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Acute phase gene expression in rainbow trout *(Oncorhynchus mykiss)* after exposure to a confinement stressor: a comparison of pooled and individual data.

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Abstract

This study set out to investigate whether differential expression of genes for acute phase proteins in rainbow trout (*Oncorhynchus mykiss*) could be induced by confinement stress, a non-invasive method of activating the neuroendocrine stress response. In addition, a second objective was to assess the variation in gene expression between individual fish within the population of stressed fish in an attempt to identify APP genes having uniform and consistent changes in expression during stress. The liver was chosen for this investigation as it is the primary site of acute phase protein synthesis. Relative expression of the eight genes including transferrin, fibrinogen-like protein 2 (flp2), α-1-anti-proteinase-like protein (α1-antiprot), leukocyte cell-derived chemotaxin 2 (LECT2), pentraxin, serum amyloid A (SAA), haptoglobin (Hp), and differentially regulated trout protein 1 (DRTP1) was analysed by quantitative real-time PCR (qPCR) over 5 experimental time points spanning the course of a week. The results showed that the expression of three genes, SAA, haptoglobin and DRTP1, were most altered as a result of exposure to confinement stress. A correlation was identified between the expression of haptoglobin and DRTP1. Gene expression analyses in individual fish found that the transcript levels of haptoglobin and DRTP1 genes varied much less between individuals than was the case for SAA. The increase of haptoglobin and DRTP1 gene expression and its

uniformity in response to stress make these genes potential biomarkers for stress in trout.

1. Introduction

Induction of the acute phase response in fish is most frequently associated with the activation of the inflammatory response, typically in response to a pathogen challenge (Bayne *et al*., 2001; Gerwick *et al*., 2007; Martin *et al*., 2006). The acute phase response proteins (APPs) are regarded as having general functions in opsonisation and trapping of micro-organisms and their products, in activating complement, in binding cellular remnants, in neutralising enzymes, scavenging free haemoglobin and radicals and in modulating the immune response (Gurys *et al.*, 2005). In humans and experimental animals, physical and psychological stress has been found to elevate plasma interleukin-6 and acute phase protein (APP) levels (Deak *et al*., 1997; Nukina *et al*., 2001). Although the induction of the acute phase response is typically associated with the activation of the inflammatory response, the focus of this study was to investigate whether confinement stress could induce differential gene expression of acute phase proteins in rainbow trout.

Confinement is a well-established non-invasive model stressor (Pottinger and Pickering, 1992) capable of eliciting a neuroendocrine stress response in rainbow trout. The magnitude of this response in fish is commonly determined by the measurement of the plasma hormone cortisol (Pottinger and Carrick, 2001; Hosoya *et al*., 2007; Cairns *et al*., 2008; Fast *et al*., 2008). Although these initial increases in hormone and metabolite levels may be indicative of the activation of the stress response, they may not reflect the true nature of what is occurring in the fish, particularly during long-term stress when these markers have returned to baseline levels.

The advent of new molecular techniques, such as suppression subtractive hybridisation (SSH) and microarray technology, have lead to large-scale analysis of differential gene regulation being possible. These techniques have been used in many recent studies to investigate the acute phase response in fish after bacterial challenge in rainbow trout (*Oncorhynchus mykiss*) (Bayne *et al*., 2001; Gerwick *et al*., 2007)

and in Atlantic salmon (*Salmo salar*) (Tsoi *et al.*, 2004; Ewart *et al*., 2005; Martin *et al*., 2006).

A panel of eight candidate genes was chosen for evaluation of acute phase response induction. These genes included transferrin, a member of the fibrinogen superfamily - fibrinogen-like protein 2 (flp2), α-1-anti-proteinase-like protein, pentraxin, leukocyte cell-derived chemotaxin 2 (LECT2), serum amyloid A (SAA), differentially regulated trout protein 1 (DRTP1) and haptoglobin. The acute phase protein transferrin, fibrinogen and α-1-anti-proteinase-like protein and haptoglobin, were selected after they were identified as potential differentially regulated genes from previous work carried out on rainbow trout in our laboratory (Cairns *et al*., 2008). SAA and pentraxin are characterised as major acute phase proteins. The plasma concentration of the major acute phase proteins in mammals can increase up to 1000-fold during an acute phase response. Two other less well characterised genes selected for analysis were LECT2 and DRTP1. Expression of the LECT2 gene has been reported to increase markedly during the acute phase response in zebrafish (*Danio rerio*) (Lin *et al*., 2007) while the DRTP1 gene has been shown to be differentially regulated during the acute phase response in salmon (Martin *et al*., 2006) and trout (Bayne *et al*., 2001; Gerwick *et al*., 2007). The induction of DRTP1, in common with other acute phase proteins, has NF-κB and C/EBPβ as important transcription factors involved in its induction (Talbot *et al*., 2008).

The primary aim of this study was to identify alterations in gene expression that resulted from confinement stress with the goal of identifying potential biomarkers for stress. In addition, we sought to determine whether a correlation existed between the level of plasma cortisol and acute phase gene expression during long-term confinement stress. This study provided a comparison between the gene expression analysis of pooled samples with that of individual fish and demonstrates the potential problems in performing pooled analysis in teleost fish.

2. Materials and Methods

2.1 The confinement procedure

The confinement stress experiments were carried out at the Natural Environment Research Council (NERC) Centre for Ecology and Hydrology facility at Windermere, Cumbria in May 2004. Female rainbow trout (Borrowdale strain) were sourced locally and allowed to acclimatise in 1000 L outdoor circular tanks for three weeks prior to the start of the experiment. The fish were 1+ years in age, 250-350g in weight and 28-32 cm in length. Prior to the start of the confinement the fish were fed once daily with commercial trout pellets (Skretting Standard Expanded 40) at the manufacturer's 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. Confinement conditions of 65 g/L were established in 50 L rectangular tanks while the control fish were maintained in the 1000 L tanks at a density of 10 g/L. A constant flow of lake water at ambient temperature (11^{o}C) was supplied to the control tanks at a rate of 25 L/min and to the confined tanks at 15 L/min. The photoperiod was natural.

