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A cDNA microarray assessment of gene expression in the liver of rainbow trout (*Oncorhynchus mykiss*) in response to a handling and confinement stressor.

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Abstract

A purpose-designed microarray platform (Stressgenes, Phase 1) was utilised to investigate the changes in gene expression within the liver of rainbow trout during exposure to a prolonged period of confinement. Tissue and blood samples were collected from trout at intervals up to 648 h after transfer to a standardised confinement stressor, together with matched samples from undisturbed control fish. Plasma ACTH, cortisol, glucose and lactate were analysed to confirm that the neuroendocrine response to confinement was consistent with previous findings and to provide a phenotypic context to assist interpretation of gene expression data. Liver samples for suppression subtractive hybridization (SSH) library construction were selected from within the experimental groups comprising "early" stress (2-48h) and "late" stress (96-504h). In order to reduce redundancy within the four SSH libraries and yield a higher number of unique clones an additional subtraction was carried out. After printing of the arrays a series of 55 hybridisations were executed to cover 6 time-points. At 2h, 6h, 24h, 168h and 504h 5 individual confined fish and 5 individual control fish were used with control fish only at 0h. A preliminary list of 314 clones considered differentially regulated over the complete time course was generated by a combination of data analysis approaches and the most significant gene expression changes were found to occur during the 24h to 168h time period with a general approach to control levels by 504h. Few changes in expression were apparent over the first 6h. The list of genes whose expression was significantly altered comprised predominantly genes belonging to the biological process category (response to stimulus) and one cellular component category (extracellular region) and were dominated by so-called acute phase proteins. Analysis of the gene expression profile

in liver tissue during confinement revealed a number of significant clusters. The major patterns comprised genes that were up-regulated at 24 h and beyond, the primary examples being haptoglobin, β-fibrinogen and EST10729. Two representative genes from each of the six k-means clusters were validated by qPCR. Correlations between microarray and qPCR expression patterns were significant for most of the genes tested. qPCR analysis revealed that haptoglobin expression was up-regulated approximately 8-fold at 24 h and over 13-fold by 168 h.

Keywords: rainbow trout, *Oncorhynchus mykiss*, stress, microarray, gene expression

1. Introduction

The vertebrate stress response is a highly conserved suite of neuroendocrine, physiological and behavioural adjustments that, when evoked, enhance the individuals likelihood of survival when exposed to destabilising and challenging circumstances (Johnson et al., 1992). The stress response in fish has been the subject of much interest, not only in an evolutionary and comparative context, but also because of the perceived impact of stress on the welfare and productivity of aquacultured fish (Pottinger and Pickering, 1997). Aquacultured fish species are essentially non-domesticated (Price, 1999; Vandeputte and Launey, 2004), few generations removed from the wild type, and therefore respond inappropriately to relatively benign events that occur unavoidably within the aquaculture environment, such as routine husbandry procedures, transport and related activities. They may also be adversely affected by holding conditions that impose levels of inter-individual interaction or other behavioural conditions that are at odds with the normal life-history requirements of the species (Huntingford, 2004). Where activation of the stress response is intermittent or prolonged the adverse outcomes for growth, reproductive function and immunocompetence are well-documented (Bonga, 1997).

Selective breeding of aquacultured fish to improve economically important traits such as fecundity and growth rate is an established practice (Gjoen and Bentsen, 1997; Hulata, 2001). There is now increasing effort being directed at evaluating the feasibility of selective breeding for a reduced stress response, as a means to improve performance across a range of traits (Pottinger, 2000; Pottinger and Carrick, 1999; Wang et al., 2004). In order for this approach to become practicable, we require a greater understanding of

the genetic basis of the stress response than is currently available - relying solely on phenotypic performance markers is imprecise – and we require genetic markers of desirable stress-related traits.

The aim of the present study was to utilise a purpose-designed microarray platform to investigate the changes in gene expression that occur within a single tissue, the liver, in rainbow trout during prolonged exposure to stressful conditions. A well-established non-invasive model stressor, prolonged confinement (Pottinger and Pickering, 1992), was used to elicit a neuroendocrine stress response in adult rainbow trout and a range of phenotypic endpoints was measured to provide a contextual basis for the interpretation of the microarray data. Analysis of gene expression in the liver tissue of the stressed fish was addressed by: (1) constructing a series of cDNA libraries enriched for genes differentially regulated during stress; (2) applying cDNA microarray technology to investigate the global response of the liver to the stressor, and (3) seeking to identify genes and processes that might ultimately provide markers of stress responsiveness.

2. Materials and Methods

2.1 Experimental animals

Ten days prior to the start of the study 360 adult rainbow trout (CEH 2000, unselected, mixed-sex; mean weight ± SEM: 236 ± 4.3 g) were distributed evenly between 20 holding tanks (18 fish/tank). Each tank (circular, glassfibre, 1000 l) was supplied with a constant flow of lake water (25 l/min) at ambient temperature. Prior to the start of the time-course study the fish were fed commercial food (Skretting Standard Expanded 40) at

the manufacturers recommended rate. During the study food was withheld from both the control and confined fish because of the potentially confounding effects of stress-induced appetite suppression.

2.2 Protocol for confinement stress

Twelve fish from each of ten of the holding tanks were transferred by dip net to pairs of confinement tanks (6 fish/tank, 50 l capacity, 15 l/min flow through) at intervals. The transfers were carried out at appropriate intervals to allow the sampling and processing of fish at 0, 1, 2, 4, 6, 24, 48, 96, 168, 336, 504 and 648 h after the onset of confinement. In addition, at each of these time-points 6 fish each from two of the ten remaining undisturbed holding tanks were sampled, providing 12 stressed and 12 control fish, from duplicate holding tanks/confinement tanks, at each time point. This protocol necessitated the repeated sampling of control tanks, however the interval between successive episodes of disturbance was considered to be of sufficient duration, and the degree of disturbance so minor, as not to impinge on the results of the study.

2.3 Sample collection and tissue processing

At each sample point, 6 fish were netted rapidly from the holding/confinement tank and transferred to an anaesthetic/sedative solution (2-phenoxyethanol; 1:2000; Sigma-Aldrich). Blood samples were collected from the caudal vessels into syringes, then the fish were weighed, measured and killed by spinal section. Each syringe contained EDTA (1.5 mg/ml blood; Sigma-Aldrich) as anticoagulant and the antiprotease aprotinin (3000 KU/ml blood; Sigma-Aldrich). Tissues (liver, skin, head kidney, brain, pituitary) were

dissected rapidly and transferred either to labelled cryotubes, or wrapped in foil, before being snap frozen in liquid N_2 and stored at -80^{o}C. Blood samples were held on ice before being centrifuged at 4^{o}C. Plasma was aspirated and stored frozen (-20^{o}C) in 100 µl aliquots for subsequent analysis.

2.4 Hormone and metabolite analysis and statistical evaluation

Analyses of conventional indicators of the neuroendocrine stress response were carried out in order to support the interpretation of array data, and to confirm that the physiological status of the fish was as intended. Plasma corticotropin (ACTH) and cortisol levels were determined by RIA (Balm and Pottinger, 1993; Pottinger and Carrick, 2001) and plasma glucose and lactate levels were determined spectrophotometrically (Sigma Diagnostics procedures nos. 510 and 735). The hormone and metabolite data were subjected to analysis of variance (ANOVA, GENSTAT) with time and treatment (stressed, control) as factors and fish within tanks as blocking structure. Significant differences were determined using the estimated SE of the differences between means derived from the ANOVA. Where mean and variance were found to be interdependent, the data were log-transformed prior to analysis.

2.5 RNA isolation

Liver samples for SSH library construction were selected from within the experimental groups on the basis of blood cortisol levels. Only unstressed fish with cortisol levels < 15 ng/ml were used for the control group, while fish with cortisol levels > 25 ng/ml were

147 selected from among the stressed fish. Only female fish were selected for RNA isolation
148 to avoid expression anomalies that might be due to sex differences.
149
150 Total RNA was isolated using the RNeasy Maxi Kit (Qiagen). Routinely 0.3-0.5g liver
151 tissue yielded 1-2.5mg total RNA. An on-column DNase treatment step was incorporated
152 to yield samples predominantly free of contaminating DNA. The integrity of the RNA
153 was checked by separating 5μg of RNA on formaldehyde denaturing gels. Only samples
154 that showed no degradation after probing with an α^{32}P-labelled β-actin control cDNA, as
155 indicated by a sharp band with no 'tailing' were used for microarray analysis.
156 Subsequently all samples were re-analysed on the Agilent Bioanalyser: RNA Integrity
157 Numbers (RIN) averaged 9.72 ± 0.38. RNA was quantified by UV spectrometry at
158 260nm (Shimadzu UV-1601). Following evaluation of blood cortisol levels it was
159 decided to divide both stress and control samples into "early" (2-48h) and "late" (96-
160 504h) groups. Each group was represented by 9-13 fish. Poly A^+ RNA was isolated from
161 pooled RNA samples with affinity columns using the Oligotex mRNA Kit (Qiagen). The
162 average yield of mRNA from 1mg of liver total RNA was 2%. Integrity and enrichment
163 of the poly A^+ RNA was checked again using a β-actin probe.
164
165 For microarray screening, 5 samples of stressed and 5 samples of control fish were
166 obtained for each of the time points 0 h (control only), 2h, 6h, 24h, 168h and 504h.
167 Although only females were employed, there was no selection on the basis of cortisol
168 levels. The quality of all these samples was verified by probing northern blots with β-

169 actin. Because an insufficient number of high quality RNA preparations were available
170 the 48h and 96h time points were excluded.
171
172 *2.6 SSH library construction*
173 Four suppression subtractive hybridization (SSH) libraries were constructed using the
174 PCR-Select cDNA Subtraction Kit (BD Biosciences). These corresponded to 'early' and
175 'late' fish groupings (see previous section) and both the forward and reverse subtractions:
176 each subtraction was a stress group with its corresponding control group. SSH was
177 performed following the manufacturer's protocol on 2 µg poly A^+ RNA. An aliquot of
178 the secondary PCR product (2 µl) was cloned into pCR2.1 vector using the TA Cloning
179 Kit from Invitrogen, and transformed into chemically competent TOP10 *E. coli*
180 (Invitrogen). The efficiency observed was $1 \cdot 10^5$ colonies/µg of DNA (5,000 colonies per
181 transformation). Twenty-five white colonies were selected from each library and
182 subjected to PCR. Insert sizes varied from 200 bp to 1,300 bp with 97% of clones having
183 inserts. In order to reduce redundancy within the four SSH libraries and yield a higher
184 number of unique clones an additional subtraction was carried out. The procedure was
185 based on labelling the products of one library (or libraries) with biotin, hybridising these
186 with a second library (or libraries), and cloning the unhybridised DNA after magnetic
187 separation with streptavidin-tagged magnetic beads (Carninci et al., 2000). Three
188 subtractions (libraries RTGL5-7) were carried out on the four liver SSH libraries
189 (RTGL1-4).
190
191 *2.7 Sequence analysis*

192 At the same time that samples were being prepared for PCR, duplicate plates of glycerol
193 stocks were sequenced (The Sanger Centre, Hinxton, Cambs.). Sequences were edited to
194 remove vector and adaptor sequences, and cleaned and filtered before clustering and
195 annotation by the SIGENAE information system (INRA Toulouse, France). Cleaning
196 involved masking of poor quality bases and low complexity sequences such as polyA
197 sequences. Filtering removed contaminating sequences (bacteria, yeast) and allowed only
198 sequences with more than 100 bases of high quality sequence to pass. Contigs were
199 annotated by comparison to the SwissProt database using the Blastx program
200 (http://www.ncbi.nlm.nih.gov/BLAST/). In addition, because of the 3' bias to SSH
201 libraries, sequences were also analysed and compared to a variety of nucleic acid
202 databases using the EST-Ferret program (Li, W., University of Liverpool;
203 http://legr.liv.ac.uk/)
204
205 *2.8 Micoarray hybridisations*
206 PCR (96-well) was carried out in detergent-less conditions in 30 mM Tricine, pH 8.0
207 containing 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTPs and 0.2μM of each nested primer
208 (same primers as used in the final stage of the SSH library construction). The reaction
209 volume was 75 μl and 2.5 units of Biotaq (Bioline, UK) were used per reaction.
210 Templates were the glycerol stocks of the SSH clones. Quality of PCR products was
211 assessed on ethidium bromide-stained agarose gels: single products of approximately
212 equal concentration were required. Printing of the arrays was carried out at the University
213 of Liverpool without further purification of PCR products.
214

