768 clones were sequenced. All four libraries were found to be highly redundant; Ten fragments comprised *c.* 40% of all sequences. BLASTx analyses gave significant hits (e-value < e^{-20}) for 79 (54%) of the 145 different sequences, though only one of these (catfish fatty-acid binding protein) appeared to be directly related to lipid metabolism.

Smoltification responsive genes

Four tissue-specific SSH libraries enriched in genes up-regulated in brain, pituitary, kidney and gill of smolts (seawater phase) in comparison to parr (freshwater phase) were made. Tissues were dissected from parr and smolt in November / December 2002 and April / May 2003. A total of 380 clones from each of the four libraries was sequenced. Between 32% and 50% of the sequenced clones were identified by BLASTx sequence homology searches. All libraries had considerable redundancy (28% brain, 56% gill, 56% kidney, 86% pituitary). Significantly, the single most abundant sequence in the gill subtractive library corresponded to Na^+/K^+ ATPase whose levels of activity are a key indicator of smoltification status.

Candidate and other genes

A third, minor source of cDNAs for the array, was a small collection of full length genes or gene fragments in plasmid constructs that were already possessed by the partners. These included both candidate genes and also other genes with no known relevance to the specific traits of interest (Table II). Being better characterised than the EST clones these constructs were potentially useful as reference genes on the microarray. Of note, with regard to the research reported in this paper, is the inclusion of three cDNA fragments (the ORF and two 3' UTR fragments) from both (Δ5 FAD and Δ6 FAD) Atlantic salmon fatty acyl desaturase genes.

CLUSTER ANALYSIS AND PROBE SELECTION

All sequence data derived from the above resources, together with 57K Atlantic salmon sequences available *in silico* from Genbank in July 2004, were clustered using the TGI clustering tools (Pertea *et al*., 2003). The process of clone selection for the cDNA microarray is summarised in Fig. 2. Approximately equal numbers (*c.* 9 K) of contigs and singletons were identified as having accessible clones. Since the overall number (*c.* 18 K clones) was comfortably within the printing capacity of the microarray spotter no further clone selection / refinement was undertaken. A single representative clone from each contig was selected for inclusion on the microarray. Where possible a SALGENE clone was selected in preference to an SGP clone, since the entire SALGENE resource was archived at the printing site (ARK Genomics, Roslin Institute). Also, clones were selected from non-normalised or normalised libraries in preference to SSH derived cDNAs in order to take advantage of longer transcripts.

MICROARRAY FABRICATION

Bacterial cultures were grown from archived samples and cDNA inserts amplified directly from these using vector-specific primers. Initially various primer sets were used, dependent on the specific vector employed. However, a generic set (BSKS-F: CGATTAAGTTGGGTAACGC; BSKS-R: CAATTTCACACAGGAAACAG) was found to work with all but one vector. For pBK-CMV constructs, T3 (AATTAACCCTCACTAAAGGGA) and T7 (TAATACGACTCACTATAGGG) primers were employed. PCR reactions (50 μL) were purified by passing them through a 384-well multiscreen filter plate (Millipore). Amplicons were electrophoresed through a 1% agarose gel, and ethidium bromide stained. Only PCR products producing a clear singlet band were considered for spotting. Amplicons were quantified using PicoGreen assay (Invitrogen). Features were printed onto amino-silane coated glass slides (Corning GAPS II) using a MicroGrid II printer (Genomic Solutions). DNA was resuspended in printing buffer (150 mM sodium phosphate buffer, 0.1% SDS, pH8.0) to a concentration of *c.* 150 ng $mL^{-1}$and spotted with a 48 pin tool (Biorobotics 10K pins). Mean spot diameter was 110 microns. The slide format comprised 48 sub-arrays, each consisting of 27 columns and 28 rows. Each cDNA was printed in duplicate, with duplicate features being printed non-adjacently within the same sub-array. A number of control features were printed across the microarray; sonicated Atlantic salmon genomic DNA (96 features); sheared salmon sperm DNA (*Oncorhynchus* derived – Sigma; 96 features); four different SpotReport® (Stratagene) controls – namely PCR product 1-3 (Cab, RCA, rbcL genes from *Arabidopsis thaliana*), and human β-actin PCR fragment (20 features each) and spotting buffer (192 features). In addition each sub-array had two Cy3 spots (landing lights) located at the upper left corner to aid orientation of the slide during grid placement and spot finding procedures. The remaining 1826 locations on the 36,288 spot grid were left blank. Following printing DNA spots were fixed by baking at 80^{o}C for 2 h. Prior to hybridisation microarray slides were treated using succinic anhydride and 1-methyl-2-pyrrolidinone (Sigma) to block unbound amino groups (slide manufacturer's recommended protocol) and

denatured by incubation in 95°C MilliQ water for 2 min. Slides were then rinsed twice in isopropanol, centrifuged to dry and stored in a desiccated environment until required. Details of the TRAITS cDNA microarray have been submitted to the ArrayExpress platform (www.ebi.ac.uk/arrayexpress) under accession number A-MEXP-664. The GAL file is also available for inspection from the TRAITS website (www.abdn.ac.uk/sfirc/salmon/). All clones used on the microarray are archived in 384 well plates as glycerol stocks in two locations (ARK Genomics facility, Roslin Institute UK; SGP Genetics laboratory, University of Oslo).

EST ANNOTATION

Gene identification was carried out by BLAST searches (Altschul *et al*., 1990) of appropriate databases (NCBI nr nucleotide and protein databases) and interrogation of the TIGR Atlantic salmon gene index, release 3.0, (biocomp.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=salmon). Gene ontology identifiers were obtained through Blast2GO (Conesa *et al*., 2005).