2.2 Sample collection and tissue processing

Control and confined fish were sampled at each time point, with the exception of 0 h when only control fish were sampled. Fish were sampled 8 h, 24 h, 48 h, and 168 h after the onset of confinement. Six fish were sampled for each condition (2 replicate tanks x 3 fish), five of which were used for real-time analysis. After removal from the experimental tanks fish were immediately anaesthetised in a solution of 2-phenoxy-ethanol (1:2000). Blood samples were collected from the caudal vessels using a 4.5 ml Monovette containing Lithium-Heparin beads (Sarstedt S-Monovette with Multi-adapter). The fish were weighed, measured and killed by spinal section. Blood samples were held on ice before being centrifuged at 4 ^{o}C and the plasma separated and stored at -20 ^{o}C. Liver tissue was extracted rapidly and minced before being placed in RNA*later*® (Sigma). Liver tissue was stored at -80 ^{o}C until required.

2.3 Plasma cortisol assay

Cortisol is routinely used as a measure of the neuroendocrine stress response. Plasma cortisol levels in individual fish were determined by radioimmunoassay (Pottinger and Carrick, 2001) to confirm the endocrinological status of the fish.

2.4 RNA extraction

RNA was extracted from liver tissue using the RNeasy® Maxi Kit for Total RNA Isolation (Qiagen) as detailed in the manufacturer's instructions. The quantity and quality of the RNA was determined using the 2100 Bioanalyser (Agilent). RNA integrity numbers (RIN) of 8 and higher were taken as a measure of acceptable RNA quality.

2.5 Primer design

Primers were designed using Vector NTI Suite 6 (Invitrogen) and commercially synthesised (MWG Biotech, Germany). All primers used in this study are listed in Table 1.

2.6 Quantitative real-time PCR (qPCR)

For the qPCR analyses, 50 ng of total RNA was used as template. RNA pools representing 5 fish from each sampling time point (control and confined) were generated by pooling 10 ng of total RNA from each of the 5 fish per condition. For the qPCR analyses of individual fish 50 ng of total RNA per fish was used as template. Quantitative PCR assays were performed in the Mx3000P™ Real-Time PCR System (Stratagene, La Jolla, CA, USA) using the RT-SYBR Green Kit (Qiagen). PCR reactions were carried out in a 20 µl total reaction volume consisting of 10 µl of 2X QuantiTect® SYBR Green RT-PCR master mix, 0.2 µl QuantiTect® RT mix, 1 µl of 10 µM sense and anti-sense gene specific primers, 2.8 µl dH_2O and 5 µl of total RNA template (10 ng/µl). The thermal profile of the Mx3000P™ system (Stratagene) was set up to include a reverse transcription step of 50 ^{o}C for 30 min, 95 ^{o}C for 15 min, followed by 40 cycles of 94 ^{o}C for 15 s and 60 ^{o}C for 30 s and 72 ^{o}C for 30 s. In order to detect the presence of non-specific amplification, control reactions containing all the reaction components except for the template were included for each primer set. At the end of each amplification a DNA melt curve analysis was performed using a ramping rate of 1 ^{o}C/min over a temperature range of 60 to 95 ^{o}C. Specificity of the PCR reaction was confirmed by the detection of a single peak. The β-actin gene was used as an internal standard for the qRT-PCR analysis after no significant differences were found between the means of the control

and confined pools of RNA at any time point (Student's *t*-test, two-tailed). The relative expression was calculated according to an established method (Plaffl, 2001) where the expression levels for the non-confined and confined fish were calculated with respect to a 0 h control.

2.7 Statistics

A Student's *t*-test (unpaired and two-tailed) was used to compare gene expression between control and confined fish at specific time points. The Mann-Whitney test was used when variances between groups were unequal. Following the Kolmogorov-Smirnov test for normality, the Pearson correlation test was carried out using GraphPad Prism (version 4.00 for Windows, GraphPad Software, San Diego California USA). *P* values less than 0.05 were accepted as significant.

3. Results

3.1 Plasma cortisol

Cortisol is a key element of the neuroendocrine stress response and provides a reliable indicator of activation of the hypothalamic-pituitary-interrenal axis. Plasma cortisol levels rose rapidly following the onset of confinement to reach a level that was consistently sustained throughout the experiment. The level of cortisol in the plasma of control fish ranged from 0 to 8.1 ng/ml (mean 1.54 ± SD 1.92 ng/ml, n = 25) compared to plasma concentrations in the confined fish which ranged from 6.2 to 200.7 ng/ml (mean 61.6 ± SD 56.1 ng/ml, n = 20) confirming that a neuroendocrine stress response was elicited in the confined individuals (Fig. 1).

3.2 Relative gene expression in pooled samples over time

Comparisons of the relative gene expression levels of transferrin, fibrinogen-like protein 2 (flp2), α-1-anti-proteinase-like protein and pentraxin by qPCR were carried out between pools of control and confined fish (5 fish per pool; Fig. 2). The results of this analysis showed that confinement stress did not increase gene expression of any of these four genes. The results showed no differences in transferrin gene expression between control and confined fish at any point during the course of the confinement period. A decrease in fibrinogen-like protein 2 (flp2) expression in stressed fish relative to controls was evident at 24 h, however, this decrease was small (0.3 fold

less in the confined than in the control). With regard to α-1-anti-proteinase-like protein, there was no difference found in the gene expression between confined and non-confined fish at any time point except for 168 h where a 2 fold decrease was evident in the confined fish when compared to the controls at the same time point. No change in pentraxin expression was found at 8 h and 24 h however, the level of expression was found to be lower in the stressed fish than control fish at the later time points of 48 h and 168 h. Relative to the non-confined fish the difference at 48 h (1.9 times less in the confined fish) was slightly more than was found at 168 h (1.6 times less in the confined compared to the control).

Comparisons of relative levels of gene expression were made between control and confined pools of fish (5 fish per pool) for LECT2, SAA, haptoglobin and DRTP1 (Fig. 3). The results of these analyses show that increases in gene expression in the stressed fish were evident for all four genes, most notably at the later time point of 168 h. An increase in the expression of leukocyte cell-derived chemotaxin 2 (LECT2) gene was found in confined fish, relative to unstressed controls, at 8 h and 168 h. These expression changes were small with the increase at 8 h slightly less than 2 fold, while that at 168 h was slightly more than 2 fold. Increases in SAA gene expression in confined fish were found at 8 h and 168 h whereas decreases in SAA expression were evident in the confined fish at 24 h and again at 48 h. The greatest difference in SAA between the treatment groups was evident at 168 h. With all calculations made relative to the 0 h control (Plaffl, 2001) the relative SAA expression in the control at 168 h was found to be 0.15 that of the 0 h group (i.e control fish were approximately 7 fold down-regulated relative to 0 h) whereas in the confined fish SAA expression was found to be 12 fold up-regulated. This difference between the control and the confined pool of fish equates to an 80 fold up-regulation of SAA as a result of confinement stress at 168 h. For haptoglobin an increase in expression was only found at 168 h of confinement where haptoglobin mRNA expression was 4.3 fold higher in the confined fish relative to the paired control. With regard to DRTP1, increases were found in the relative gene expression of this gene at all time points with the exception of 24 h. The amount of the DRTP1 transcript found in the confined pools relative to their respective controls at 8 h and 48 h was approximately 3 fold higher while at 168 h it was approximately 12 times higher.