A reference design approach was taken for the microarray hybridisations: all experimental samples (10 μg) were labelled with one of the two dyes and compared to a reference sample (10 μg) labelled with the second dye that consisted of a pool of all the RNA samples used in the experiment. Denatured RNA (10 μg) was reversed transcribed with 75 units Stratascript reverse transcriptase (Stratagene, UK) in a total volume of 20 μl containing RT buffer, 10 mM DTT and 1.25 mM amino-allyl dUTP/dNTP (1:1) mix. The reaction was primed with both an anchored oligo dT primer and a random primer (V9) at 0.25 μg/μl and incubated at 42^{o}C for 1 h before making a second addition of 50 units Stratascript reverse transcriptase. After a further 1 h at 42^{o}C the reaction was stopped with 0.45M EDTA in 1M NaOH and neutralised with 0.75M HEPES, pH 7.4 containing 0.75M sodium acetate. The cDNA was purified on a Nucleospin column (Macherey-Nagel), dried on a vacuum concentrator and resuspended with 5 μl 0.1M $NaHCO_3$, pH 9.0. cDNAs (experimental and reference) were labelled with Cy5 and Cy3 dyes (Amersham GE, UK) using the indirect labelling procedure as directed by the supplier. After combining Cy5 and Cy3 labelled cDNAs and removal of unincorporated dyes on a Nucleospin column (Macherey-Nagel), samples were resuspended to 100μl with ArrayHyb Low Temp Hybridisation Buffer (Sigma, UK) at 65^{o}C.

Slides printed at the University of Liverpool were denatured by immersion in boiling 18MΩ water for 1 min. Denatured, labelled target cDNA was applied to the microarray slides, covered with a lifter slip (Erie Scientific) and the slides were incubated in a hybridisation chamber (Genetix) at 50^{o}C for 16 h. After hybridisation all slides were washed manually in 1x SSC, 0.1% SDS, 1mM DTT (2 x 5 min), 0.1xSSC, 0.1% SDS,

1mM DTT (2 x 5 min), rinsed briefly in 18MΩ water (all solutions pre-warmed to 55^{o}C) and immersed briefly in room temperature isopropanol. Slides were centrifuged to dryness and scanned immediately on a ScanArray Express HT scanner (Perkin Elmer). Genepix software (GRI Ltd, UK) was employed to facilitate data acquisition from the images. Any spots in poor areas of the slides (smears, dust specks, poor spot morphology) were manually flagged as 'bad'.

A series of 55 hybridisations were planned to cover six time-points. At 2 h, 6 h, 24 h, 168 h and 504 h: five individual confined fish and five individual control fish (biological replicates) were used. Only control fish were required for the 0 h time point. Although the hybridisations utilised several batches of slides, these were distributed across conditions and time points to minimise the possibility of bias arising.

2.9 Microarray analysis

Stage 1 of the analysis in GeneSpring involved an overview of all samples in order to highlight or remove spurious samples. Viewing of the Cy5/Cy3 ratios in GeneSpring allowed a subjective appreciation of poor slides that could be dismissed from the analysis. Principal Component Analysis (PCA) was also used to examine variation across the samples with time and with animal: again this indicated slides to exclude from the analysis. Normalisation (after dye swapping) used the Lowess normalisation method even though this approach should not strictly be used on clones when the majority are expected to be differentially regulated. Stage 2 of the analysis involved cleaning up the data by filtering out genes that showed large variability in their Cy5/Cy3 ratios and/or by filtering

261 out genes with near baseline raw signal intensities. Stage 3 involved the identification of
262 potential differentially regulated genes by use of one-way ANOVA.
263
264 *2.10 Quantitative RT-PCR analysis*
265 Total RNA samples with RNA Integrity Numbers (RIN) averaging 9.72 ± 0.38 were used
266 for qRT-PCR. cDNA was synthesized from 5 μg of total RNA and 500 ng of polydT
267 primer in a reaction volume of 40 μl using SuperScript™ III reverse transcriptase
268 (Invitrogen) following the supplier's protocol. Primers were designed for qRT-PCR for
269 all selected 12 candidate genes plus two housekeeping genes using Vector NTI
270 Advance™ software (Invitrogen). Size of the amplicons to be produced ranged between
271 100-150 base pairs. qPCR reactions were set up as follows: 10 μl of QuantiTect SYBR
272 Green PCR Kit (Qiagen), 0.5 μM final concentration of primers, 5 μl of cDNA template
273 at a dilution of 1:12.5 and to a final volume of 20 μl with RNase-free water. The
274 Mx3000P® QPCR system (Stratagene) was used for performing the qPCR reaction. The
275 program used for qPCR was 95°C for 15 min, 40 cycles of 95°C for 15 s, annealing at 51
276 – 60°C (depending on primers) for 30 s and extension at 72°C for 30 s. Dissociation
277 curves were examined at the end of the PCR reaction to check for unspecific
278 amplification and primer-dimers.
279
280 Relative changes in the expression of candidate genes were calculated using the method
281 described by Pfaffl (2001). To assess the PCR efficiency of each gene, standard curves
282 were created by serial dilution of standard cDNA preparations.
283

3. Results

3.1 Hormone and metabolite levels during confinement

The changes in plasma ACTH, cortisol, glucose and lactate levels in control and confined fish over the duration of the study are shown in Fig. 1 (a – d).

[Insert Figure 1]

3.1.1 ACTH

Overall, ANOVA revealed plasma ACTH levels to be higher in confined than control fish ($P<0.001$: Fig. 1a). This difference was most pronounced between 24 h and 96 h after the onset of confinement. Plasma ACTH levels in confined fish increased significantly between time 0 and 1 h ($P<0.01$) although a high level of variation among ACTH values in control fish means that no significant difference between control and confined fish was detected at 1 h. There was no significant difference between control values at 0 h and 1 h. The peak in plasma ACTH that was observed at 48 h after the onset of confinement in stressed fish coincides with disturbances in both glucose and lactate in the confined group and suggests that these fish experienced a stressful event that was imposed upon the ongoing confinement. Inspection of the data reveals that the increase was accounted for primarily by the fish within one of the two confinement tanks sampled at this time. It may be the case that agonistic social interaction within this tank was particularly pronounced.

3.1.2 Cortisol

ANOVA revealed highly significant effects of both time ($P<0.001$) and treatment ($P<0.001$) on plasma cortisol levels with confined fish displaying overall higher plasma

cortisol levels than unconfined individuals (Fig. 1b). Plasma cortisol levels rose rapidly following the onset of confinement to reach a level that was consistently sustained until declining between 96 and 336h. Between 336h ad 648h plasma cortisol levels in control and confined fish were statistically indistinguishable. It is noteworthy that little direct correlation between plasma cortisol and plasma ACTH levels was evident, either for treatment groups or individual fish (data not shown). In addition, although not evident from Fig. 1, there was a marked difference in plasma cortisol levels between fish from the two confinement tanks sampled at 48 h, consistent with similar observations for the other determinands. For most of the study period, mean plasma cortisol levels in the control groups remained below 10 ng/ml. However, between 2h and 6h mean levels rose from 5.7 to 38.7 ng/ml before declining again to 6.5 ng/ml by 48h. This is coincident with some minor disturbance of ACTH levels in the control fish and may reflect an unattributable disturbance to the fish in the tanks sampled on these occasions. These tanks had not been sampled previously.

3.1.3 Glucose

Plasma glucose levels changed significantly with both time and treatment ($P<0.001$: Fig. 1c). There was no evidence of an early elevation in plasma glucose levels following transfer to the confinement tanks but levels began to rise after 6 h to reach a peak at 48 h, before declining to baseline levels between 168 and 336 h. An increase in plasma glucose levels at 48 h in the confined fish was coincident with peaks in plasma ACTH and lactate levels, probably for the reasons noted above. There was a steady and significant

(P<0.001) decline in plasma glucose levels in the control fish during the course of the study, presumably related to the absence of food.

3.1.4 Lactate

Significant variation was evident in lactate levels in both control and confined groups (Fig. 1d). The ANOVA reported significant treatment (P<0.001) and time (P<0.001) effects which appeared to have two major components. A significant elevation in plasma lactate levels was evident in confined fish within 1 h of the start of the study (P<0.001). In addition, there was a gradual but significant (P<0.01) increase in lactate levels in control fish between 4 h and 48 h such that lactate levels in confined fish were significantly lower than those in controls for the latter phase of the confinement period. Furthermore, there was a two-fold increase in lactate levels in confined fish at 48 h (P<0.001), at the same time as increases in plasma ACTH and glucose were observed.

3.2 Sequence analysis

The array consisted of 21120 spots most of which were unknown sequences at the time of microarray printing. Of these, 1011 were liver clones isolated from the four confinement stress SSH libraries and 621 were liver clones isolated after the redundancy subtraction from these same four libraries (section 2.6). The complete array will be described elsewhere but an overview of its composition is presented in figure 2. In addition to the 1632 liver clones from confinement stress there were also 1536 pituitary clones and 1152 brain clones from fish exposed to a confinement stressor. Liver clones from fish exposed to a temperature stressor (3072), hypoxia stressor (1536) and pathogen exposure (960)

were also represented. There were additional clones from the brain tissue of fish exposed to temperature (3072) and hypoxia stressors (1536), muscle clones from temperature (3072) and hypoxia stressor-exposed fish (1536), and gill (980), head kidney (960) and mixed tissue (460) clones from pathogen exposed fish. Only a subset of the liver clones (769 clones of the 1632 clones relating to confinement stress) was sequenced generating 507 quality sequences made up of 247 different contigs, 49 of which were singletons. The level of redundancy overall was ~51% for the liver confinement libraries.

[Insert Figure 2]

A number of the liver clones had been generated by a redundancy subtraction procedure described in section 2.6. One of the redundancy-subtracted libraries (RTGL5) was a direct subtraction from the 'forward late' library (RTGL3) and there were approximately equal numbers of clones with data from each. The redundancy-subtracted library produced 35% more unique clones than the source library. Furthermore, two highly represented genes (apolipoprotein A-I-1 and EST16605) were reduced in representation from 23% to 3% and from 16% to 8% respectively in the redundancy-subtracted library.

3.3 Characterisation of expression profiles.

A list of clones (964) considered to be differentially regulated over the complete time course was generated by a combination of data analysis approaches. Instead of restricting the analysis to one very stringent statistical test, lists of genes whose expression was altered by exposure to confinement were generated by a number of tests and how these lists overlapped was taken into account before deciding on the most relevant genes. The

two main approaches were an ANOVA over time with multiple test correction (Benjamini and Hochberg False Discovery Rate) and a t-test comparing stressed samples with their corresponding controls. Analysis of the confinement stressor by microarray analysis clearly demonstrated that the most significant gene expression changes occurred over the 24 h to 168 h time period with a general return to control levels by 504 h. Changes in expression levels over the first 6 h were evident for only a few genes.

As stated above most of the clones were unknown at the time the arrays were printed and as a result many genes were represented multiple times on the array. This allowed examination of the reproducibility of the array. The genes most frequently represented were the haptoglobin precursor (101x), EST10729 (28x), CIRBP (21x) and complement C3-1 (71x). There were however 110 instances of the haptoglobin precursor on the whole array and 9 instances not showing significance. On inspection of the expression patterns it was clear that all 110 instances showed the same clear expression profile. Similarly all instances of EST10729 show a common expression pattern. On the other hand, there were over 1000 instances of complement component C3-1 on the whole array yet only 71 of these clones were identified as significantly differentially expressed. That is, the expression pattern for all instances of complement component C3-1 showed no common pattern suggesting the detection of changes in expression level of this gene is probably due only to its high representation on the array.