SENSITIVITY OF MICROARRAY PROTOCOL

Feed trial

The effect of replacing fish oil (FO) with vegetable oil (VO) at a replacement level of 75% was investigated in Atlantic salmon in a trial conducted over an entire two-year production cycle (Torstensen *et al.*, 2005). Briefly, the two diets were fed to triplicate tanks / cages at Marine Harvest Ltd. facilities at Invergarry (freshwater) and Loch Duich, Lochalsh (seawater), Scotland. Atlantic salmon fry were distributed randomly into 6 tanks (3m x 3m, depth 0.5m) at a stocking level of 3000 / tank, and weaned onto extruded feeds containing 20% added oil which was either FO (capelin oil) or a VO blend, containing rapeseed, palm and linseed oils in a 3.7 : 2 : 1 ratio, replacing 75% of the

277 FO. This VO blend was formulated to mimic fish oil in saturated and monounsaturated fatty acid
278 content *but* with C_{18} PUFA replacing n-3 HUFA. Fish were fed the diets described above for one year
279 until seawater transfer, at which point fish (mean weight ~ 50g) were transferred into 5m × 5m net
280 pens at 700 fish / pen. The fish were fed the same diet in seawater as in freshwater although the
281 dietary oil levels were increased to 25% (3mm pellet) rising to 32% (9mm pellets) through the year-
282 long seawater phase. The diets aimed to be practical, and were formulated and manufactured by
283 Skretting ARC, Stavanger, Norway according to current practices in the salmon feed industry. All
284 diets were formulated to satisfy the nutritional requirements of salmonid fish (NRC, 1993)
285
286 *Sample preparation*
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288 Fish fed on each diet were sampled at two timepoints during freshwater rearing (at 36 & 52 weeks
289 post-hatch, the latter just one week before transfer to sea) and at a further two time-points in seawater
290 (at 55 and 86 weeks post-hatch). Twenty four liver samples per dietary treatment and timepoint were
291 collected. Total RNA was isolated by organic solvent extraction (TriReagent, Sigma) following the
292 manufacturer's protocol. Spectrophotometry (Nanodrop) and electrophoresis (Bioanalyser 2100,
293 Agilent Technologies) were used to quantify and assess quality of the RNAs respectively. For the
294 transcriptomics analysis, equal amounts of RNA from four individuals (2 males + 2 females) were
295 pooled to produce six biological replicates per diet per time-point. Each pooled RNA sample was
296 further cleaned by mini spin-column purification (RNeasy, Qiagen) and was re-quantified and quality
297 assessed as above.
298
299 *Experimental design*
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301 Each biological replicate was co-hybridised in a two dye experiment with a single pooled reference
302 sample. This design permits valid statistical comparisons across both diets and time-points to be

made. The pooled reference sample comprised equal amounts of RNA from each of the 24 biological replicate samples. A dye-swap procedure was incorporated to mitigate selective binding and scanning artifacts. Thus the entire experiment comprised 96 separate hybridisations (2 diets × 4 time-points × 6 biological replicates × 2 dye-swaps).

Labelling and hybridisation protocols

Due to the large number of hybridisations, not all hybridisations could be completed at the same time. Samples were therefore randomised and processed in two batches one week apart. RNA was reverse transcribed and labelled with either Cy3 or Cy5 fluors using the FAIRPLAY II cDNA indirect labelling kit (Stratagene) according to the manufacturer's instructions. Briefly 20μg total RNA was reverse transcribed after being primed with oligo dT, which incorporated aminoallyl-dUTP into the synthesised cDNA strand. The RNA template was then hydrolysed using 1M NaOH for 15 min and neutralised with 1M HCl. The cDNA was NaAce / ethanol precipitated overnight. cDNA pellets were washed in 80% ethanol and air dried before being resuspended in 5 μL 2X coupling buffer. Once the cDNA had fully dissolved (after at least 30 min) 5 μL of either Cy3 or Cy5 dye was added and the samples incubated in the dark for 30 min. The Cy3 and Cy5 dyes (Amersham-Pharmacia) were dissolved in 45μL DMSO prior to being added to the coupling buffer. To remove unincorporated dye, the labelled cDNA (total volume 10μL) was passed through a SpinEX column (Qiagen). Dye incorporation was assessed by separating 1μL of the sample on a mini agarose gel and visualising fluorescent products on a microarray scanner (Perkin Elmer ScanArray 5000XL). No pre-hybridisation step was required. For hybridisation the remainder of each labelled cDNA (7-9 μL; 16-30 pmol each dye) was added to 85 μL hybridisation buffer (UltraHyb, Ambion), 10 μL poly$(A)_{80}$ (10 mg mL^{-1}; Sigma) and 5 μL ultrapure BSA (10 mg mL^{-1}; Ambion). The hybridisation mixture was heated to 95^{o}C for 3 min, then cooled to 60^{o}C before being applied to the microarray. Hybridisations (48 slides per day) were performed on a Gene TAC Hyb Station (Genomic Solutions) for 16 h at

45°C. Slides were then automatically washed with 2×SSC, 0.5% SDS for 10 min at 60°C; 0.2×SSC, 0.5% SDS for 10 min at 42°C; and finally 0.2 X SSC 10 min at 42°C. Slides were then manually rinsed in isopropanol and dried by centrifugation before being scanned.

Data acquisition and analysis

Hybridised slides were scanned at 10 μm resolution using a Perkin Elmer ScanArray Express HT scanner. BlueFuse software (BlueGnome) was then used to visualise and identify features. Following a manual spot removal procedure and fusion of duplicate spot data (BlueFuse proprietary algorithm) the resulting intensity values and quality annotations were exported into the GeneSpring GX version 7.3.1 (Agilent Technologies) analysis platform. Data transformation, normalisation and quality filtering were as follows: 1) all intensity values less than 0.01 were set to 0.01; 2) a 'per spot per chip' intensity dependent (Lowess) normalisation was undertaken using software defaults (20% smoothing / cutoff 10); 3) data were filtered using a BlueFuse spot confidence value > 0.1 in ≥ 24 slides and BlueFuse spot quality of ≥ 0.5 in ≥ 24 slides. This gave a final list of 11,800 genes which were eligible for statistical analysis. Experimental annotations complied fully with MIAME guidelines (Brazma et al., 2001).

qRT-PCR validation

qRT-PCR was performed as described by Villeneuve *et al.* (2005). Primer details are given in Table III. Relative expression ratios were statistically compared between diet samples following normalisation against three housekeeping genes, using REST software (Pfaffl, 2001; Pfaffl *et al.* 2002). Five thousand random allocations were performed and differences were considered to be significant at $P<0.05$.