3.3 Relative gene expression in individual fish over time

The three genes (SAA, haptoglobin and DRTP1) with the greatest changes in gene expression in the pooled analyses were further analysed in the individual fish making up each of the pools in the previous qPCR analyses (Fig. 4). The objective of this part of the study was to assess the variation in expression levels between fish within a pooled population and to attempt to identify genes, which have uniform and consistent pattern of expression across individual fish within a sample population.

3.3.1 SAA

The mean level of SAA gene expression in the control fish at 8 h was 0.3 fold that of the 0 h controls (i.e. on average SAA gene expression was 3-4 fold down-regulated compared to the average fish at 0 h.). In the confined fish at the same time point, although 4 fish showed down-regulation relative to the 0 h fish (Fish 7, 8, 9 and 10 showed average down-regulation of 1-2 fold) one fish (Fish 6) had a much higher expression of SAA (4.1 fold) that the other fish in the group (Table 2). This result would suggest that the small but significant difference seen between the control and the confined fish in the pooled analysis at 8 h (Fig. 3) can be attributed to Fish 6 alone. At the 24 h time point two of the five control fish (Fish 12 and 13) were found to have elevated transcript levels of SAA (7.2 fold and 11.8 fold, respectively). In the confined fish at the same time point the mean of the 5 confined fish was 1.3 fold up-regulated. This result would suggest that the significant decrease in the relative expression of SAA between the control and the confined fish in the pooled analysis can be attributed to these two control fish (Fish 12 and Fish 13). At 48 h a similar pattern of expression was seen with one control fish (Fish 22) showing a very large increase in SAA expression (93 fold). The mean down-regulation of the other 4 control fish at 48 h, excluding Fish 22 from the group was 5 fold. The confined fish taken together at this time showed a mean up-regulation of 1.7 fold. This result would suggest that the significant decrease in the relative expression of SAA between the control and the confined fish in the pooled analysis at 48 h can again be attributed to one fish (Fish 22). At 168 h the mean relative expression of SAA in the control fish was 1.1 fold up-regulated. While four of the five control fish had reduced levels of SAA compared to the 0 h control one fish (Fish 35) had an elevated expression level of 4.9 fold. In the confined fish at the same time point, the SAA gene expression of

two fish (Fish 38 and 39) was up-regulated 2.1 fold and 1.3 fold respectively, with the other three fish in the group were found to have very elevated levels of SAA (Fish 36 - 18.4 fold, Fish 37 - 41.7 fold, and Fish 40 - 221.4 fold).

Statistical significance for differences in SAA transcript levels between the control and confined groups of individual fish was found only at 168 h when a non-parametric *t*-test (Mann-Whitney) was used ($P = 0.0317$). If the one control group fish from each of the 24 h, 48 h and 168 h time points (Fish 13, Fish 22 and Fish 35, respectively) is considered an outlier and excluded from the analysis a significant difference in gene expression could be demonstrated at 48 h ($P = 0.0159$) and again at 168 h ($P = 0.0159$). Although significant differences could be demonstrated in SAA gene expression as a result of confinement, there was no consistent pattern to the SAA expression. This was most evident at 168 h with two fish exhibiting little or no change in expression (Fish 38 and 39), two fish showing large increases in expression (Fish 35 and 36) and one fish showing a very substantial increase in expression (Fish 40).

3.3.2 Haptoglobin

At the 8 h time point the relative expressions of haptoglobin for all fish, whether control or confined, were similar with the means of the five control fish and confined fish being 0.8 fold and 0.7 fold, respectively (i.e. slightly down-regulated compared to the 0 h fish). In the control fish at 24 h, as was seen with SAA, one fish (Fish 13) had higher levels of haptoglobin than the other four control fish at that time point (4.4 fold up-regulated relative to the 0 h fish). Despite this, the mean of the 5 fish in the confined group was still slightly higher than that of the control group (1.3 fold for the control compared to the confined which was 1.8 fold). At 48 h in the control fish, as was seen with SAA, Fish 22 had a higher level of expression relative to the other fish in the group. However, including Fish 22 the mean of the control group of individuals was 1.4 fold. The increase in the relative expression of haptoglobin in the confined fish at this time was very consistent, with all fish in the confined group having a 2 fold or higher increase in expression (range 2.2 to 4.5, mean 3.5). At 168 h time point, four of the five confined fish had higher levels of haptoglobin than what was seen in the control fish. The mean of the control fish as a group was 0.4 fold while the mean of the confined fish as a group was 1.9 fold, representing a 4.7 fold up-regulation.

Statistical analysis of the 5 individual control and confined fish at each time point found that significance was reached only at 168 h (P = 0.0019). No significant difference was found between the control and confined fish at 8 h (P = 0.7956). However, analysis in the absence of the outliers from the 24 h and 48 h control groups (Fish 13 and Fish 22, respectively) resulted in significance at both 24 h (P = 0.0159) and 48 h (P = 0.002).