A shorter list of 314 differentially expressed clones was generated using the more stringent Bonferroni multiple test correction. Of these clones 138 (44%) provided quality

sequence information. Uninformative clones were the result of a combination of factors: not all clones were selected for sequencing, some selected clones gave no sequence information, some gave poor quality sequence and some gave less than 100 bases of quality sequence. The 138 clones were composed of 39 different contigs with identification of 28 genes, leaving unidentified a number of EST contigs and singletons. The list of significant genes comprised predominantly genes originally isolated from liver and brain libraries. When compared to the whole array (953 annotated genes) using the GOTree Machine (GOTM) program (Zhang et al., 2004), no biological process category was over-represented at levels 4 or 5. However, in a broader context (i.e. levels above 4 and 5) the "Response to stimulus" category was over-represented ($P<0.01$). Also in the broader context, the cellular component category, "Extracellular region" was over-represented ($P<0.01$). Genes that fall into these categories include: (response to stimulus) haptoglobin, complement factor H-related protein 1, fibrinogen beta polypeptide, complement component factor H, cold-inducible RNA binding protein and (extracellular region) haptoglobin, complement factor H-related protein 1, fibrinogen beta polypeptide, procollagen typeVIII alpha 1, inter-alpha (globulin) inhibitor H2 and secreted phosphoprotein 2. There are, however, too few annotated genes (24) in the list of 314 differentially expressed genes to carry out significant Gene Ontology analysis of confinement stress in the liver.

A k-means clustering of the 964 clone list suggested six main expression patterns (Figure 3). The main group (Set 1 - 332 clones) showed up-regulation at 24 h and 168 h and a return towards the control level by 504 h. The seminal gene in this list was the

haptoglobin precursor but it also included fibrinogen (beta and gamma polypeptides). A second group (Set 2 - 94 clones) also showed up-regulation at 24 h and 168 h and a return towards the control level by 504 h. Despite this similarity the component sequences of Sets 1 and 2 were clearly different – for example, Set 2 did not include any of the 110 instances of haptoglobin on the array, instead, the seminal set member was complement factor H. Set 3 (131 clones) was maximally down-regulated at 168 h but showed some down regulation of several members as early as 2 h (RpL7, TC8053 and TC7970). The seminal member of this group was CIRBP (cold-inducible RNA-binding protein) but it also included alpha-1-antitrypsin homolog, RpL35 and AMBP protein precursor. Set 4 (107 clones) was similar to Set 3 in being down regulated over 24 h and 168 h: in fact down regulation of these genes in the stressed group at 168 h and 504 h was more pronounced when compared to expression levels in the control samples which were markedly up-regulated relative to the earlier time points. This is clearer in TC8200 than 14-3-3 (see Figure 4). There is no clear characteristic sequence in this group but the tissue origins of the clones in this set were heavily biased towards brain rather than liver libraries. Set 5 (58 clones) showed immediate down regulation at 2 h, followed by up regulation over 6-24 h, then a second down regulation at 168 h before returning to normal at 504 h. The seminal member was alpha1-acid glycoprotein (orosomucoid) but the set also included serum albumin. The final set (Set 6 -103 clones) also showed early down-regulation over 2-6 h then a steady up-regulation from 24 h to 504 h. The seminal members of Set 6 were EST10729 and ceruloplasmin. In this set control samples showed a similar expression pattern to that in stressed fish but generally of a lesser magnitude.

[Insert Figure 3]

Overall, the expression patterns that were seen in the stressed fish were also seen, though to a more muted extent in the 6 h and 24 h control samples. This corresponds to the unattributable elevation of plasma cortisol levels in the ostensibly undisturbed control fish described above and evident in figure 1b.

To determine whether the pattern of gene expression in response to confinement varied between individual fish an ANOVA was carried out for specific time points. Analysis of haptoglobin (all instances on the array) at 168h showed that fish 191 was clearly very different from fish 190. Fish 191 was notable within this group of confined fish in that its plasma cortisol levels were atypically low (16.5 ng/ml) compared to those of the others in the group (295.4 ng/ml, 179.9 ng/ml and 44.7 ng/ml for fish 190, 188 and 181 respectively). At this time point the ranking of the individual haptoglobin responses matched that of the cortisol ranking.

3.4 Quantitative PCR

To validate the microarray results two genes were selected from each of the six k-means cluster groups for real time RT-PCR (qPCR). Primer sequences were generally based on actual clone sequences on the array (with reference to the contig) rather than homologous database sequences (but see Table 1). The 2 h time point was omitted from the qPCR experiment as the microarray suggested expression changes were small over the first 6 h. Generally the same five fish (both control and stress) were used in the qPCR experiment as were used in the microarray experiment. Two housekeeping genes (β-actin and 18S ribosomal RNA gene) were shown to remain at a consistent level between the control and

stress groups, and over time. However, there was some inter-individual variability in the expression of these genes and therefore both genes were averaged for use in normalising expression levels. All expression levels were related to the 0 h control using a calculation method based on differences in Ct and amplification efficiency (Pfaffl, 2001).

All control samples gave a very similar expression pattern with a general rise after 24 h through 168 h and 504 h. Although changes in gene expression patterns detected over time in the stressed fish generally showed good correlation between qPCR and microarray methods (see below) the magnitude of expression change was often reported by qPCR as being greater than that detected on the microarray. Sets 1 and 2 showed quite similar expression patterns with maximum expression over 168 h and 504 h: this maximum appeared to be reached somewhat later in Set 2. Maximum relative changes in expression detected by qPCR were ~13-fold and ~3.5-fold compared to ~2.2-fold and ~1.8-fold by microarray for haptoglobin and β-fibrinogen respectively (both Set 1). Sets 3 and 4 also showed up-regulation with time, however expression appeared to increase in two steps, from 0 h to 6 h then 24 h to 504 h with a plateau of expression between 6 h and 24 h. The maximum expression levels in these two sets at 504 h were very similar between the stress and control groups with at least CIRBP suggesting a late down-regulation in the stress group compared to the control group. Set 5 showed maximum expression levels at 6 h but quite an irregular pattern over time in both control and stress groups. Set 6 genes were up-regulated over time with a slight tendency to dip between 6 h and 24 h before increasing again through 504 h.

As mentioned above the magnitude of expression changes as measured by microarray were more muted than those detected by qPCR, however the patterns of expression generally correlated well between both methodologies. Correlations were determined for each fish individually and were found to be significant ($P<0.05$) for 8 of the 12 genes examined with correlation coefficients for haptoglobin and EST10729 of 0.67 and 0.90 respectively. Neither of the two genes of Set 5 correlated significantly between methodologies but both genes of Sets 1, 2 and 6 did correlate significantly.

4. Discussion

The elevation of plasma ACTH and plasma cortisol in the confined fish clearly indicated that the stressor employed in these studies activated the hypothalamic-pituitary-interrenal axis in a manner consistent with previous studies using this approach (Pottinger et al., 1994). Plasma cortisol levels in the confined fish displayed a profile typical of rainbow trout subjected to a chronic stressor with significantly elevated levels during the early stages of confinement being followed by an acclimatory return to baseline levels within 336 h. Among the control fish the range of mean plasma cortisol values observed (0.8 – 38.7 ng/ml) was greater than is normally typical of unstressed trout due in large part to elevated levels in several consecutive samples between 2 h and 48 h after the start of the study. There is no clear explanation for this and we assume that some unaccounted for disturbance of these tanks took place during the sampling period. The six control tanks that were sampled at 4, 6 and 24 h when cortisol levels were most elevated were sampled again at 96 h, 336 h and 504 h respectively at which time levels were typical of undisturbed fish. To ensure a contrast between stressed and unstressed individuals

control fish liver samples for the SSH exercise were selected from individuals whose plasma cortisol levels at the time of sampling did not exceed 15 ng/ml.

Exposure of trout to prolonged confinement is characteristically accompanied by disturbances in plasma glucose levels and plasma lactate levels (Trenzado et al., 2003) and both responses were evident in the present study. Perhaps surprisingly, no short-term elevation of plasma glucose was detected initially in the confined fish, although levels were elevated during the 24h – 168h period. Short-term glucose elevation is a widely observed response of fish to acute stressors so the absence of this response is puzzling. It is possible that the severity of the initial stressor was insufficient to cause a change in circulating catecholamine levels (Perry and Bernier, 1999), considered to be the mediator of stress-induced hyperglycaemia in fish. The short period of hyperlactemia that occurred in confined fish early in the time-course was presumably associated with the respiratory consequences of chasing, netting and transfer to the confinement tanks and was rapidly ameliorated within 2 – 4 hours. The occurrence of slightly elevated plasma lactate levels in the control fish between 4h and 168 h is not easily explained but does coincide with the anomalous elevation of plasma cortisol in the control fish. Overall, the experimental data indicate that the response of these fish to confinement was consistent with previous findings and provided a suitable vehicle for further investigation of gene expression in the liver.

The analysis of gene expression in liver tissue from confined fish that is presented here is part of a larger EU study "STRESSGENES: A Functional Genomics Approach to

Measuring Stress in Fish Aquaculture" (Q5RS-2001-002211) that includes analysis of gene expression during exposure to a confinement stressor in other tissues (head kidney, brain, pituitary and gill) and additional stressors (temperature shift, hypoxia, salinity stress and pathogen exposure) in a range of relevant tissues. For each tissue SSH libraries were constructed and 'pre-arrays' were hybridised to eliminate unchanging genes. Initially arrays comprising unselected clones (Phase 1 arrays) were constructed followed by one array using a selected clone set (Phase 2 array). For the experiment reported here a Phase 1 array was used: a consequence of this is that there was not full sequence analysis of the clones.

As noted above, the list of genes whose expression was significantly altered comprised predominantly genes originally isolated from liver and brain libraries and these fell largely into one biological process category (response to stimulus) and one cellular component category (extracellular region). Analysis of the gene expression profile in liver tissue during confinement revealed a number of significant clusters. The major patterns comprised genes that were up-regulated at 24 h and beyond, the primary examples being haptoglobin, fibrinogen, TC8442, ceruloplasmin and EST10729. Haptoglobin expression was up-regulated at 24 h and transcripts maintained this high level through to the 168 h sample. After 504 h (21 days) continuous confinement expression levels approached but had not yet reached control levels. Quantification of gene expression levels by qPCR confirmed the microarray expression pattern and showed an up-regulation of approximately 13-fold over the zero time point control and approximately 8-fold over the paired control. Of all the genes validated by qPCR this was

559 the greatest change in the magnitude of expression observed but this does not rule out the
560 possibility of greater changes in other genes that were not scrutinized with qPCR – for
561 example, TC17071 appeared more differentially regulated on the microarray than many
562 of the haptoglobin clones. Although not evident from the microarray, haptoglobin was
563 up-regulated 4-5-fold by 6 h. In functional terms, haptoglobin is a prominent acute phase
564 protein in man and in most mammalian species studied. Circulating levels of this protein
565 can change by between 2-fold to over 10-fold depending on the species (Petersen et al.,
566 2004). In fish (*O.mykiss*) elevated plasma levels of haptoglobin have been identified after
567 a variety of bacterial and viral challenges, but changes in haptoglobin levels have not
568 previously been linked to a non-immunological stressor (Bayne et al., 2001). In rainbow
569 trout, 24 h after receiving a bacterin injection, up-regulation of haptoglobin at the
570 transcript level on an oligo-microarray was limited (2-fold) but qPCR showed large
571 variation in individual fish response (Gerwick et al., 2006). In other studies on rainbow
572 trout, there was no significant change in brain haptoglobin expression following a
573 handling stress (Krasnov et al., 2005a) in liver haptoglobin expression after toxicant
574 exposure (Krasnov et al., 2005b) or in aflatoxin-induced hepatocellular carcinoma (Tilton
575 et al., 2005). In pigs haptoglobin levels are not elevated in response to cold, heat or
576 transport stressors (Hicks et al., 1998). Haptoglobin is however elevated in disease and
577 subclinical states suggesting that in pigs at least it is a good indicator of the health status
578 of the animal (Petersen et al., 2004). In calves stress related to housing on a slippery floor
579 raised levels of the acute phase protein serum amyloid A but not that of haptoglobin
580 (Alsemgeest et al., 1995).
581