RESULTS

MICROARRAY FEATURE OVERVIEW

cDNAs derived from fifteen different tissue sources are represented among the 16,950 Atlantic salmon gene features printed on the TRAITS / SGP cDNA microarray (Table I). Of these, approximately 9% are from SSH libraries. Brain cDNAs predominate (15% of total). This reflects the large number of ESTs generated for this tissue, due to 1) brain libraries being included in all three of the EST resources available, 2) increased sequencing effort being focused on these libraries due to perceived diversity of gene expression within brain tissue, 3) availability of a normalised brain EST library. BLASTx homology searches of contig and singleton sequences (Table IV) revealed close to 40% of features having a weak hit (e-value $\geq e^{-10}$) or no hit at all to the NCBI non-redundant protein database. Among the 10,399 features with a significant BLASTx hit (e-value $< e^{-10}$), 6762 (65%) nominally different genes were identified. GO annotations were obtained for 7749 features and can be accessed from the TRAITS website.

DIETARY LIPID EXPERIMENT

The main findings of this microarray-based investigation will be reported in detail elsewhere. Here, data from preliminary analyses are presented to illustrate the degree of robustness and sensitivity that the microarray / experimental design achieved.

Hybridisation data were analysed by two-way ANOVA, which examined the explanatory power of the variables 'time-point' and 'diet' and the interaction between the two and incorporated a Benjamini-Hochberg (1995) multiple test correction ($P \leq 0.05$). This analysis identified 4,142 features showing significant differential expression over the year long experimental time course,

demonstrating considerable temporal changes which may be related to a large number of biological factors. In contrast only 15 significant diet-responsive features were detected (and a further 10 features with significant time-point × diet interactions) Of the 15 significant diet-associated genes, 10 with BLASTx or other known sequence homologies had functions associated with either HUFA or cholesterol biosynthetic pathways (Table V). The differential expression of these genes in these samples was confirmed by qRT-PCR. The identity of the remaining five of the 15 probes remains to be established.

To explore the consequences of analysing smaller numbers of microarrays, the significance of expression of the nine Δ5 / Δ6 fatty acyl desaturase (FAD) probes present on the microarray was used as a proxy indicator for the performance of a given experimental design. Two strategies were employed to reduce microarray number, 1) omission of dye swap, 2) reduction of biological replicates. Two-way ANOVAs were undertaken (without multiple test correction) and gene lists ordered by ascending (diet) P-value. The position of the nine probes in each of the lists is shown in Table VI. In a full analysis incorporating all six biological replicates and a dye swap (12 replicate microarrays per condition) the nine desaturase probes were all present in the top 25 of this list. Reducing microarray numbers, whilst retaining a dye swap, decreased apparent sensitivity, but only markedly when the number of biological replicates was reduced to three. At this replication level the results of the analyses appeared to be sensitive to the particular biological replicates selected, as demonstrated by the duplicate analyses (biological replicates 1-3 vs 4-6). Omission of a dye swap gave mixed results depending upon the dye selected for the pooled reference sample. With a Cy5 pooled reference the results (all nine desaturase probes in top 27 of list) closely matched those of the full 12 microarray design. However, with a Cy3 pooled reference only three probes were in the top 25 of the list. One probe was not in the list at all and others had dropped as low as position 284.

406 Expression profiles derived from the nine FAD probes over the four time-points and for both diets
407 are depicted in Fig. 3. Two distinct patterns are apparent, which, from 3' UTR probe results, clearly
408 correspond to expression of the two different FAD genes (Δ5 and Δ6). However, the expression
409 profiles derived from two of the Δ5 FAD probes (the ORF PCR fragment and full length EST) mimic
410 those of the Δ6 FAD probes.

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414 **DISCUSSION**

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416 TRAITS / SGP MICROARRAY FABRICATION

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418 The TRAITS / SGP cDNA microarray was conceived as a preliminary tool, contributing towards the
419 goal of developing a more focused DNA chip for routine health and performance monitoring in
420 Atlantic salmon. A number of existing EST collections were used as the basis for its design and
421 construction, and whilst this approach had obvious advantages in reducing the time-frame and costs
422 associated with resource development, it also made microarray fabrication all the more challenging.
423 Not only did probe preparation involve a range of different host / vector combinations, but also, the
424 need to track and annotate clones from different library resources compounded the difficulties. Most
425 of the ESTs available comprised 5' end reads. Although this improved the likelihood of successful
426 probe annotation, use of mainly 5' sequence data will inevitably have compromised the cluster
427 analysis as not all the clones will have been full length. The extent of gene redundancy on the
428 microarray can only be confidently established from analysis of 3' end sequence data. Presently,
429 there are no plans to re-sequence the microarray resource.

430

431 Enriching the microarray for potential trait-specific genes has proven to be a worthwhile strategy. In
432 a number of studies (Martin *et al*., 2007; and in preparation), SSH derived probes have been

identified as significant responders in immune, protein catabolism, and smoltification studies. However, in lipid metabolism studies to date, no probes from SSH clones (or contigs containing SSH clones) have been identified as responding differentially between diets. One possible explanation for the apparent failure of the SSH procedure in this case follows from the subsequent microarray analysis of the dietary lipid experiment reported here. Overall fold changes in diet-responsive genes were found to be quite low, with the greatest changes being only 3-fold, and there were no data to support differential expression of these genes at the two time-points (50 & 53 weeks) used for SSH library construction.

