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SHORT COMMUNICATION

Individual measurement of gene expression in blood cells from Rainbow trout *Oncorhynchus mykiss* (Walbaum)

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ABSTRACT

Most studies that monitor a biological parameter over time in fish are based on the culling of animals and subsequent tissue sampling or the use of complicated surgical procedures such as cannulation of the dorsal aorta. The former method suffers from the large inter-individual variability typically seen in outbred fish, whilst the latter requires highly skilled operators and is not possible with small fish. We describe here a novel and simple method based on non-lethal collection of blood samples from the same individual, allowing improved data quality and reduction in number of animals used without the need for surgical procedures. The frequency and volume of blood collected repeatedly was adjusted to limit the decrease in the percentage of blood packed cell volume (PPCV). The stress response evaluated by measuring the expression of the heat shock proteins 90αb1, 70KDa and the cytochrome p450 family 17 A1 in blood cells by qPCR. Expression levels increased during the PPCV decline and returned to their basal level after adjustment of the sampling procedure. This study demonstrates that a non-lethal sampling procedure can be used for salmonid fish and gene expression in blood cells can be monitored over time from the same individual.

KEYWORDS: Non-lethal sampling; Single animal monitoring; In vivo experiment; Haematocrit; Real time PCR.

INTRODUCTION

The majority of fish experiments are based on destructive sampling whereby a number of animals are killed sequentially to monitor biological parameters over time. Not only is this model very costly in number of animals used but it also works under the assumption that all individuals are synchronised in treatment effect/response. A typical example is infectiology where a population of fish are

infected with a live pathogen and the progression of disease and host response are monitored over time. Multiple factors will affect the pathogen growth kinetics between two different individuals even though they share the same tank and the same dose of pathogen is administered at the same time. Besides, fish livestock used in experimental work exhibit large inter-individual variability, a situation which is less problematic in rodents used for medical research where there is possibility to use inbred lines exhibiting a higher genetic and phenotypic uniformity (Festing, 1979). The practice in fish experimentation differs from that in terrestrial large veterinary animals or laboratory rodents. In the latter, it is common practice to collect blood non-lethally or in some cases to perform biopsies (Flutter et al., 2000; Parasuraman et al., 2010). Whilst cannulation of the dorsal aorta is possible as a means to sequentially blood sample fish, it is a surgical procedure that requires a high degree of skill and training and consequently is typically avoided in preference for terminal sampling.

In order to improve the quality of the data generated by fish experiments and to reduce the number of animals used, a novel experimental model was pioneered in the present study where a small blood sample was collected repeatedly over the experimental period using a simple sampling procedure. The experimental design including sampling interval and blood collection was optimised for salmonids. In addition, a method to measure gene expression in blood cells from minute whole blood samples was adapted and used to investigate stress-related gene expression in relation to the bleeding regime. Three genes were selected for analysis of the stress response at the cellular level; heat shock protein 90 kDa alpha (cytosolic) class B member 1 (HSP90ab1), heat shock cognate 70 kDa protein (HSP70) and cytochrome P450- family 17, subfamily A- polypeptide 1 (cyp17a1). Heat shock proteins (HSPs) consist of a family of proteins with a range of different molecular weights (Lindquist & Craig 1988). They are constitutively expressed within non-stressed cells to relatively high levels and play important roles in protein folding, transport, cellular redox state, protein turnover and other homeostatic functions (Sonna et al, 2002). Their induction has been observed in response to many different stressors including anoxia, toxins, protein degradation, hypoxia, and damage due to infection (Roberts et al., 2010). Stressors can cause cellular protein damage followed by unfolding, exposure of hydrophobic elements and aggregation of the proteins. HSPs induced under stress conditions act as “chaperones” where they bind to partially unfolded proteins to stabilise them (Sonna et al, 2002). HSP70 has been found to either block (Mosser et al., 2000) or promote (Ran et al., 2004) apoptosis in mammals while HSP 90 is involved in maintaining structure of the proteasome (Imai et al., 2003), a protein complex responsible for degrading damaged proteins within the cells. Cyp17a1 is a member of the cytochrome P450 enzyme family which is involved in the synthesis of lipids including cholesterol and steroids (Miller & Bose, 2011). It is a key enzyme in the synthesis pathway of progestins, mineralocorticoids, androgens, oestrogens, and glucocorticoids (such as cortisol).

MATERIALS AND METHODS

Rainbow trout were purchased from Almond bank (Perthshire) and transported to the aquarium facility in in the School of Biological Sciences, University of Aberdeen. Fish were maintained in 1 m diameter aerated fibreglass tanks supplied with a continuous flow of recirculating freshwater at 12 ± 1 oC. Fish

were fed twice daily on standard commercial pellets (EWOS). After a week of acclimation, all the fish were anaesthetised with 0.08 g/l MS222 (Sigma-Aldrich). Weight (average weight 255.2±33.4 g) and length (average length 26.6±1.1 cm) were measured and labelled using Passive Integrated Transponder (PIT) tags (Biomark). A week after, all animals were anaesthetised identified using a PIT-tag reader (Biomark) and a small blood sample (100 µl) was collected from the caudal vein using a 29G needle (29G1 BD Microlance 3) on a 1 ml syringe (BD Bioscience). The needle was removed and the whole blood was immediately transferred into heparin coated vaccutainers (Midmeds LtD) and kept on ice. Subsequently, blood samples were collected at days 2, 4, 6, 9, 14, 21 and 28. The total amount of blood withdrawal was just above 10 % total blood volume estimated at approximately 3 % of total body weight for rainbow trout (Conte et al., 1963). The Haematocrit was measured using the method from Fujimoto (2000). After measurement of the PPCV, blood (approx. 50 µl) was recovered from the haematocrit capillary using a syringe needle (18G1 BD Microlance 3) and a long capillary glass Pasteur pipette (Sigma-Aldrich), combined to the remaining blood and centrifuged for 30 s at 12,000 g at room temperature. The plasma was then collected and stored at -80 °C for future use. The blood cell pellet was vortexed for 10 sec and 30 µl of whole blood cells was gently pipetted and mixed with 600 µl RLT buffer (RLT, Qiagen RNeasy Miniprep kit) plus 1 % (v/v) β-mercapto-Ethanol (Sigma-Aldrich), and stored at -80°C until RNA purification.