582 The main role of haptoglobin appears to be in conserving haemoglobin, haem and iron
583 after their release from damaged red blood cells and the prevention of oxidative stress.
584 Scavenging iron is also believed to be important in preventing the growth of iron-
585 requiring bacteria (Bullen, 1981). Genes coding for proteins such as albumin,
586 ceruloplasmin, hepicidin, lactoferrin, transferrin, ferritin and hemopexin might be
587 expected to play a similar role. Differential regulation of both ceruloplasmin and albumin
588 was identified in this study (though controls and stress were very similar) but neither
589 hepcidin nor lactoferrin were selected for the array (suggesting no treatment-related
590 change in their expression levels) and neither ferritin nor transferrin showed any clear
591 change on the microarray. Transferrin has been described as both a positive and negative
592 acute phase protein in different species and there is a suggestion that it is a positive acute
593 phase protein under an inflammatory stimulus in trout (Bayne and Gerwick, 2001). Apart
594 from alterations in haptoglobin itself there was minimal evidence in the present study that
595 exposure to a prolonged stressor produced alterations in other factors responsible for iron
596 metabolism/storage.
597
598 Serum albumin and alpha 1-acid glycoprotein (orosomucoid) fall within the same
599 expression cluster (Set 5 in Figure 3) and are again considered to be acute phase proteins
600 in several mammalian species (Gabay and Kushner, 1999). On the microarray both
601 transcripts demonstrate a very early (2 h) down-regulation, followed by rapid up-
602 regulation by 6 h and then a second more prolonged down-regulation over 168 h.
603 However, neither of these gene expression patterns detected on the microarray correlated
604 closely with the pattern determined by qPCR. Both genes were up-regulated at 6 h and

although orosomucoid expression in stressed fish showed a downward trend from 6 h to 504 h, serum albumin showed no tendency for reduced expression over the later time points. (Relative to the paired control samples, which exhibited some variation with time, serum albumin did show a downward trend). The absence of the 2 h time point from the qPCR does not preclude the possibility of an early 2 h down-regulation. Serum albumin is normally considered a negative acute phase protein as it decreases under an acute phase stress. Here, however, the expression pattern as it relates to the abundance of RNA transcript seems much more complex. Whether this is a consequence of message levels not necessarily reflecting protein levels, or that confinement stress is not a severe enough stressor, or that stress responses as characterised in mammals are not wholly applicable to non-mammalian vertebrates, is unclear from the present study. It should be noted that to treat the full period of confinement as the imposition of a homogeneous stressor upon the fish is probably a gross simplification. The nature of the stressor as perceived by the fish is likely to alter over time, from being primarily a disturbance/novelty/unfamiliar environment combined with spatial restriction in the first few hours after transfer to the confinement tank, to a primarily social stressor after more time has elapsed. Acclimation to the confinement environment is quite rapid when fish are held in isolation, but requires increasingly longer as the size of the interacting group increases (Pottinger and Pickering, 1992).

Orosomucoid (ORM1), like many acute phase proteins, does not have a well-defined role although it is considered both anti-inflammatory and immunomodulatory. It is reported to interact with serum albumin (Krauss et al., 1986), which possibly explains the broadly

similar gene expression patterns observed for the two transcripts, and with plasminogen activator inhibitor type 1 (PAI-1). When orosomucoid is upregulated (6 h), PAI-1 (a serpin) is maintained in a highly active form: proteinase inhibitor activity is therefore maximal over this period before it falls quite dramatically over the 24 h to 168 h period. High levels of proteinase inhibitory activity may allow the build up of the acute phase proteins and subsequent reduction of this activity may facilitate the removal of the acute phase proteins and return to a pre-challenge steady-state. The very early down regulation (at 2 h, seen on the microarray) may be the consequence of some immediate need for protease activity – possibly to do with activation of complement or blood clotting cascades. The absence of correlation in Set 5 may suggest that this cluster is weakly assigned.

Consistent with this speculation is the fact that another group of functionally related proteins that appear from the microarray to be down-regulated late in the stress process are the protease inhibitors alpha-1-antitrypsin homolog and alpha-2-HS glycoprotein precursor (a cysteine protease inhibitor). Both are down-regulated through 24 h and 168 h. Although qPCR shows up-regulation of alpha-1-antitrypsin homolog over 6 h and again at 504 h, the net effect is one of down-regulation compared to the paired controls. Alpha2-macroglobulin (A2M) is a major acute phase protein in many species but has not been identified as such in trout (Bayne and Gerwick, 2001). Like serum amyloid A (see below) it does not appear in an identifiable form on the array. Alpha2-macroglobulin is also an antiproteinase inhibitor acting on enzymes such as trypsin, thrombin and collagenase. It is believed to interact with alpha1-microglobulin/bikumin precursor

(AMBP). Interestingly AMBP was identified as a significantly differentially regulated gene and was found in the same cluster (Set 3) as alpha-1-antitrypsin homolog.

Differences in immune system function between mammals and fish are very significant. Fish are dependent on innate, non-lymphoid mechanisms to defend themselves against infection and trauma, whereas mammals exploit an adaptive immune system based upon T- and B-cells. Fish are therefore much more dependent than mammals on a non-specific response focusing on defensive proteins dissolved in body fluids and possibly have adapted their functions accordingly. There are some immune-related genes that are, perhaps surprisingly, missing from the list of differentially expressed genes obtained during the present study. Serum amyloid A does not appear in an identifiable form on the array: its absence from the liver confinement SSH libraries suggests it is not a significant part of the non-specific stress response in trout. The pentraxins, although represented on the array, show no significant change in expression. Two clearly different pentraxins are on the array: one is the published SAP-like pentraxin (Jensen et al., 1997) but the second has almost equal homology to human serum amyloid P and human C-reactive protein (see accession nos. CR944257 and CR944502). Although not significant, the SAP-like pentraxin appears to be down-regulated from 6 h and maximally at 168 h whereas the other pentraxin appears to be up-regulated at 168 h. If there is an acute phase response to a prolonged non-invasive stressor in trout it does not follow the pattern found following a pathogenic challenge.

Also of interest is the relative absence of differentially regulated genes associated with gluconeogenesis and glycogenolysis in the liver confinement study. Intuitively, an early up-regulation and a later reversal of this process might be anticipated, in order to support the hyperglycaemic response normally seen in stressed fish. It is of course possible that the severity of the confinement stressor is not substantial enough to require changes in the expression levels of key genes involved in gluconeogenesis and instead post-translational modifications (phosphorylation, etc) are sufficient to regulate the process. Indeed the absence of an immediate rise in blood glucose levels in this confinement experiment might explain why gluconeogenesis- and glycogenolysis- related genes were not to the fore in the microarray analysis. A gene identified as a glycogen synthase kinase binding protein (zebra fish; *Danio rerio*) is clearly upregulated at 24 h and 168 h though the relevance of this event is presently unclear. It is known that mammals mount qualitatively different acute phase responses to different stimuli and indications from the literature are that fish do likewise.

Krasnov et al (2005a) used similar methods to those employed in the present study to investigate the effects of handling stress on gene expression in both brain and head kidney of rainbow trout. Although this earlier study found changes in some genes in common with the present study (troponin C, immunoglobulin epsilon receptor, 14-3-3) and these expression patterns over time appear similar, the majority of the genes for which significant alterations in expression were reported are quite different. This is probably in part due to the different tissues studied and in part due to the choice of clones in the microarray design, but it may also indicate that the response to a short handling

696 stress (Krasnov et al., 2005a) is quite different to that arising from a continuous
697 confinement stressor (this study). Previous studies that have examined gene expression in
698 trout liver tend to relate to toxicological challenge (Krasnov et al. 2005b; Hook et al.,
699 2006; Tilton et al., 2007). What most of these studies have in common with each other
700 and with the current study is that the effects on gene expression are small, generally
701 approximating to 2-3 fold although changes quantified by qPCR are often larger than
702 those detected by microarray. A bacterin challenge shows very large changes in liver
703 expression of hepcidin and diacylglycerol *O*-acyl-transferase neither of which are seen to
704 respond to confinement stress yet haptoglobin and DRTP1 are up-regulated in response to
705 both stressors (Gerwick et al., 2007). There appear to be clear distinctions in gene
706 expression patterns between different stressors: a gene that may be strongly up- or down-
707 regulated in response to one stressor may not be responsive to a different type of stressor.
708 This is exemplified by the diverse nature of the response to a group of model toxicants
709 (Hook et al., 2006).
710
711 In general the qPCR results confirmed the microarray results which therefore suggests the
712 latter analysis can be used confidently to provide a "global" picture of the gene
713 expression response to prolonged confinement stress in fish. Some patterns did conflict
714 but this was probably a result of the weaker clusters where expression changes were quite
715 small on the array. It has been reported previously that correlations between microarray
716 and qPCR are best when expression changes are 2-fold or more (Morey et al., 2006). It
717 should also be noted that only two genes were checked by qPCR from each cluster and
718 that better correlations would have been achieved if only the most differentially