There are no plans to radically improve upon the probe set in the TRAITS / SGP microarray. In its existing format there is still spare capacity for feature printing, and additional candidate gene probes have, and will, be added on an *ad hoc* basis. For example, probes for 12 genes associated with lipid metabolism and 10 immune-related genes, not known to be on the microarray, have recently been included to facilitate two specific experimental studies. Over the past few years printing technology has advanced significantly. Currently the TRAITS / SGP microarray is being printed using non-contact inkjet based technology (ArrayJet Ltd), which delivers more consistent spot and slide uniformity than contact pin printing, and which should improve the reliability of generated microarray data and increase the sensitivity of detection.

DIETARY LIPID EXPERIMENT

By identifying candidate genes, the results of this experiment provide reassurance of the clone-tracking accuracy of the microarray. They also confirm that the selected microarray design and fabrication technology, together with the experimental methodology employed, provide the capacity for sensitive detection of differential expression. The fold change differences in expression of Δ5

458 FAD and Δ6 FAD between fish fed VO *vs* fish fed FO based diets closely match those obtained by
459 qRT PCR analysis in a previous study (Zheng *et al.*, 2005*b*).
460
461 Microarray analyses are expensive and time consuming to perform and there is often financial
462 pressure to minimise the number of microarrays used in an experiment. Here, reducing the slide
463 number from 12 to 8 (while retaining the dye swap) had little apparent effect on the ability to detect
464 significant differential expression in FAD genes known to show a variable response according to
465 diet. Using just six slides per condition (one per biological replicate and no dye swap) gave different
466 outcomes according to the dye / target combination used. When the experimental sample was
467 labelled with Cy3 and the pooled reference with Cy5, the results were comparable with the full 12
468 slide (including dye swap) analysis. However, there was much less agreement when the experimental
469 sample was Cy5 labelled and the pooled reference was Cy3 labelled. The reason for this marked dye-
470 dependent disparity remains to be established. However, it has been noted in this and other related
471 studies conducted by TRAITS partners that 1) pooled reference samples produce higher background
472 intensities and 2) the Cy3 channel consistently displays higher background values compared to the
473 Cy5 channel. The combination of these factors may, at least in part, account for this phenomenon.
474 The relevance of these observations to other studies is difficult to assess. The results are largely
475 dependent on the homogeneity of the system under study and the absolute and relative expression
476 levels of any differentially responding genes. For similar reasons, it is also difficult to critically
477 assess published studies more generally. It is likely that the use of sex-balanced pools for
478 experimental biological replication in this study, rather than individual samples, contributed to the
479 discriminatory capabilities of this microarray experiment. Where cost considerations are an
480 important factor, interrogation of reduced numbers of microarrays may be the only viable option
481 available. If background fluorescence can confidently be controlled, omitting a dye swap, as opposed
482 to reducing biological replication, may be a preferable route to follow in such cases.
483

The FAD expression profiles demonstrated the potential for obtaining artifactual results due to cross-hybridisation of similar transcripts. Expression profiles derived from two Δ5 FAD probes (the ORF PCR fragment and full length EST) closely matched those obtained from all Δ6 FAD probes. While the 3' UTRs are very distinct (Δ5 FAD 3' UTR = 1072 bp; Δ6 FAD 3' UTR = 457 bp; sequence similarity *c.* 30%), the two ORFs are very similar to their Δ6 counterparts (both 1365 bp; *c.* 95% sequence similarity). It has also been reported from qRT-PCR analysis, that Δ6 FAD gene expression is approximately four-fold higher than Δ5 FAD expression, in liver tissue of farmed salmon fed on fish oil (Zheng *et al*,. 2005*b*). Thus it would appear that cross-hybridisation of the more abundant Δ6 FAD transcript targets is masking the true expression profiles derived from these two Δ5 FAD probes. The failure of the microarray analysis to correctly distinguish between Δ5 FAD and Δ6 FAD expression profiles in all cases clearly illustrates one of the inherent weaknesses of cDNA microarray-based studies *i.e.* the inability to distinguish between highly similar message transcripts. This is of particular relevance in interpreting transcriptomic data from salmonid species, since fish of the family Salmonidae have undergone a relatively recent whole-genome duplication *c.* 25-100 million years ago (Allendorf & Thorgaard, 1984), such that simultaneous expression of duplicate genes is a commonly observed phenomenon.

OTHER STUDIES.

As part of the original funded project, the TRAITS partners have already used the cDNA array to explore transcriptomic responses in experiments targeting the four key traits identified as being important for sustained salmon aquaculture *i.e.* a) supply of dioxin-free highly unsaturated oils for the salmon diet, b) protein growth efficiency, c) infectious disease, and d) a long and complex lifecycle. In addition to the diet work outlined in this paper, other research at Stirling University, funded by the European Commission, is comparing gene expression both within and between families of fish fed on fish oil or vegetable oil-based diets. Other studies, investigating the

transcriptomic response of Atlantic salmon to infection by infectious pancreatic necrosis virus (IPNV) in both seawater and freshwater environments are similarly in progress. Researchers at Aberdeen University are using the cDNA microarray to study the immune response in Atlantic salmon *in vivo* following infection by *A.salmonicida* and *in vitro* to examine the response to recombinant fish cytokines. Additionally, short-term starvation trials have been used to explore protein catabolism pathways. Finally the Cardiff partner has identified genes and gene pathways from three tissues (brain, gill and kidney) that are involved in the parr-smolt transformation. The TRAITS / SGP microarray has also been supplied to an Australian research group to examine gene expression responses to amoebic gill disease. To date its performance has not been critically assessed for use with other closely related species. Cross-species hybridisation between salmonids has been shown to be extremely high (Rise *et al*., 2004; von Schalburg *et al*., 2005), suggesting that microarrays could be used with similar confidence for both Atlantic salmon and rainbow trout due to the high level of sequence homology between the two species.