3.3.3 DRTP1

At 8 h, the mean DRTP1 expression in the five control fish as a group was more elevated than the 0 h control (2.7 fold up-regulated). Three of the fish in this control group had elevated gene expression levels of greater than 3 fold. The mean DRTP1 expression of the confined fish as a group was 6.0 fold up-regulated (compared to the 0 h control). Even taking the elevation of the control fish into consideration there was still a 2 fold up-regulation of DRTP1 in the confined fish at 8 h. At 24 h, the mean of the control fish as a group (13.6 fold) was higher than the mean of the confined group at the same time (11.9 fold). As was seen at the same time point with the SAA and haptoglobin, Fish 13 was also found to have had elevated levels of DRTP1 (60.7 fold) compared to the other fish in the same group. The mean expression of the control group excluding Fish 13 (n = 4) was 2 fold. Therefore, in the absence this one control fish there was approximately 6 times more DRTP1 found as a result of confinement. Again at 48 h, one fish (Fish 22) in the control group displayed DRTP1 expression levels considerably above the other control fish. Fish 22, in addition to having elevated levels of DRTP1, also had elevated gene expression levels of SAA and haptoglobin. The mean of the control group including Fish 22 (n = 5) was 10 fold, whereas excluding Fish 22 the mean of this group (n = 4) was 2.4 fold. The mean of the confined group was 17 fold with all five fish in the confined group exhibiting levels in DRTP1 above the 0 h control fish. In the absence of Fish 22 expression levels of DRTP1 were 7 times higher in the confined group compared to the control group. At 168 h, again one control fish (Fish 35) had an elevated level of DRTP1. The mean for the control group including Fish 35 (n = 5) was 3.5 fold, whereas excluding Fish 35 from the group (n = 4) the mean was 1.0 fold. The levels of DRTP1 expression in the confined fish at the 168 h time point were higher than any of the

other earlier time points, with the mean for the group (n = 5) being 35.2 fold. In the confined group four of the five fish exhibited substantial increases in DRTP1 gene expression, with two fish having greater than 25 fold increases (28.7 fold and 29.7 fold for Fish 39 and 40, respectively) and the other two fish in this confined group having greater than 50 fold increases (54.5 fold and 58.5 fold for Fish 36 and 37, respectively).

Using a Student *t*-test a significant difference in gene expression between the control and the confined fish was found only at 168 h ($P = 0.0159$ using the Mann Whitney test). By removing the outliers from each of the control groups (Fish 13 at 24 h, Fish 22 at 48 h and Fish 35 at 168 h) it was possible to reach significance at 48 h ($P = 0.0159$) in addition to 168 h.

4. Discussion

The sustained elevation of plasma cortisol in the confined fish indicated that confinement conditions of 65 g/L were sufficient to activate the hypothalamic-pituitary-interrenal axis in a manner consistent with other studies using this model stressor (e.g. Cairns *et al*., 2008). The primary objective of this study was to investigate whether activation of this neuro-endocrine response was associated with the up-regulation of genes involved in the acute phase response. Although the mechanism of APP induction in response to stress is yet to be elucidated, activation of the hypothalamic-pituitary-interrenal axis in fish by stress may be the trigger of systemic or local (intra-pituitary) cytokine production, resulting in hepatic APP synthesis and subsequent release of the acute phase proteins into the bloodstream (Murata *et al*., 2004).

Transferrin is a plasma protein involved in iron delivery. Transferrin plays a role in the innate immune system. In the mucosa, where transferrin binds up the free iron, an environment is created which is unsuitable for bacterial survival (Langston *et al*, 1998). In trout after challenge with *Vibrio anguillarum* (Bayne and Gerwick, 2001; Bayne *et al*., 2001) transferrin has been reported to act as a positive acute phase protein. It acts as a positive APP in rabbits and rats (Schreiber *et al*., 1989), yet transferrin is classified as a negative acute phase protein in some mammals. A recent study investigating gene expression in the brain and head kidney of rainbow trout in

response to handling stress (netted for 2 minutes once a day, over 5 days) using microarray analysis found transferrin to be down-regulated in the head kidney (1.54 fold) (Krasnov *et al*., 2005). In the present study where confinement was used as a stressor, it was not surprising to find that there was no significant changes in transferrin gene expression found in the liver of rainbow trout.

Fibrinogen plays a role in homeostasis by providing a substrate for fibrin formation, tissue repair and the migration of inflammatory related cells (Thomas, 2000). Fibrinogen-like protein 2 (flp2), fibrinogen (beta chain) and fibrinogen (gamma A chain) are members of the fibrinogen super-family. Analysis of flp2 found that this protein did not act as a positive acute phase gene in trout under the present experimental conditions. In a recent study in which a similar confinement stressor was employed and gene expression was examined in the liver, fibrinogen (beta chain) was shown to increase over time (6 h and 24 h) exhibiting a peak in expression at 168 h (Cairns *et al*., 2008). Another study identified fibrinogen (gamma A chain) as an immune-related gene in a suppression subtraction hybridisation cDNA library from trout liver after fish were challenged with *Vibrio anguillarum* (Bayne *et al*., 2001). From the latter two studies fibrinogen has been implicated in the acute phase response however, the amplification of different genes of this super-family would appear to be associated with different stimuli.

The α-1-anti-proteinase-like protein gene showed no differential expression at 8 h, 24 h or 48 h. At the later time of 168 h the level of α-1-anti-proteinase-like protein was found to have decreased by 2.0 compared to the control. This down-regulation at 168 h was consistent with earlier observations in a similar confinement stress study (Cairns *et al*., 2008).

The classical members of the pentraxin family C-reactive protein (CRP), serum amyloid P component (SAP) and hamster female protein (FP) are primarily synthesised in the liver. CRP is characterised by a high calcium dependent affinity for phosphocholine, a common component of fungal and bacterial polysaccharides and cell membranes (Tillett and Francis, 1930). Serum amyloid protein-like pentraxin homologues are circulating lectins whose ligands include agarose, carbohydrate

moieties, and phosphoethanolamine, a binding affinity shared with CRP. In this study, pentraxin primers were designed to a SAP-like pentraxin sequence (GenBank accession no. BE665049) (Jensen *et al*., 1997). Pentraxin gene expression was found to be significantly decreased (approximately 2 fold) at the later times of 48 h and 168 h suggesting a possible role for SAP-like pentraxins as negative acute phase proteins during periods of prolonged confinement. Pentraxin, both at the level of protein and mRNA expression has been reported as a negative acute phase protein in trout after activation of the acute phase response by bacterial challenge (Jensen *et al*., 1997; Gerwick *et al.*, 2007).

LECT2 has recently been identified as an acute phase gene in zebrafish (*Danio rerio*) where a dramatic induction of the LECT2 gene was found after challenge with *Aeromonas salmonicida* and *Staphylococcus aureus* (1344 fold and 51 fold, respectively) (Lin *et al*, 2007). Although increases in LECT2 expression were found as a result of confinement at 8 h and 168 h, these increases were no more than 2 fold in magnitude.