719 expressed genes were validated. Ultimately,, protein levels (both in each relevant tissue
720 and in plasma) will have to be measured to get a clear indication of what is the full
721 physiological response of the fish to confinement stress. In many cases though this will
722 await the production of antibodies with specificity to fish proteins.
723
724 Overall, the present study indicates that while the primary role of the liver during acute
725 and chronic stress may be linked with the provision of energy, this role is not reflected in
726 the patterns of gene expression. Instead, the up-regulation of genes in trout during
727 exposure to a non-invasive stressor seems primarily to be associated with the non-specific
728 defence. The results perhaps highlight the need for a multifaceted approach to the
729 analysis of complex physiological processes such as the stress response where
730 examination of neither expression profiles nor key indicator endpoints alone provides a
731 full picture of the events taking place.
732
733
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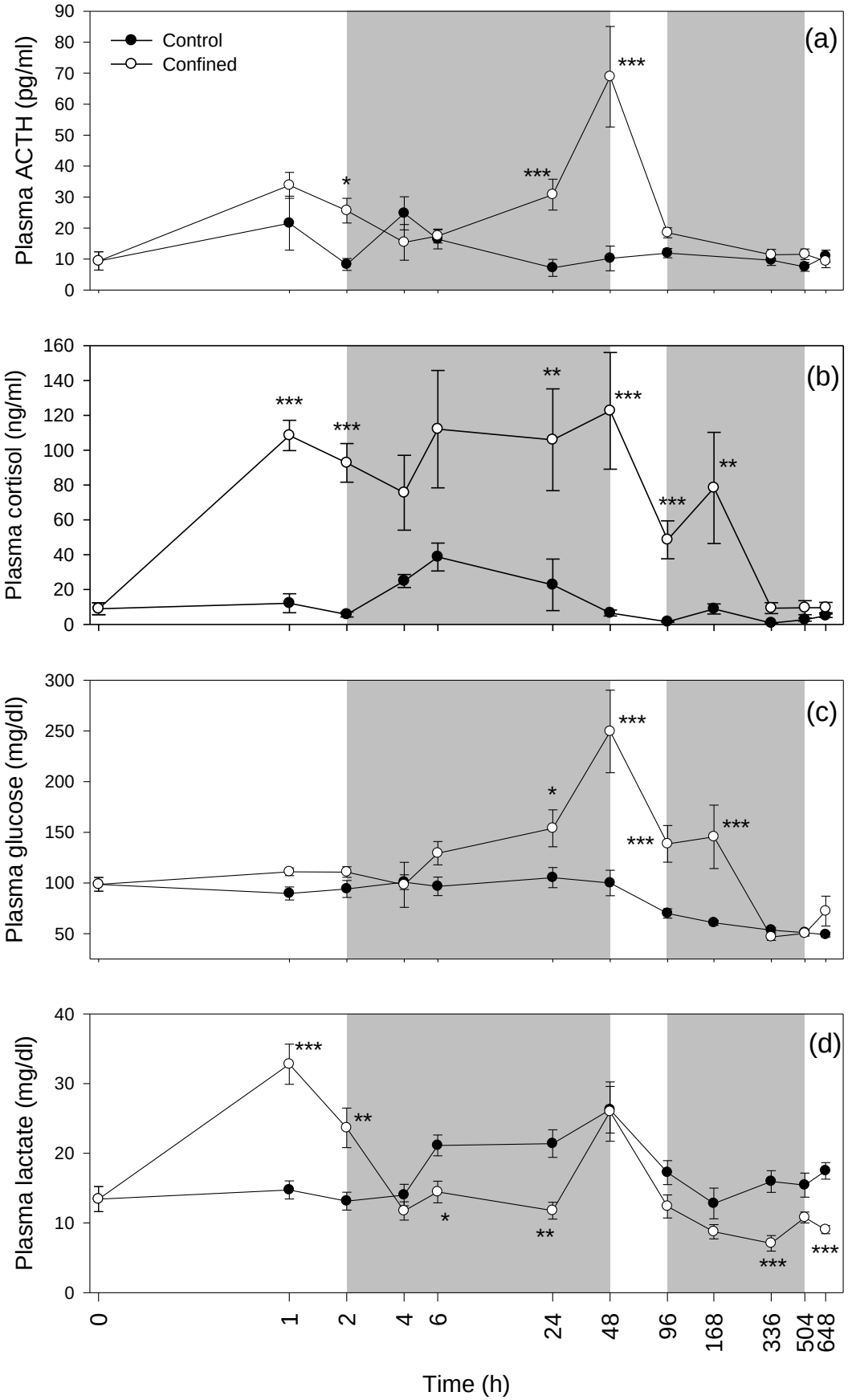
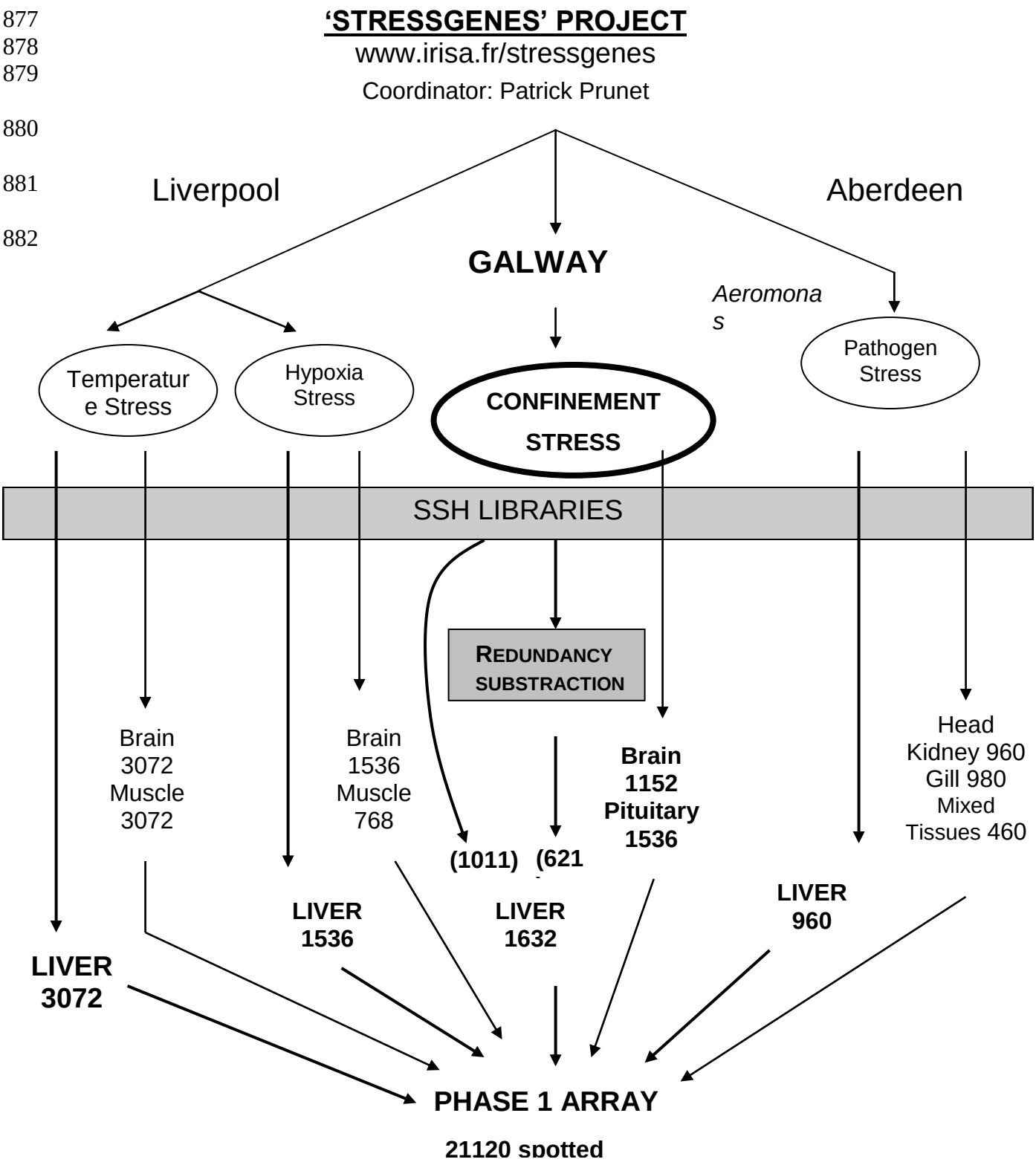
871 Table 1: Primers used for quantitative real-time PCR

Gene	Accession Number	Forward Primer	Reverse Primer
Haptoglobin	CT566464	5'TCCTTCGATAAGACCTGTGCC3'	5'GGAGACTGGCTGATGTCTCA3'
Fibrinogen beta chain	CR944658	5'TGGGTATTCAGGAACAGCCG3'	5'TCGCCTGGAGTCCAGTTATC3'
Complement factor H	CR944614	5'AAGCAAGCTGTCCTGATCCT3'	5'AAACTCAGTGTAAACTGTCTGCTG3'
TC8442	CR94440	5'GGCTCAGCTCATTCAGCAGT3'	5'GCTTTCCCTTCTAGTCTCACTCC3'
α-1-Anti-trypsin homolog	CR944146	5'TGTCTTCATGGGCAAAGTCA3'	5'TGCCACTTGTTAAAGCGTGA3'
CIRBP	CT567069	5'GTGTTGTTGTTGACCGGATG3'	5'TTGGAAATGAATGGCTGACA 3'
14-3-3 Protein β/α	AY370880*	5'TCCGATGTCCACAGAGTCAG3'	5'AAAACGGCATTTGATGAAGC3'
TC8200	CR944318	5'GTCAAACTGCTCAATGAACCA3'	5'CCTTCGGCAATCAGATGAA3'
Orosomucoid	CR944165	5'CTGGAGAAATGGGATGAGGA 3'	5'TGGCAGACTGAGACAATCCA3'
Serum albumin 1	TC120523*	5'TGGTTGCTGAGAGTGCAGAG3'	5'TGTAGGCTGGGCAGGTAGAT3'
EST10729	CT566588	5'CCTCTTTCAGTGGTGGTGGT3'	5'CAAGGGGTCTCCTCATTCTG3'
Ceruloplasmin	CT565372	5'TGGATGAACGTGGAGCATTA3'	5'TCATGCATTGTCAGTGGTCA3'
β-actin	AJ438158*	5'ATGGAAGATGAAATCGCCGCA3'	5'TGGCCCATCCCAACCATCAC3'
18S Ribosomal RNA	AF308735*	5'ACCACCCACAGAATCGAGAAA3'	5'GCCTGCGGCTTAATTTGACT3'

872 *Primers to candidate genes were generally designed to specific clones on the array with
873 reference to contigs, but where there was any ambiguity NCBI (AY370880) and TIGR
874 (TC120523) database sequences were used.
875