FUTURE DIRECTIONS

The final stage of the TRAITS project, *i.e.* development and validation of a focused oligonucleotide array is currently in progress. Approximately 1000 differentially responding genes have been identified from cDNA microarray interrogations by the TRAITS partners and unique 70mer oligonucleotides designed and synthesised for these and appropriate control genes. These form the basis of the TRAITS / SGP secondary oligochip, the performance of which is currently being evaluated. Irrespective of the outcome, the TRAITS / SGP cDNA microarray will continue to be a valuable tool and be available for use to the wider scientific community. Enquiries regarding purchase / use of this microarray should be directed to ARK Genomics (www.ark-genomics.org) in the first instance.

Acknowledgments

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References

Adzhubei, A. A., Vlasova, A. V., Hagen-Larsen, H., Ruden, T. A., Laerdahl, J. K., Høyheim, B. (2007). Annotated expressed sequence tags (ESTs) from pre-smolt Atlantic salmon (*Salmo salar*) in a searchable data resource. *BMC Genomics* **8,** 209. doi:10.1186/1471-2164-8-209

Allendorf, F. W. & Thorgaard, G.W. (1984). Tetraploidy and the evolution of salmonid fishes. In *Evolutionary Genetics of Fishes* (Turner, B.J., ed.), pp. 1–46. New York: Plenum Press.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990). Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403-410.

Benjamini, Y. & Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerfulapproach to multiple testing. *Journal of the Royal Statistical Society* **B57**, 289-300.

561 Bonaldo, M. D. F., Lennon, G. & Soares, M. B. (1996). Normalisation and subtraction: two
562 approaches to facilitate gene discovery. *Genome Research* **6,** 791-806.
563
564 Brazma, A., Hingamp, P., Quackenbush, J., Sherlock, G., Spellman, P., Stoeckert, C., Aach, J.,
565 Ansorge, W., Ball, C. A., Causton, H. C., Gaasterland, T., Glenisson, P,. Holstege, F. C. P., Kim,
566 I. F., Markowitz, V., Matese, J. C., Parkinson, H., Robinson, A., Sarkans, U., Schulze-Kremer, S.,
567 Stewart, J., Taylor, R., Vilo, J. & Vingron, M. (2001). Minimum information about a microarray
568 experiment (MIAME) - toward standards for microarray data. *Nature Genetics* **29**, 365-371.
569
570 Cairns, M. T., Johnson, M. C., Talbot, A. T., Pemmasani, J. K., McNeill, R. E., Houeix, B., Sangrador-
571 Vegas, A. & Pottinger, T. G. (2007). A cDNA microarray assessment of gene expression in the liver
572 of rainbow trout (*Oncorhynchus mykiss*) in response to a handling and confinement stressor
573 *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*
574 doi:10.1016/j.cbd.2007.04.009
575
576 Conesa, A., Gotz, S., Garcia-Gomez, J. M., Terol, J., Talon, M. & Robles, M. (2005). Blast2GO: a
577 universal tool for annotation, visualization and analysis in functional genomics research.
578 *Bioinformatics* **21**, 3674-3676.
579
580 Ewart, K. V., Belanger, J. C., Williams, J., Karakach, T., Penny, S., Tsoi, S. C. M., Richards, R. C. &
581 Douglas, S. E. (2005). Identification of genes differentially expressed in Atlantic salmon (*Salmo*
582 *salar*) in response to infection by *Aeromonas salmonicida* using cDNA microarray technology.
583 *Developmental and Comparative Immunology* **29**, 333-347.
584

585 Hagen-Larsen, H., Laerdahl, J. K., Panitz, F., Adzhubei, A. & Høyheim, B. (2005). An EST-based
586 approach for identifying genes expressed in the intestine and gills of pre-smolt Atlantic salmon
587 (*Salmo salar)*. *BMC Genomics* **6**, 171. doi:10.1186/1471-2164-6-171
588
589 Hastings, N., Agaba, M. K., Tocher, D. R., Zheng, X., Dickson, C. A., Dick, J. R. & Teale, A.J.
590 (2004). Molecular cloning and functional characterization of fatty acyl desaturase and elongase
591 cDNAs involved in the production of eicosapentaenoic and docosahexanoic acids from α-
592 linolenic acid in Atlantic salmon (*Salmo salar*). Marine Biotechnology **6,** 463-474.
593
594 Jordal, A.-E. O., Torstensen, B. E., Tsoi, S., Tocher, D. R., Lall, S. P. & Douglas, S.E. (2005).
595 Dietary rapeseed oil affects the expression of genes involved in hepatic lipid metabolism in
596 Atlantic salmon (*Salmo salar* L.). *Journal of Nutrition* **135,** 2355-2361.
597
598 Koskinen, H., Pehkonen, P., Vehniainen, E., Krasnov, A., Rexroad, C., Afanasyev, S., Molsa, H. &
599 Oikari, A. (2004). Response of rainbow trout transcriptome to model chemical contaminants.
600 *Biochemical and Biophysical Research Communications* **320**, 745-753.
601
602 MacKenzie, S., Iliev, D., Liarte, C., Koskinen, H., Planas, J. V., Goetz, F. W., Molsa, H., Krasnov, A.
603 & Tort, L. (2006). Transcriptional analysis of LPS-stimulated activation of trout (*Oncorhynchus*
604 *mykiss*) monocyte / macrophage cells in primary culture treated with cortisol. *Molecular*
605 *Immunology* **43**, 1340-1348.
606
607 Marsden, M. J., DeVoy, A., Vaughan, L., Foster, T. J. & Secombes C. J. (1996). Use of a genetically
608 attenuated strain of *Aeromonas salmonicida* to vaccinate salmonid fish. *Aquaculture International*
609 **4**, 55–66.
610