SAA is the major APP for horses and ruminants but because of the difficulty of measuring serum SAA levels its use as a diagnostic test in veterinary medicine has been limited (Eckersall, 1999). The induction of SAA mRNA in fish has been demonstrated in Arctic char following infection with *Aeromonas salmonicida* (Jensen *et al*., 1997*),* and in carp after tissue injury (Fujiki *et al*., 2000), infection with *Trypanoplasma borreli* (Saeij *et al*., 2003) or infection with *Icthyophthirius multifilis* (Gonzalez *et al*., 2007). The induction of SAA in the liver and several extra-hepatic tissues has been reported in rainbow trout after the induction of the acute phase response either by natural infection with *Flavobacterium psychrophilum* or by challenge with pathogen-associated molecular patterns (PAMPs) including LPS or CpG oligonucleotides (CpG ODN) (Villarroel *et al.*, 2008). Interestingly, SAA was not identified as being differentially expressed in trout liver after bacterial challenge with *Listonella* (Gerwick *et al*., 2007) or after confinement stress (Cairns *et al*., 2008). In the latter case the gene was not represented on a microarray constructed from suppression subtractive hybridisation libraries related to a number of stressors.

In the present study, using pooled samples increases in SAA gene expression were found at 8 h and 168 h (P = 0.0022 and P = 0.0022, respectively) with decreases in SAA expression evident at 24 h and again at 48 h (P = 0.0022 and P = 0.0022, respectively). However, from the second set of analyses carried out on the individual fish that went to make up each pool, one outlier was identified in each of the 24 h, 48 h and 168 h control groups (Fish 13, Fish 22 and Fish 35, respectively). The inclusion of this one control fish outlier in the pool of 5 fish led to a misleading interpretation of the results. By excluding these fish from the statistical analyses a different interpretation of gene expression in the remaining fish (n = 4 for the control and n = 5 for the confined groups) was apparent. With regard to SAA significant differences in gene expression between the control and confined groups of individual fish were demonstrated at 48 h (P = 0.0159) and at 168 h (P = 0.0159). However, there was no consistent pattern to SAA expression. This was most evident at 168 h with two fish exhibiting little or no change in expression (Fish 38 and 39), two fish showing large increases in expression (Fish 35 and 36) and one fish showing a very substantial increase in expression (Fish 40).

With regard to SAA, the interpretation of data generated from pooled analysis might be inaccurate due to the enormous variability in the expression of this gene that is evident in certain individuals in a group. Another recent study where SAA gene expression was also investigated by quantitative PCR on individual fish (Raida and Buchmann, 2009) reported fold increases 6000 fold in SAA after primary i.p. infection with *Yersinia ruckeri*. Although the induction of SAA is clearly an indication of infection, the standard deviation of the individuals in each group was very broad. After 3 days of infection the number of SAA transcripts detected in the liver of the infected trout (n = 5) was 3053(1599-5828), at 7 days was 166.6 (4.5-6169.4) and at 14 days was 210(74.4-593.4). The present study and other studies that investigated the induction of SAA in teleost fish (Jensen *et al*., 1997; Fujiki *et al*., 2000; Saeij *et al*., 2003; Gonzalez *et al*., 2007; Villarroel *et al*., 2008; Raida and Buchmann, 2009) have shown that it SAA is a very inducible gene in response to a variety of stressors in different teleost species. The approach taken in this study where the gene expression was investigated at the level of the individual rainbow trout is a relatively new approach (Raida and Buchmann, 2009; Gerwick *et al*., 2007) and

demonstrates how individual variation can potentially lead to inaccurate interpretation of data if the data had been generated using pooled samples.

Haptoglobin, by binding to haem, regulates iron levels. Haptoglobin is important for rapid hepatic clearance of free haemoglobin from the plasma. Haptoglobin is a major APP in ruminants where circulating levels are negligible in normal animals, but increase over 100-fold on immune stimulation (Conner *et al*., 1988; 1989). Haptoglobin in dogs is a constitutive serum protein, and its increase is very modest during disease. Human haptoglobin is a constitutively secreted plasma protein that exhibits only a moderate increase during APR. Haptoglobin has been identified as acute phase protein in the liver of rainbow trout after exposure to a bacterin (*Listonella*) where the induction of the mRNA was shown to increase 2 fold by microarray analysis (Gerwick *et al*., 2007). Activation of the stress response by netting and suspension in air for 30 seconds followed by exposure to continuous 3 h low water stressor resulted in a 1.83 fold induction of haptoglobin mRNA in the liver of juvenile rainbow trout (Momoda *et al*., 2007).A recent study in which similar confinement conditions were established over a period of a month (504 h), found haptoglobin expression in trout liver to be elevated throughout the time course of the confinement (6 h, 24 h, 168 h and 504 h) (Cairns *et al*., 2008). In the present study, in the pooled analysis an elevation in haptoglobin gene expression as a result of confinement was only evident at 168 h (4.3 fold) (P = 0.0075). However, analysis of the individual fish from the 24 h, 48 h and the 168 h time points excluding the one outlier from each of these control groups (Fish 13 at 24 h, Fish 22 at 48 h and 35 from the 168 h) found that the differences between the control and confined group now attained significance (P = 0.021, P = 0.002 and P = 0.0159, respectively).

In addition to its role in the clearance of free haemoglobin, various immunoregulatory effects can be attributed in part to the binding of haptoglobin to CD11/CD18 receptor on effector cells (El-Ghmati *et al*., 1996). The binding of haptoglobin to this receptor mediates crucial leukocyte adhesion functions such as chemotaxis, phagocytosis, and adhesion to endothelium, aggregation and cell-mediated cytotoxicity. Haptoglobin also has an inhibitory effect on granulocyte chemotaxis, phagocytosis and bactericidal activity (Rossbacher *et al*., 1999) and may inhibit mast cell proliferation (El-Ghmati *et al*., 2002), prevent spontaneous maturation of epidermal Langerhans cells (Xie *et*

al., 2000) or suppress T-cell proliferation (Arredouani *et al*., 2003). Haptoglobin produced by liver cells in mice in response to interleukin-6 (IL-6) has been demonstrates to modulate the inflammatory response induced by endotoxins (Arredouani *et al*., 2005). The multiple and varied immunoregulatory effects of haptoglobin may provide an explanation for the up-regulation of this acute phase gene in during instances of both invasive and non-invasive stress.