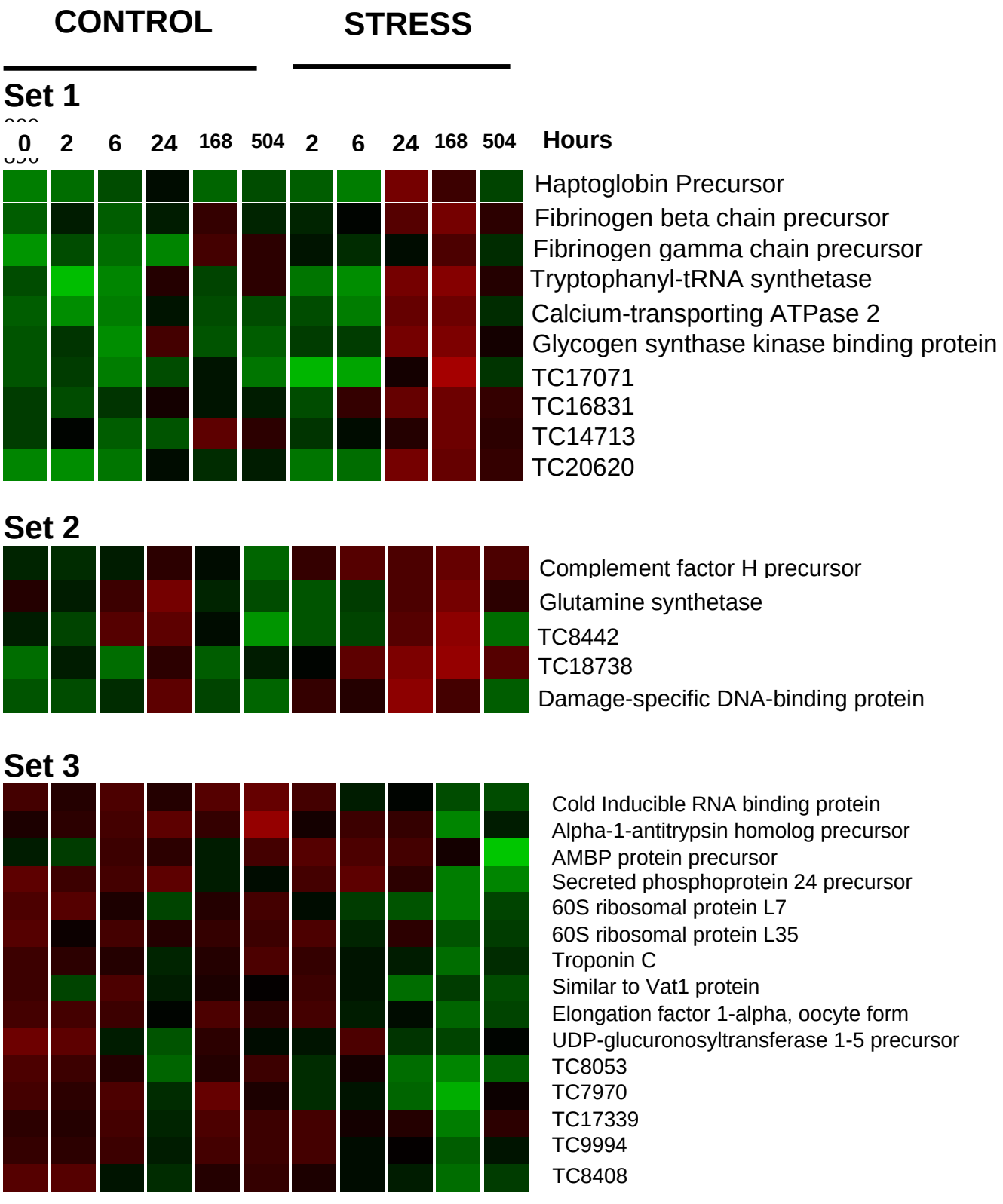


876 Cairns et al. Figure 1.



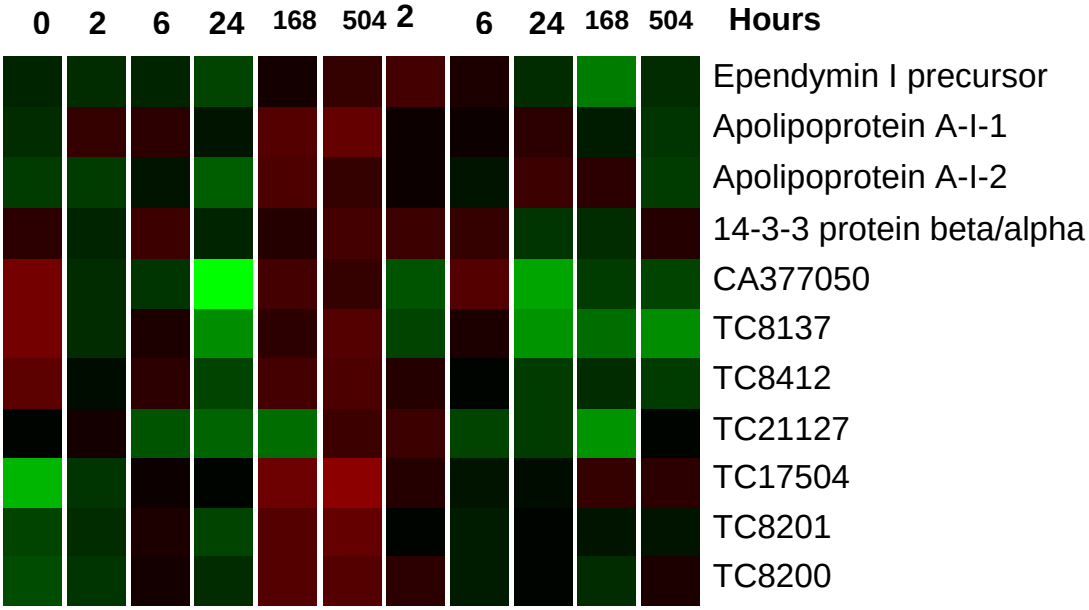
Cairns et al. Figure 2

Figure 3: Cairns et al.,

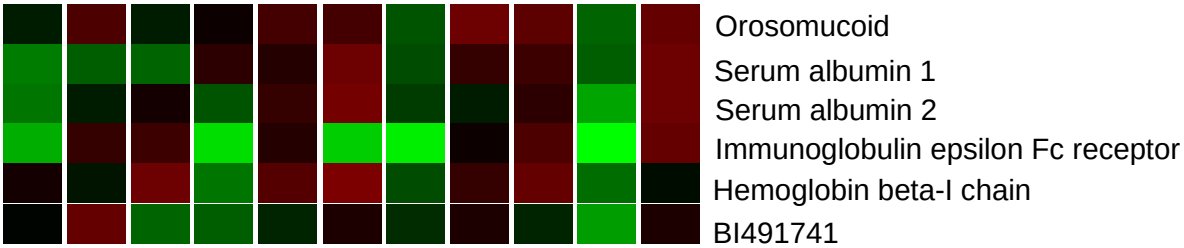


Scale:

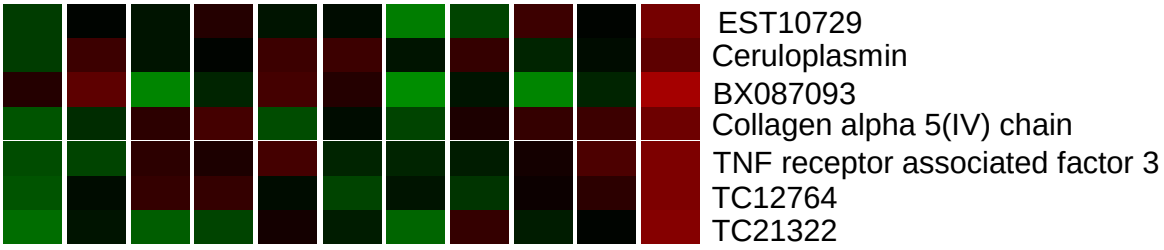
Set 4



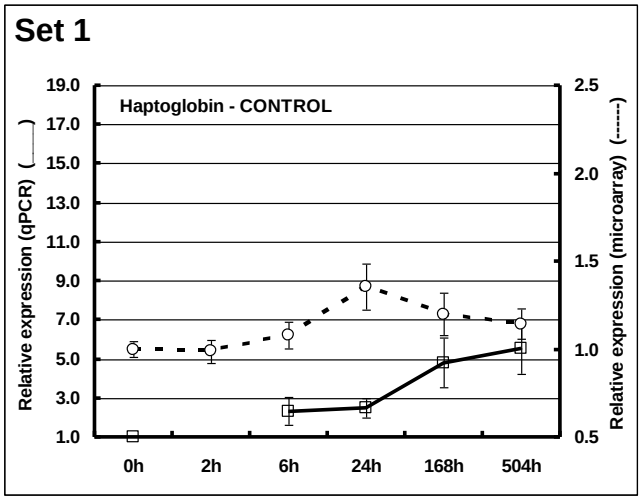
Set 5

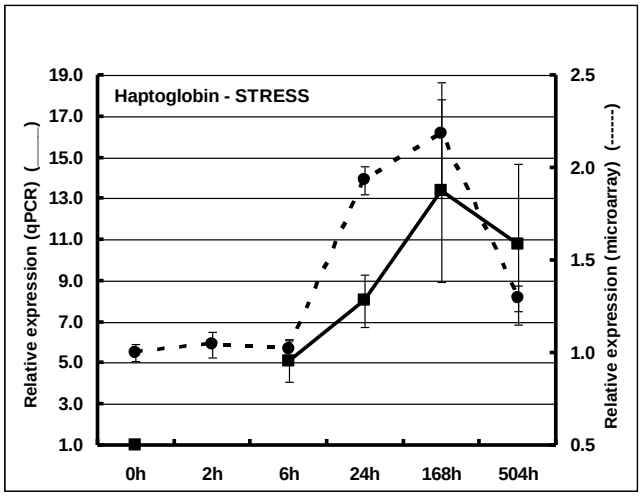


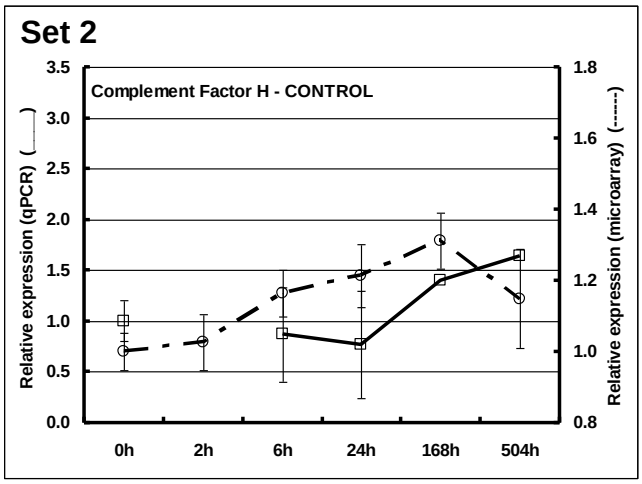
Set 6

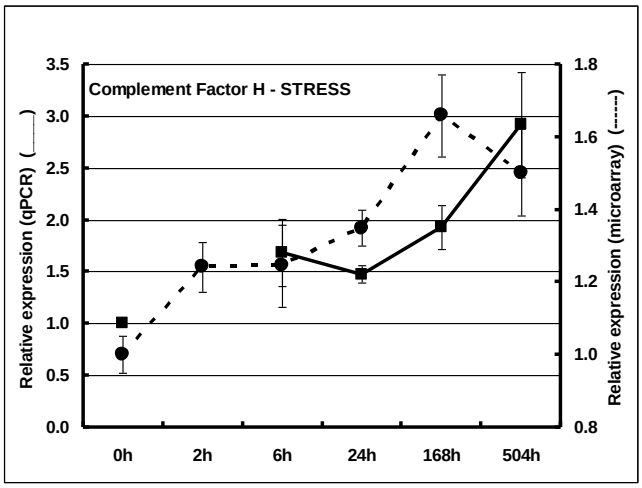


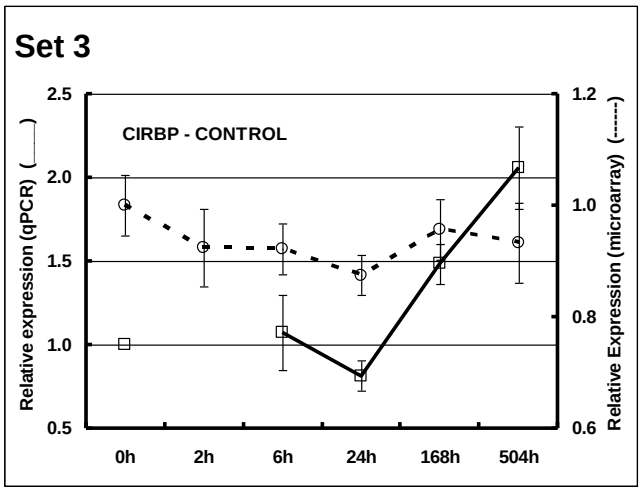
912
913

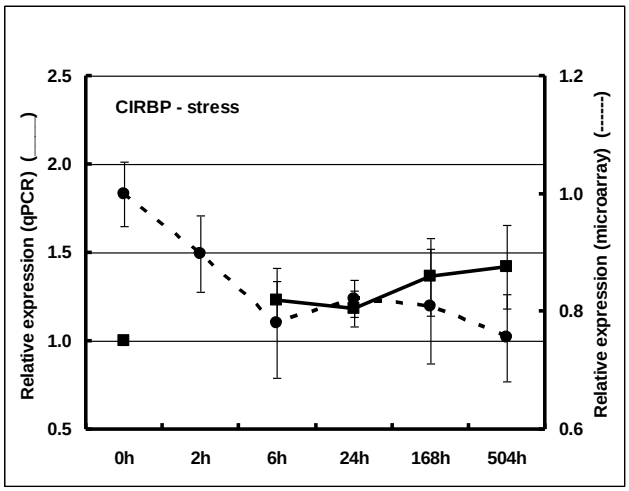

Set 1
Haptoglobin - CONTROL
Relative expression (qPCR) (___)
Relative expression (microarray) (------)
19.0
17.0
15.0
13.0
11.0
9.0
7.0
5.0
3.0
1.0
2.5
2.0
1.5
1.0
0.5
0h
2h
6h
24h
168h
504h


Haptoglobin - STRESS
Relative expression (qPCR) (___)
Relative expression (microarray) (------)
19.0
17.0
15.0
13.0
11.0
9.0
7.0
5.0
3.0
1.0
2.5
2.0
1.5
1.0
0.5
0h
2h
6h
24h
168h
504h

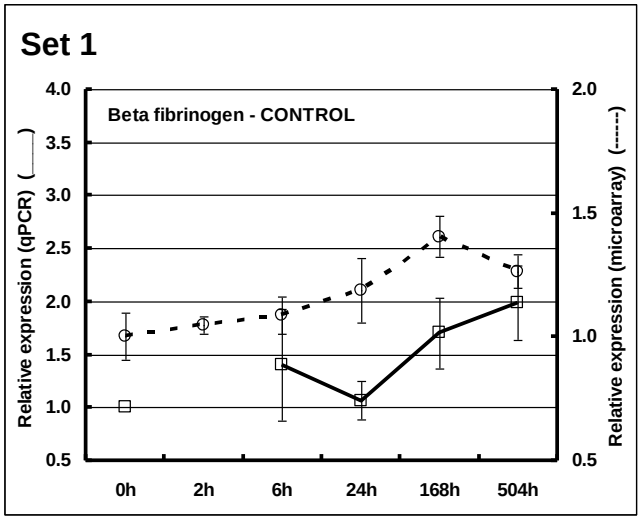

Set 2
Complement Factor H - CONTROL
Relative expression (qPCR) (___)
Relative expression (microarray) (------)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1.8
1.6
1.4
1.2
1.0
0.8
0h
2h
6h
24h
168h
504h