611 Martin, S.A., Caplice, N.C., Davey, G.C. & Powell, R. (2002). EST-based identification of genes
612 expressed in the liver of adult Atlantic salmon (*Salmo salar*). *Biochemical and Biophysical*
613 *Research Communications* **293**, 578-585.
614
615 Martin, S. A. M., Blaney, S. C., Houlihan, D. F. & Secombes, C. J. (2006). Transcriptome response
616 following administration of a live bacterial vaccine in Atlantic salmon (*Salmo salar*). *Molecular*
617 *Immunology* **43**, 1900-1911.
618
619 Martin, S. A. M., Taggart, J. B., Seear, P. J., Bron, J. E., Talbot, R., Teale, A. J., Sweeney, G. E.,
620 Hoyheim, B., Houlihan, D. F., Tocher, D. R., Zou, J, & Secombes CJ. (2007). Interferon type I and
621 type II responses in an Atlantic salmon (*Salmo salar*) SHK-1 cell line using the salmon
622 TRAITS/SGP microarray. *Physioogical Genomics* doi:10.1152/physiolgenomics.00064.2007.
623
624 National Research Council (NRC). (1993). *Nutrient Requirements of Fish.* Washington DC: Nation
625 Academy Press.
626
627 Pertea, G., Huang, X. Q., Liang, F., Antonescu, V., Sultana, R., Karamycheva, S., Lee, Y., White, J.,
628 Cheung, F., Parvizi, B., Tsai, J. & Quackenbush, J. (2003). TIGR Gene Indices clustering tools
629 (TGICL): a software system for fast clustering of large EST datasets. *Bioinformatics* **19**: 651-652.
630
631 Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR.
632 *Nucleic Acids Research* **29**, 2002-2007.
633
634 Pfaffl, M. W., Horgan, G. W. & Dempfle, L. (2002). Relative expression software tool (REST) for
635 group-wise comparison and statistical analysis of relative expression results in real-time PCR.
636 *Nucleic Acids Research* **30**, 9-36.

637

638 Purcell, M. K., Nichols, K. M., Winton, J. R., Kurath, G., Thorgaard, G. H., Wheeler, P., Hansen, J. D.,
639 Herwig, R. P. & Park, L. K. (2006). Comprehensive gene expression profiling following DNA
640 vaccination of rainbow trout against infectious hematopoietic necrosis virus. *Molecular Immunology*
641 **43**, 2089-2106.

642

643 Rise, M. L., von Schalburg, K. R., Brown, G. D., Mawer, M. A., Devlin, R. H., Kuipers, N., Busby,
644 M., Beetz-Sargent, M., Alberto, R., Gibbs, A. R. Hunt, P., Shukin, R., Zeznik, J. A., Nelson, C.,
645 Jones, S. R., Smailus, D. E., Jones, S. J., Schein, J. E., Marra, M. A., Butterfield, Y. S., Stott, J. M.,
646 Ng, S. H., Davidson, W. S. & Koop, B. F. (2004). Development and application of a salmonid EST
647 database and cDNA microarray: Data mining and interspecific hybridization characteristics.
648 *Genome Research* **14**, 478-490.

649

650 Roberge, C., Paez, D. J., Rossignol, O., Guderley, H., Dodson, J. & Bernatchez, L. (2007). Genome-
651 wide survey of the gene expression response to saprolegniasis in Atlantic salmon. *Molecular*
652 *Immunology* **44**, 1374-1383.

653

654 Salem, M., Kenney, P. B., Rexroad, C. E. & Yao, J. B. (2006). Microarray gene expression analysis in
655 atrophying rainbow trout muscle: a unique nonmammalian muscle degradation model.
656 *Physiological Genomics* **28**, 33-45.

657

658 Sargent, J. R. & Tacon, A. G. R. (1999). Development of farmed fish: a nutritionally necessary
659 alternative to meat. *Proceedings of the .Nutrition Society* **58,** 377-383

660

661 Torstensen, B. E., Bell, J. G., Rosenlund, G., Henderson, R. J., Graff, I. E., Tocher, D. R., Lie, Ø. &
662 Sargent, J. R. (2005). Tailoring of Atlantic salmon (*Salmo salar* L.) flesh lipid composition and

663 sensory quality by replacing fish oil with a vegetable oil blend. *Journal of Agricultural and Food*
664 *Chemistry*. **53,** 10166 - 10178.
665
666 von Schalburg, K. R., Rise, M. L., Cooper, G. A., Brown, G. D., Gibbs, A. R., Nelson, C. C.,
667 Davidson, W. S. and Koop, B. F. (2005). Fish and chips: Various methodologies demonstrate utility
668 of a 16,006-gene salmonid microarray. *BMC Genomics* 6. 126. doi:10.1186/1471-2164-6-126
669
670 Villeneuve, L., Gisbert, E., Zambonino-Infante, J. L., Quazuguel, P. & Cahu, C. L. (2005). Effect of
671 nature of dietary lipids on European sea bass morphogenesis: implication of retinoid receptors.
672 *British Journal of Nutrition* **94,** 877-884.
673
674 Zheng, X., Tocher, D. R., Dickson, C. A., Bell, J. G. and Teale, A. J. (2004). Effects of diets containing
675 vegetable oil on expression of genes involved in polyunsaturated fatty acid biosynthesis in liver
676 of Atlantic salmon (*Salmo salar*). *Aquaculture* **236,** 467-483.
677
678 Zheng, X., Tocher, D.R., Dickson, C.A., Dick, J.R., Bell, J.G. and Teale, A.J. (2005*a*) Highly
679 unsaturated fatty acid synthesis in vertebrates: new insights with the cloning and characterisation
680 of a Δ6 desaturase of Atlantic salmon. *Lipids* **40,** 13-24.
681
682 Zheng, X., Torstensen, B.E., Tocher, D.R., Dick, J.R., Henderson, R.J. and Bell, J.G. (2005*b*).
683 Environmental and dietary influences on highly unsaturated fatty acid biosynthesis and
684 expression of fatty acyl desaturase and elongase genes in liver of Atlantic salmon (*Salmo salar*).
685 *Biochimica et Biophysica Acta* **1734,** 13-24.
686

686 **FIGURE LEGENDS**

687

688 Fig. 1. Overview of TRAITS strategy to generate both a general cDNA and a focused
689 oligonucleotide- microarray.

690

691

692 Fig. 2. Summary of clustering procedure and probe selection for the cDNA microarray.

693

694

695 Fig 3. Fatty acyl desaturase expression profiles (two diets over four time-points) derived from
696 microarray analysis of nine different Δ5 and Δ6 FAD probes. Blue lines denote Δ6 FAD
697 probes; red lines denote Δ5 FAD probes. Dashed red lines represent two Δ5 FAD probes
698 (ORF PCR fragment and clone bra_snb_04D02 – full length EST sequence) and show
699 expression profiles that mimic those of the Δ6 FAD probes.