DRTP1 was first identified in Brook trout (*Salvelinus fontanalis*) (GenBank accession no. AF004521) as a gene differentially expressed upon stimulation with phorbol myristate acetate (PMA), which is known to affect several ovarian processes in fish, including ovulation, prostaglandin synthesis and steroidogenesis (Lee and Goetz, 1998). DRTP1 was also identified and cloned in rainbow trout liver after challenge with *Vibrio anguillarum* (Bayne *et al*., 2001). Through the use of an oligonucleotide cDNA microarray combined with quantitative real-time PCR (qPCR) these authors further validated the increase in DRTP1 gene expression that resulted from the bacterial challenge (Gerwick *et al*., 2007). DRTP1 was also identified in Atlantic salmon where it was reported to be up-regulated in the liver, spleen and head kidney after challenge with the pathogen *Aeromonas salmonicida* by suppression subtractive hybridisation (Tsoi *et al*., 2004) which was confirmed in a further study by cDNA microarray technology (Ewart *et al*., 2005). A more recent study reported DRTP1 to be up-regulated in the liver and gill of Atlantic salmon, again after challenge with *Aeromonas salmonicida* (Martin *et al*., 2006).

In the pooled analysis of the present study DRTP1 gene expression as result of confinement was found to increase at 8 h (2.8 fold, $P = 0.0022$) with no change in expression evident at 24 h. Increases in DRTP1 expression were evident again at 48 h (2.9 fold, $P = 0.0019$) followed by a marked increase in expression at 168 h (12 fold, $P = 0.0022$). From the analyses of DRTP1 gene expression in the individual confined fish, the mean of the five fish in each group was seen to increase over time (Fig. 4). At 168 h, as a result of confinement stress, 4 of the 5 fish had major increases in the DRTP1 gene expression with two fish having greater than 25 fold and the two others having greater than 50 fold increases in DRTP1 expression.

During the course of the confinement the mean level of plasma cortisol in the individual confined fish remained relatively high (Table 2). At 8 h the mean plasma

cortisol level was 53.6 ng/ml ± SD 35.1, at 24 h it was 48 ng/ml ± SD 20.3, at 48 h it was 81.8 ng/ml ± SD 81.6 and at 168 h it was 63.2 ng/ml ± SD 76.4. Analysis of the individual confined fish found that no correlation existed between the level of plasma cortisol and the expression of SAA, haptoglobin or DRTP1. A correlation was found to exist between the expression of haptoglobin and DRTP1 at both the 8 h ($r^2 = 0.843$, $P = 0.028$) and the 24 h ($r^2 = 0.983$, $P = 0.009$) time points. Although the expression levels of these two genes were very different, with DRTP1 having higher expression levels when compared to haptoglobin, the pattern of expression was found to be very similar in the individual fish in these populations. No correlation was found to exist with SAA expression and the expression of either haptoglobin or DRTP1.

NF-κB family members are transcriptional factors that regulate the expression of a large number of target genes involved with physiological processes such as immune response, apoptosis, inflammation and progression of the cell cycle in different organisms. SAA and haptoglobin are acute phase proteins dependent on the IL-1/NF-κB signaling pathway and contain C/EBP binding sites in their promoter regions (Burgess-Beusse and Darlington, 1998). Previous work carried out in this laboratory (Talbot *et al*., 2008) using genome walking and promoter analysis identified transcription binding sites for NF-κB and C/EBPβ as important factors involved in the induction of the DRTP1 gene by the pro-inflammatory cytokine TNFα. The common signalling pathways between these three genes (SAA, haptoglobin and DRTP1) might provide some insight into the mechanism by which these genes are activated by stress.

In summary, this study highlighted the importance of individual variation in the interpretation of gene expression data. The presence of one atypical control fish in a pool of five at the 24h, 48 h and 168 h time points was sufficient to generate misleading results.

The results of this study would suggest that although SAA is a very inducible gene in trout, in order for the up-regulation to be interpreted accurately, the analysis of SAA should be performed in individual fish.

This study further confirmed that confinement stress is capable of altering the expression of two genes involved in the acute phase proteins, haptoglobin and DRTP1

In addition, in combination with previous published reports, it has been demonstrated that DRTP1 is up-regulated in stress, regardless of whether the stressor is invasive or non-invasive. However, the role of the DRTP1 protein in the stress response will remain unclear until the function of this protein is elucidated.

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Table 1. Primers used in this study

Gene	Genbank Accession Number	Primers	Primer Sequence
SAA	X99387	SAA_RT1 SAA_RT2	5'TTGTTCTGACCCTCGTTGTAGGAGC3' 5'CATGTCGCCATATGCACGCC3'
DRTP1	AF281355	DRTP1-S DRTP1-AS	5'GTAGCTTTGGAGAGGGCCTGAAATG3' 5'CAATGTCCTTGTTGCTTCCCAGC3'
Haptoglobin	AF279136	Hapto_S Hapto_AS	5'GAGATCCCAAACAATTCAGACCTGCG3' 5'CTTCCTGACAAACAAGTTCCTGCC3'
Pentraxin	BE669045	Pentraxin_S Pentraxin_AS	5'CACATGGTCATAGCAGAAATGGGC3' 5'TGGAGAGTGCACTCAATCTAACGGG3'
Transferrin	D89083	Trans_RTS Trans_RTAS	5'CCACCTCCAGGGCCATTAAATG3' 5'ATCCACCGCTATGGCATCTGCC3'
Fibrinogen-like protein 2	BE859104	Fib2_S Fib2_AS	5'ACTTCCAAGGGGTGCGGGAGTATG3' 5'TTGACCGTCTTGAAGGCCTGCC3'
alpha-1-antiproteinase-like protein	AJ558113	α1-AT_S α1-AT_AS	5'AGGCTTCACGGTCGACTTCAAGAAC3' 5'TGAAGTCAGCCTTGTGGGTCAGC3'
LECT2	AF363272	Lect2-S Lect2-AS	5'GATGGTCAAGTTTGGTCAGCTGTGC3' 5'ATGGCTGCCTTCTTCGGGTCTG3'
Beta-actin	AJ438158	Actin_RTS Actin_RTAS	5'ATGGAAGATGAAATCGCCGCAC3' 5'TGGCCCATCCCAACCATCAC3'

All primers were designed against rainbow trout sequences in the Genbank non-redundant database, with the exception of the fibrinogen-like protein 2 (flp2) and pentraxin primers which were designed against the Genbank EST database.