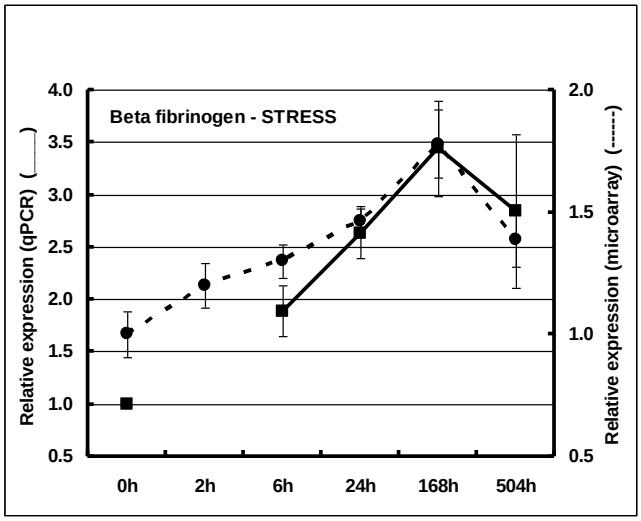

Complement Factor H - STRESS
Relative expression (qPCR) (___)
Relative expression (microarray) (------)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1.8
1.6
1.4
1.2
1.0
0.8
0h
2h
6h
24h
168h
504h

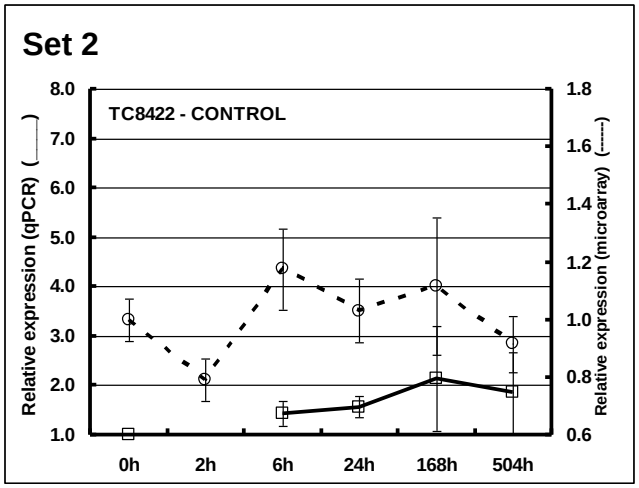

Set 3
CIRBP - CONTROL
Relative expression (qPCR) (___)
Relative Expression (microarray) (------)
2.5
2.0
1.5
1.0
0.5
1.2
1.0
0.8
0.6
0h
2h
6h
24h
168h
504h

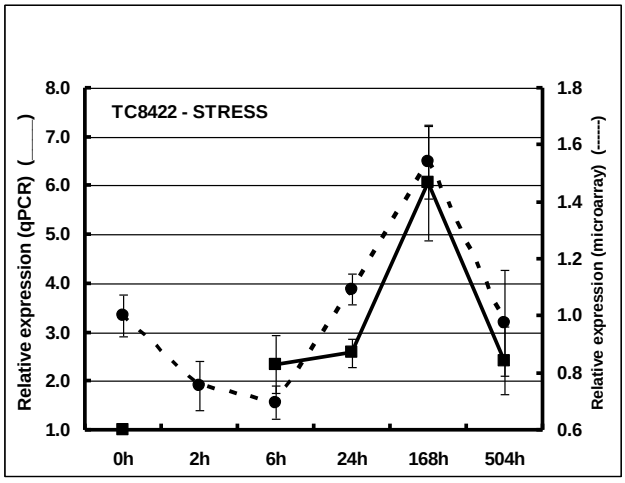

CIRBP - stress
Relative expression (qPCR) (___)
Relative expression (microarray) (------)
2.5
2.0
1.5
1.0
0.5
1.2
1.0
0.8
0.6
0h
2h
6h
24h
168h
504h

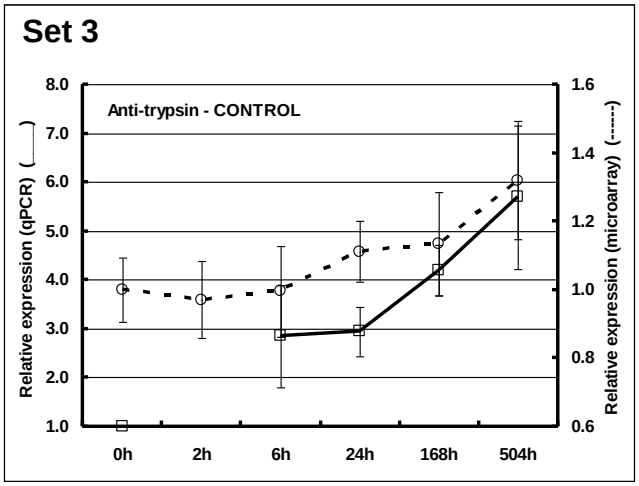
914

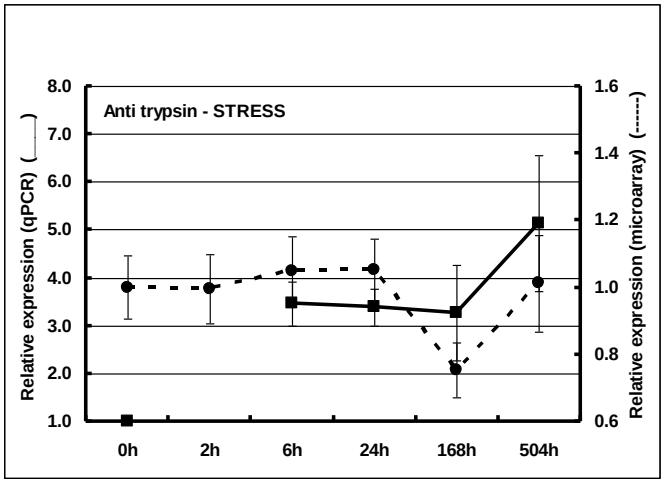

Set 1
Beta fibrinogen - CONTROL
Relative expression (qPCR) (——)
Relative expression (microarray) (------)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
2.0
1.5
1.0
0.5
0h
2h
6h
24h
168h
504h


Beta fibrinogen - STRESS
Relative expression (qPCR) (——)
Relative expression (microarray) (------)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
2.0
1.5
1.0
0.5
0h
2h
6h
24h
168h
504h

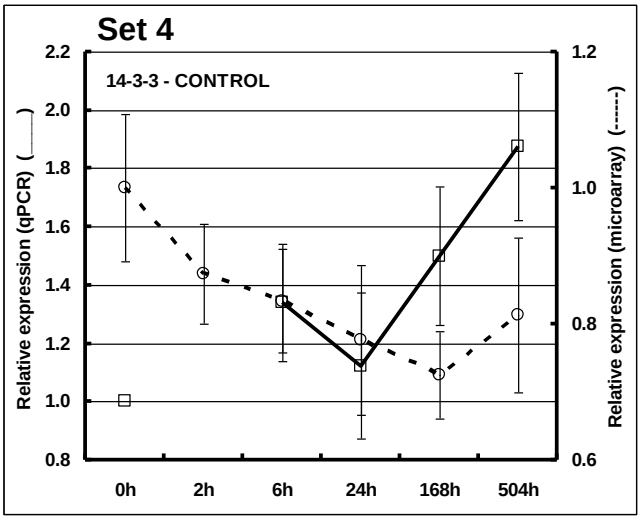

Set 2
TC8422 - CONTROL
Relative expression (qPCR) (——)
Relative expression (microarray) (------)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0h
2h
6h
24h
168h
504h

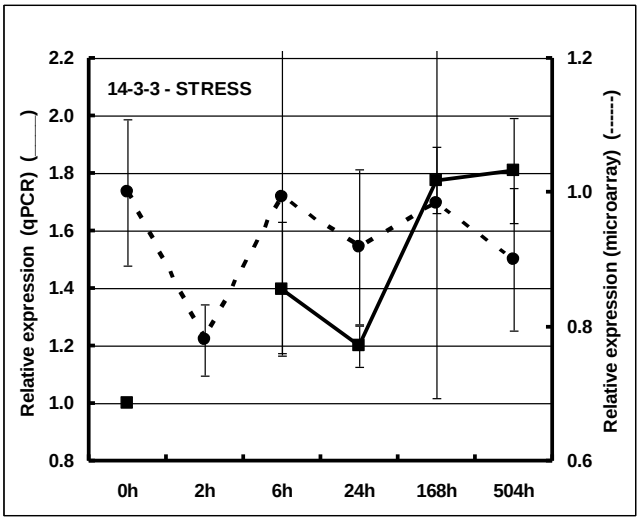

TC8422 - STRESS
Relative expression (qPCR) (——)
Relative expression (microarray) (------)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0h
2h
6h
24h
168h
504h

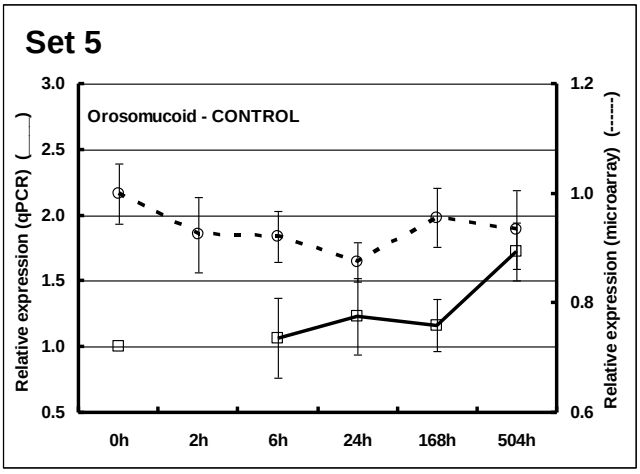

Set 3
Anti-trypsin - CONTROL
Relative expression (qPCR) (——)
Relative expression (microarray) (------)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
1.6
1.4
1.2
1.0
0.8
0.6
0h
2h
6h
24h
168h
504h

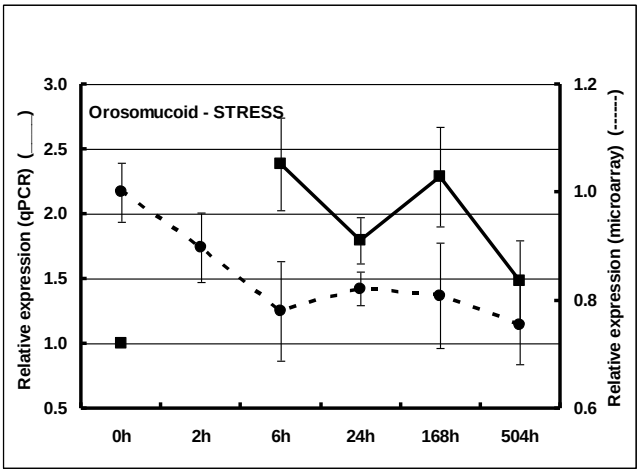

Anti trypsin - STRESS
Relative expression (qPCR) (——)
Relative expression (microarray) (------)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
1.6
1.4
1.2
1.0
0.8
0.6
0h
2h
6h
24h
168h
504h