700

700 Fig.1

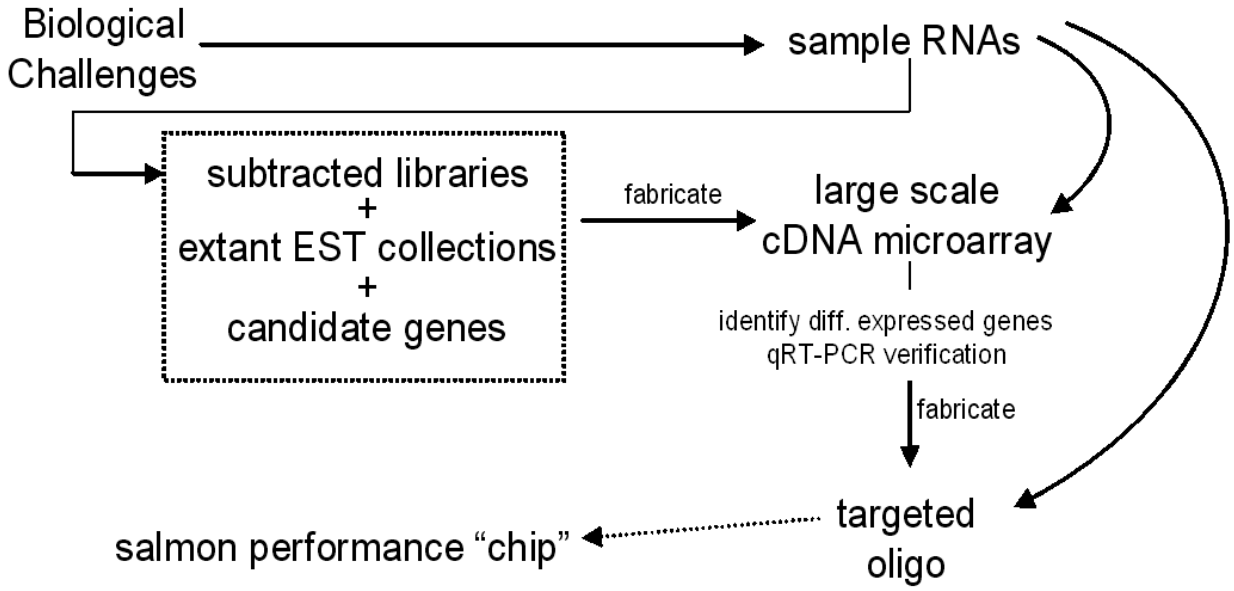

Biological
Challenges
sample RNAs
subtracted libraries
+
extant EST collections
+
candidate genes
fabricate
large scale
cDNA microarray
identify diff. expressed genes
qRT-PCR verification
fabricate
targeted
oligo
salmon performance “chip”

Fig.2.

Clustering (July 04)

Input:	**Source**	**No. Clones**	**Remarks**
	SALGENE	7,492	non-normalised; 7 tissue types; all 5' reads
	SALGENE	4,236	normalised; 2 tissue types, mix of 5' & 3' reads
	SGP	30,564	non-normalised 15 tissue types; all 5' reads
	TRAITS	3,515	SSH derived gene fragments 6 tissue types
	GENBANK	57,536	mix of 5' & 3' reads (*in silico* only)
	TOTAL	**103,343**	