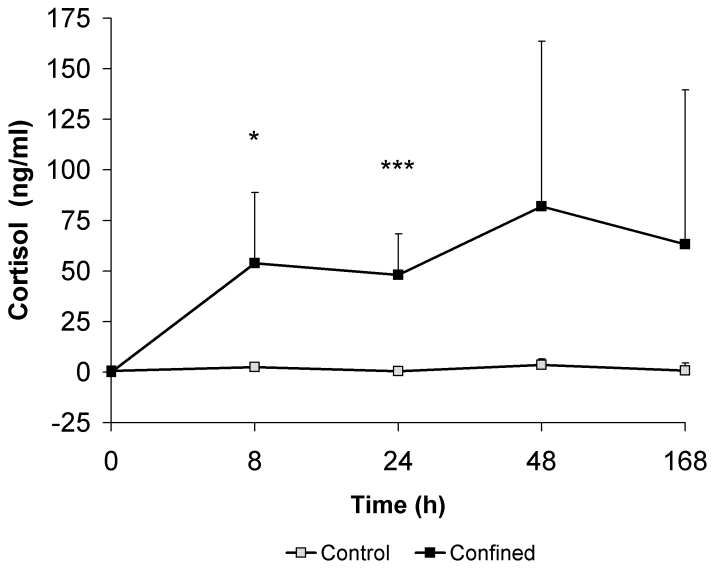

Figure 1. Total cortisol in the control and confined fish over time. Cortisol measurements were made using plasma from individual fish. The graph shows the mean value for the five individual fish (control and confined) at each of the time points (± SD). Significant differences between the control and stress groups at each time point are denoted by *: $P < 0.05$, ***: $P < 0.001$

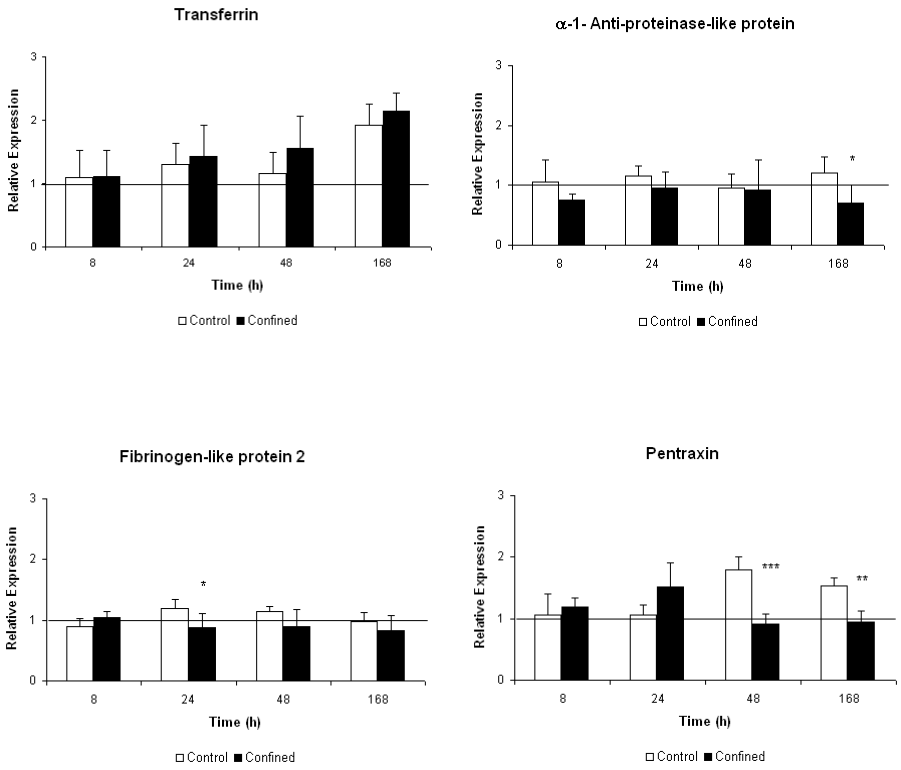

Figure 2. Hepatic gene expression of transferrin, fibrinogen-like protein 2, α-1-anti-proteinase-like protein and pentraxin relative to the 0 h unconfined control. Each bar on the graph represents a pool of either 5 control (light bars) or 5 confined fish (dark bars) and is the mean of 6 replicates ± SD. The β-actin gene was used as an internal standard for the qPCR analyses. Significant differences between control and stressed groups are denoted by *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

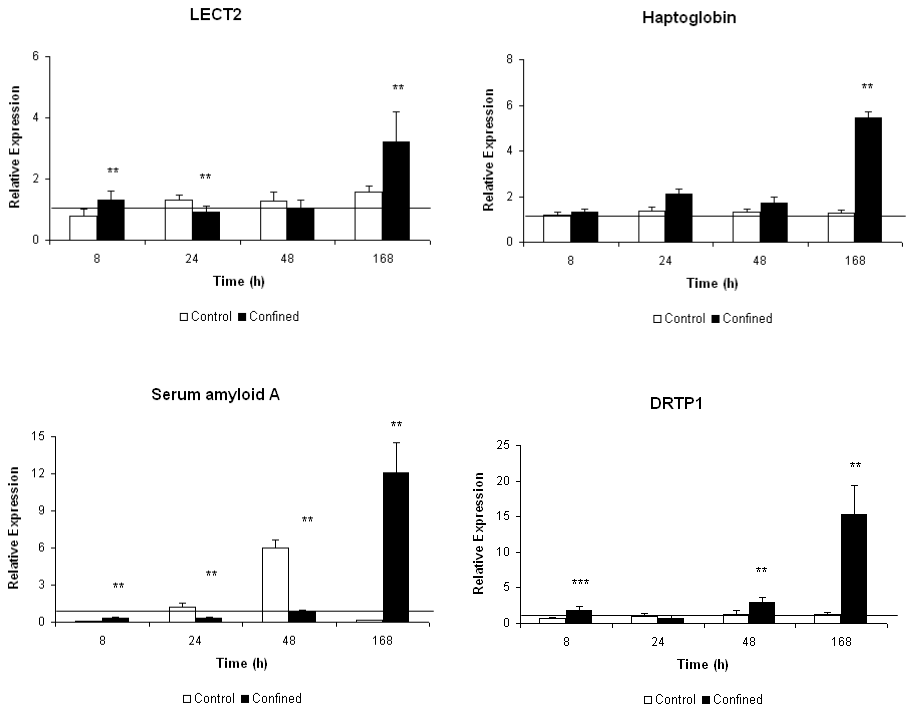

Figure 3. Hepatic gene expression of leukocyte cell-derived chemotaxin 2 (LECT2), serum amyloid A (SAA), haptoglobin and differentially regulated trout protein 1 (DRTP1) in confined and unconfined trout relative to the 0 h unconfined control. Each bar on the graph represents a pool of either 5 control (light bar) or 5 confined fish (dark bar) and was calculated using 6 replicates ± SD. The β-actin gene was used as an internal standard for the qPCR analyses. An unpaired, Student's *t*-test was used to determine significant differences in gene expression between the control and confined pools at each time point. Significant differences between control and stressed groups are denoted by: **: $P < 0.01$; ***: $P < 0.001$.