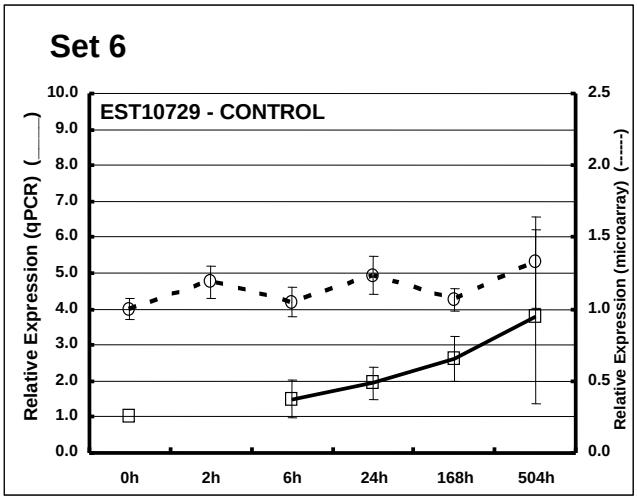
915

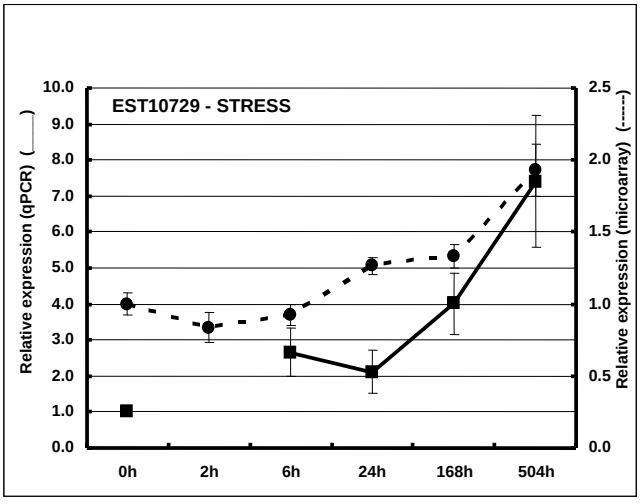

Set 4
14-3-3 - CONTROL
Relative expression (qPCR) (___)
Relative expression (microarray) (------)
0h
2h
6h
24h
168h
504h


14-3-3 - STRESS
Relative expression (qPCR) (___)
Relative expression (microarray) (------)
0h
2h
6h
24h
168h
504h

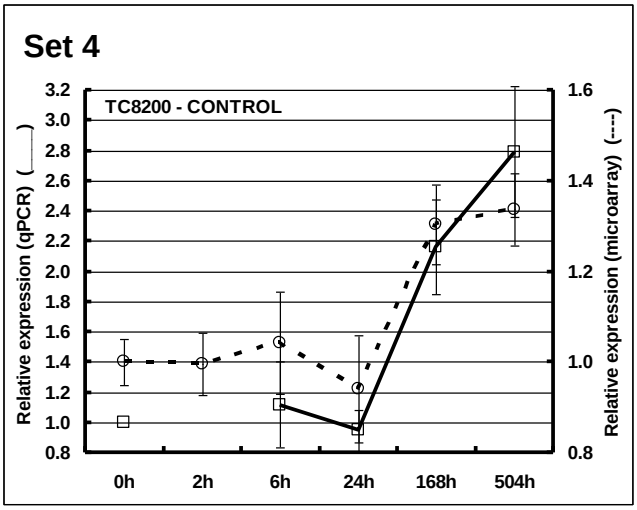

Set 5
Orosomucoid - CONTROL
Relative expression (qPCR) (___)
Relative expression (microarray) (------)
0h
2h
6h
24h
168h
504h

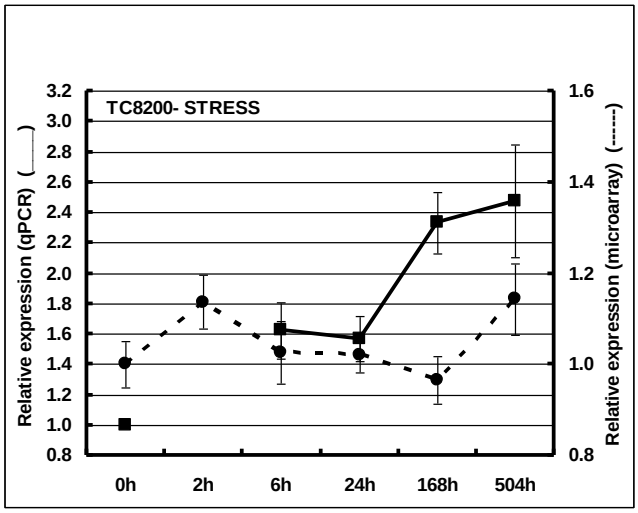

Orosomucoid - STRESS
Relative expression (qPCR) (___)
Relative expression (microarray) (------)
0h
2h
6h
24h
168h
504h

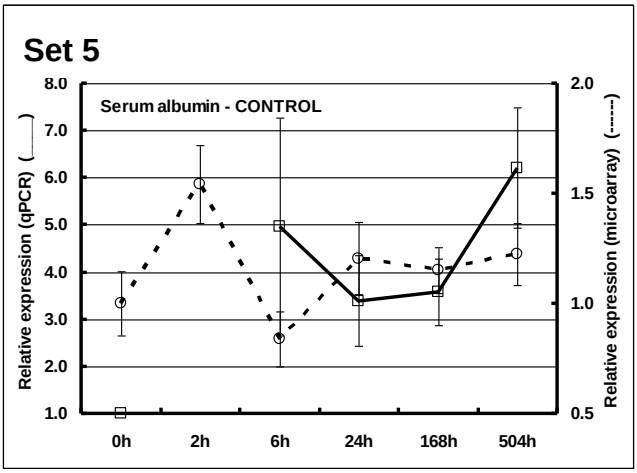

Set 6
EST10729 - CONTROL
Relative Expression (qPCR) (___)
Relative Expression (microarray) (------)
0h
2h
6h
24h
168h
504h

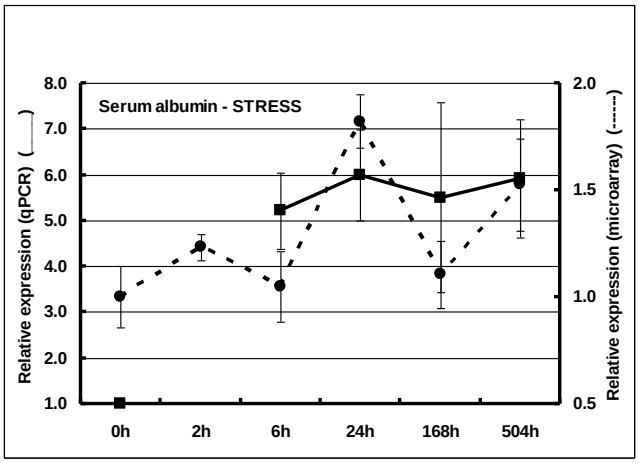

EST10729 - STRESS
Relative expression (qPCR) (___)
Relative expression (microarray) (------)
0h
2h
6h
24h
168h
504h

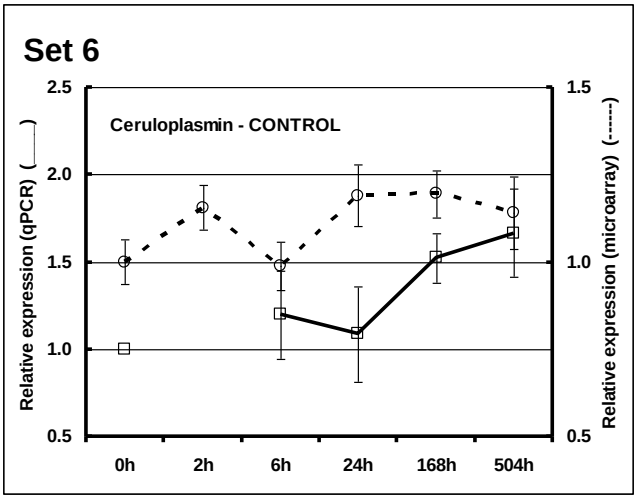
916
917


Set 4
TC8200 - CONTROL
Relative expression (qPCR) (___)
Relative expression (microarray) (----)
0h
2h
6h
24h
168h
504h


TC8200- STRESS
Relative expression (qPCR) (___)
Relative expression (microarray) (----)
0h
2h
6h
24h
168h
504h


Set 5
Serum albumin - CONTROL
Relative expression (qPCR) (___)
Relative expression (microarray) (----)
0h
2h
6h
24h
168h
504h


Serum albumin - STRESS
Relative expression (qPCR) (___)
Relative expression (microarray) (----)
0h
2h
6h
24h
168h
504h


Set 6
Ceruloplasmin - CONTROL
Relative expression (qPCR) (___)
Relative expression (microarray) (----)
0h
2h
6h
24h
168h
504h

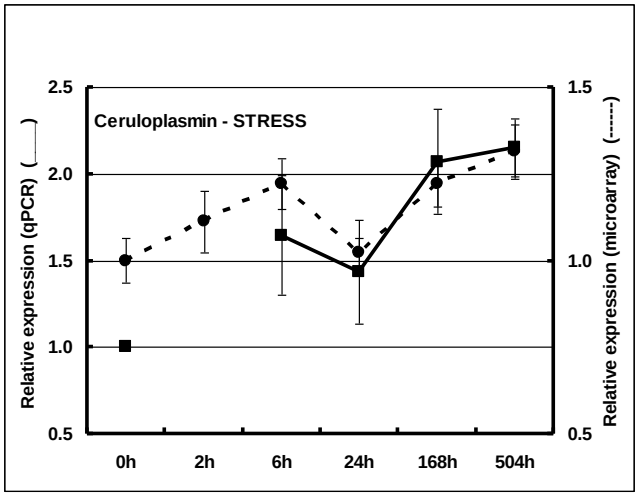

Ceruloplasmin - STRESS
Relative expression (qPCR) (___)
Relative expression (microarray) (----)
0h
2h
6h
24h
168h
504h

Figure captions

Figure 1. Levels of (a) plasma ACTH; (b) plasma cortisol; (c) plasma glucose; (d) plasma lactate in confined (○) and unconfined (●) rainbow trout over a 648 h period. Each point is the mean ± SEM of 12 fish. Significant differences between control and confined fish are denoted by: * : $P<0.05$; **: $P<0.01$; ***: $P<0.001$. The shaded areas represent the two time periods from which samples were used to construct the SSH libraries.

Figure 2. Organisation of the Phase 1 microarray.

The Phase 1 array consisted of 21120 spotted cDNAs contributed to by three partners of the "Stressgenes" project. Four stresses were represented and most of the clones were derived from liver, brain and muscle SSH libraries. Numbers provide detail on the number of clones spotted from each tissue/stress. The liver confinement libraries went through a second step of redundancy subtraction.

Figure 3. Common expression patterns in response to a confinement stressor. RNA from individual fish exposed to a confinement stressor (and unstressed controls) was hybridised in dye-swap experiments to a multi-tissue cDNA microarray. Normalised expression ratios (ln transformed) were analysed by ANOVA by time ($P<0.05$ with Benjamini and Hochberg False Discovery Rate multiple test correction) and clustered into six k-means groups. The scale ranges from 4-fold up-regulated (red) to 4-fold down-regulated i.e.0.25 (green).

941 Figure 4. Individual expression patterns for some of the key members of the confinement
942 stressor k-means cluster groups. qPCR was carried out on 5 individuals (control and
943 stressed) for two gene members of each of Sets 1-6 (see Figure 3). All qPCR was carried
944 out in triplicate and fold change determined after normalisation to housekeeping genes β-
945 actin and 18S. Microarray data is averaged data for every instance of that gene on the
946 array and is displayed as fold change relative to t = 0. qPCR data (squares and solid line)
947 is compared directly to microarray data (circles and dashed line). Stress data is in filled
948 symbols and control data in empty symbols. Vertical axes are varied to best display the
949 correlation between the qPCR and microarray data. For primer sequences and template
950 accession numbers see Table 1.
951