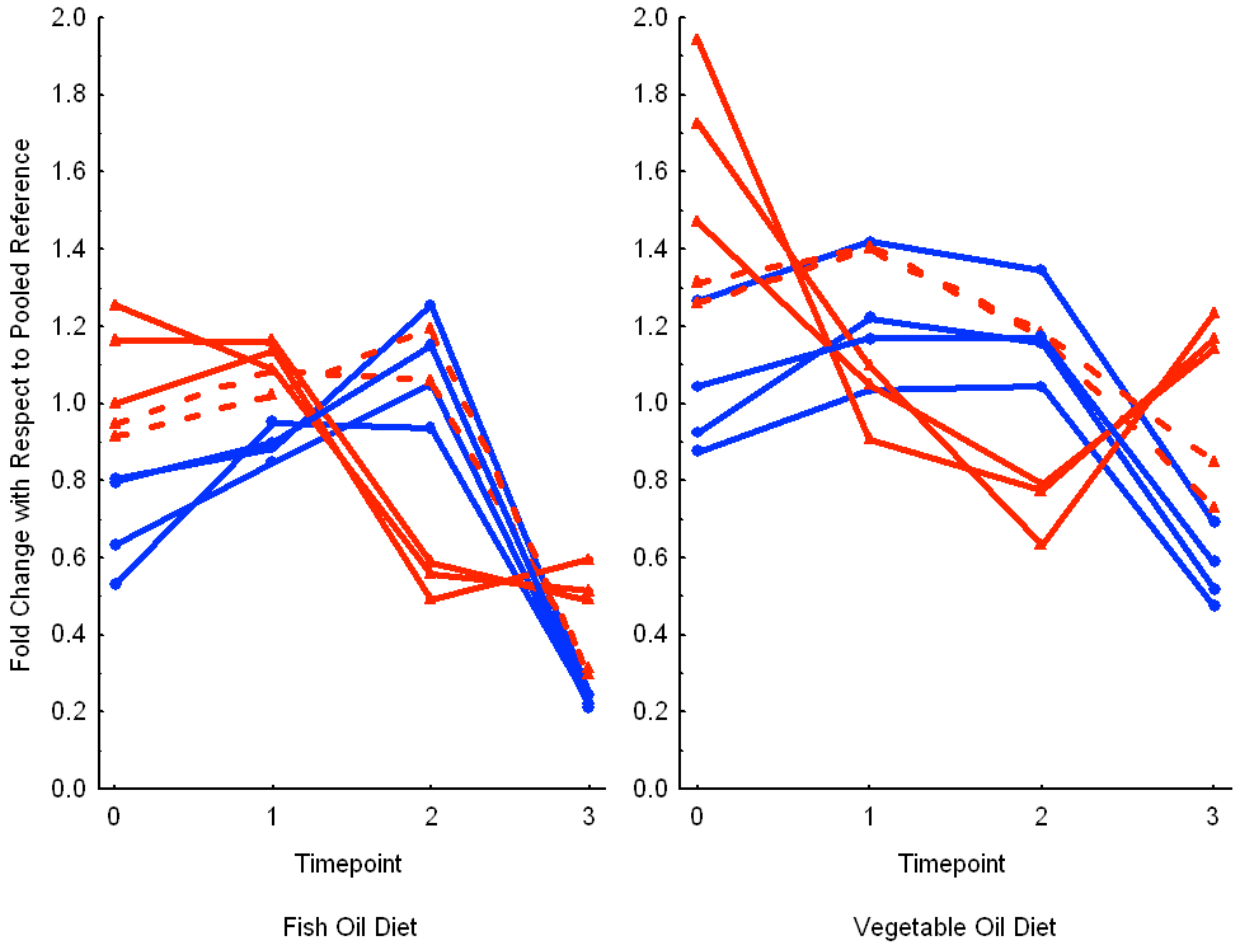
Output:	**Type**	**All**		**Available Clones**
	CONTIGS	13,555	→	9,042
	SINGLETONS	20,826	→	9,145
	TOTAL	**34,381**	→	**18,187**

Removed:	Duplicate clone data (5' & 3' reads) Clones that failed to grow Sub-optimal insert amplifications	- 1,412
Added:	TRAITS selected candidate clones	+ 31
	Later available ESTs	+ 144

GRAND TOTAL: 16,950

737 Fig.3.

739


Fold Change with Respect to Pooled Reference
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
Timepoint
Fish Oil Diet
Timepoint
Vegetable Oil Diet

740 Table I. Details of the EST libraries used to construct the TRAITS / SGP cDNA microarray.

741

Source	Tissue	Environment	Host Cells	Vector
SALGENE	Liver, Testis, Ovary	Seawater	XLOLR	pBK-CMV
SALGENE	Spleen, Kidney	Seawater	SOLR	pBlueScript II SK(-)
SALGENE	Gill, Intestine	Freshwater	XL10 - Gold	pBlueScript II SK(+) XR
SALGENE	White muscle, Brain	Seawater	SURE	pT7T3-Pac
	Liver, Kidney, Gill,		ElectroTen	
TRAITS	White muscle	Freshwater	Blue	pGEM T-easy
	Liver, Kidney, Brain,		ElectroTen	
TRAITS	Pituitary, Gill	Seawater	Blue	pGEM T-easy
SGP	Brain, Eye, Gill, Head	Freshwater	XL10 - Gold	pBlueScript II SK(+) XR
	kidney, Heart, Intestine,			
	Kidney, Liver,			
	White muscle, Ovary,			
	Skin, Spleen,			
	Swim-Bladder, Testis			

742

743

743 Table II. Pre-identified candidates and other reference genes

Gene Name	cDNA length	Notes
Apolipoprotein B	1402 bp	partial with 3'UTR
Carnitine Palmityltransferase1	823 bp	partial
Carotene dioxygenase	872 bp	partial
Estrogen receptor alpha	2900 bp	partial with 3'UTR
Growth hormone receptor	340 bp	Partial, PCR fragment
Glyceraldehyde phosphate dehydrogenase	1086 bp	full length
Heat shock protein P70	830 bp	partial with 3'UTR
Homogenistate dioxygenase	952 bp	partial with 3'UTR
Insulin-like growth factor-1	230 bp	partial
Interferon gamma	1132 bp	full length
Iinterleukin-1 beta	790 bp	ORF
NGF1-B	224 bp	partial (RACE fragment)
Pituitary specific transcription factor 1	250 bp	partial
Peroxisome proliferator-activated receptor alpha	1644 bp	full length
Peroxisome proliferator-activated receptor beta 1	1462 bp	full length
Peroxisome proliferator-activated receptor beta 2	779 bp	partial
Peroxisome proliferator-activated receptor gamma	1665 bp	full length
PUFA elongase	950 bp	ORF
Retinoic acid receptor alpha	840 bp	ORF (RACE fragment)
Retinoic acid receptor gamma	440 bp	Partial inc 5' UTR
Retinaldehyde dehydrogenase type2	922 bp	partial
TNFa induced adipose related protein	483bp	partial
Thyroid hormone receptor alpha	*c.* 2000 bp	partial inc 5' UTR
Thyroid hormone receptor beta	*c.* 900 bp	partial inc 5' UTR
Vitamin D3 receptor	360 bp	partial inc 5' UTR
Δ5 fatty acyl desaturase	408 bp	3' UTR fragment
Δ5 fatty acyl desaturase	881 bp	3' UTR fragment
Δ5 fatty acyl desaturase	1365 bp	ORF
Δ6 fatty acyl desaturase	384 bp	3' UTR fragment
Δ6 fatty acyl desaturase	401 bp	3' UTR fragment
Δ6 fatty acyl desaturase	1365 bp	ORF

744