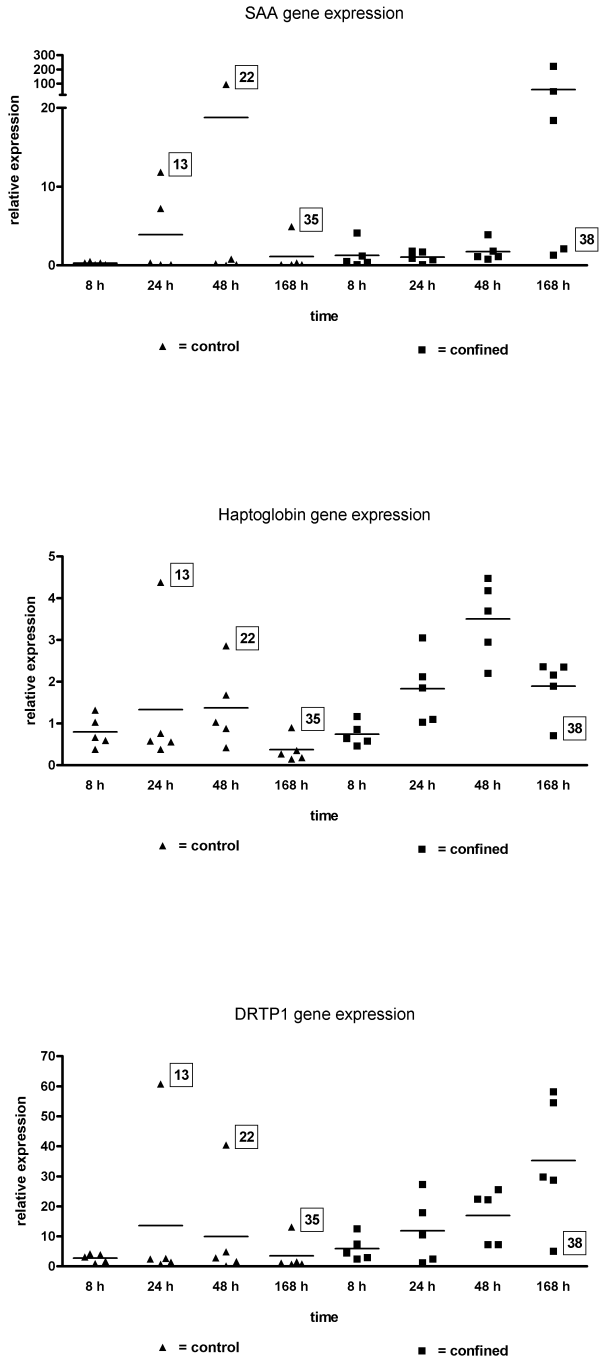

Figure 4. Relative gene expression analyses of SAA, haptoglobin and DRTP1. Five control fish (triangles) and five confined fish (squares) were analysed at 8 h, 24 h, 48 h and 168 h. The horizontal lines are representative of the median gene expression for each group. Gene expression is expressed relative to the 0 h non-confined control (n = 5 fish). Individual outlier fish are identified by their number in square boxes next to their expression symbol.

Table 2. Relative gene expression of SAA, Haptoglobin and DRTP1. Total cortisol plasma levels (ng/ml) and transcript level fold changes (relative to the 0 h control) are listed for the control and confined individual fish.

Time	Fish number - treatment	Cortisol ng/ml	Transcript fold change SAA	Haptoglobin	DRTP1
8 h	1 - control	3.2	0.1	1.3	3.1
	2 - control	0.0	0.5	0.6	3.8
	3 - control	3.8	0.1	1.0	1.7
	4 - control	1.1	0.3	0.7	4.1
	5 - control	4.2	0.2	0.4	0.9
	6 - confined	21	4.1	1.2	12.5
	7 - confined	30	0.4	0.6	2.4
	8 - confined	104	0.1	0.9	7.4
	9 - confined	76	0.5	0.6	2.9
	10 - confined	37	1.2	0.5	4.5
24 h	11 - control	0.5	0.7	0.4	0.7
	12 - control	0.7	7.2	0.6	2.6
	13 - control	0.2	11.8	4.4	60.7
	14 - control	0.1	0.3	0.8	2.5
	15 - control	0.7	0.1	0.6	1.4
	16 - confined	36	0.9	2.1	17.9
	17 - confined	18	1.7	1.8	10.5
	18 - confined	59	0.7	1.1	2.5
	19 - confined	64	0.1	1.0	1.2
	20 - confined	63	1.8	3.1	27.3
48 h	21 - control	4.9	0.1	1.0	2.9
	22 - control	0.8	92.6	2.9	40.4
	23 - control	2.0	0.8	1.7	4.8
	24 - control	1.8	0.1	0.9	1.6
	25 - control	11.4	0.2	0.4	0.2
	26 - confined	201	0.8	3.7	25.6
	27 - confined	127	1.0	4.2	22.4
	28 - confined	57	3.9	3.0	7.3
	29 - confined	10	1.8	4.5	22.2
	30 - confined	14	1.1	2.2	7.3
168 h	31 - control	0.4	0.1	0.2	0.8
	32 - control	1.7	0.1	0.4	1.5
	33 - control	0.0	0.3	0.3	0.7
	34 - control	1.4	0.1	0.2	1.1
	35 - control	0.1	4.9	0.9	13.1
	36 - confined	197	18.4	2.2	54.5
	37 - confined	33	41.7	1.9	58.1
	38 - confined	30	2.1	0.7	5.1
	39 - confined	50	1.3	2.4	28.7
	40 - confined	6	221.4	2.4	29.7

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