Total RNA from blood cells was purified using a method modified from the RNeasy Mini kit (Qiagen). The mix of blood cells and RLTb was homogenised with a Tissue Lyser using one 5 mm stainless steel bead (Qiagen) for 1 min at 25 Hz at room temperature. The remaining steps in the procedure were carried out according to the manufacturer's instructions (Qiagen RNeasy Mini kit method) and the RNA was eluted in 75 µl RNase-free water and stored at -80 °C until use. RNA was reverse transcribed to cDNA using M-MuLV Reverse Transcriptase (New England Biolabs) using oligo-d(T)16 (Applied Biosystems) as follows: 8 µl of total RNA (approx. 0.5µg), 1µl 50 µM oligo-d(T)16, 1µl 10 mM dNTPs (Applied Biosystems), 2µl PCR water (Sigma-Aldrich) were mixed and heated to 65 °C for 5 min and immediately chilled on ice. The final volume was adjusted to 20 µl by adding the following: Reverse Transcriptase buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl2,), 10 mM DTT, 0.5 mM each dNTP, 0.4U RNase inhibitor (Applied Biosystems) and 200 Units M-MuLV Reverse Transcriptase. Reactions were incubated at 37 °C for 90 min, heat inactivated at 95 °C for 5 min, diluted 5 fold with water and finally stored at -80 °C until further use. Primers for use in stress gene QPCR assays were designed from sequence data available on NCBI using the Editseq software (Lasergene) (Table 1) and obtained from Sigma-Aldrich. Primers were not specifically designed over exon-intron boundaries as genome data was not available for these genes, and many heat shock genes do not contain introns (Maquat and Li, 2001). QPCR assays were performed on LightCycler 480 system QPCR machine (Roche Applied Science) containing per reaction 4µl diluted cDNA, 5.0 µl SYBRGreen Master Mix 1 (Roche Applied Science), 0.5 µM forward and reverse primer in a total volume of 10 µl. QPCR cycling conditions were 95 °C for 10 min followed by 45 cycles of 95 °C for 30 sec, 62 °C for 30 sec,

72 °C for 45 sec. A melting curve was obtained by increasing the temperature to 99 °C at a rate of 0.11 °C/sec. Elongation factor α (ELFα) expression was used as an internal control to normalise stress gene expression levels across different samples. Expression levels for ELFα and the stress genes was calculated per Collet and Collins (2009). The expression level of the gene relative to ELF was calculated by dividing the gene expression level by ELF expression level.

Table 1. List of primers used in this study and corresponding genomic details.

Gene/Accession number/Species	Forward primer (5'-3')	Reverse primer (5'-3')	Size of product (bp)
Cytochrome P450 cyp17a1/ NM_001124747/ Om	GTAGCCCAAACAGACACCAGTATT	CCCCAAACGGCAAGTAGC	190
Heat shock protein 90kDa alpha (cytosolic) hsp90ab1/ BT043623/ Ss	TGCGCTACCACAGTTCTCAGTCC	GTCCTTGCTCTCGCCAGTG	114
Heat shock cognate 70 kDa protein putative/ BT059361/ Ss	AACGTAACACCACCATCCCAACCA	GCCCTCTCACCCTCATACACCTG	101
Elongation factor 1 alpha (ef-1aO)/ BT043567/ Ss	CCCCTCCAGGACGTTTACAAA	CACACGGCCCACAGGTACA	57

Om = Oncorhynchus mykiss; Ss = Salmo salar

RESULTS and DISCUSSION

Fish were initially bled every other day up until day 9 when the sampling regime changed to 4 day intervals, and then weekly from day 14 onwards. The change of the percentage of blood packed cell volume (PPCV or haematocrit) over the time is presented in Fig. 1A. At the first sampling point it was 33.9 % ±4.1%. After 2 days the PPCV increased (47.4% ±6.1%), whereas in samples taken at 4, 6, and 9 days it decreased dramatically reaching 10.6 ±3.4% by day 14. The PPCV increased between each sampling point from day 14 to day 28, at which time the experiment reached its endpoint (Fig. 1A) with the exception of fish 4.

The initial increase in the PPCV at day 2 may be explained by haemo-concentration, whereby a decrease in the volume of plasma in relation to the number of red blood cells may have occurred (McDonald and Milligan 2011). Just after blood collection, there was at times a small residual amount of bleeding which occurred after the procedure had been undertaken. Although this was minimised by using the smallest gauge needle possible without compromising blood quality, it was not enough to completely prevent residual bleeding. This would have contributed to the lowering of the PPCV observed after day 2. The generation of new blood cells to replace the withdrawn blood volume was unlikely to have occurred at a high enough rate when the repeat bleeds were taken every other day. This is also supported by the proportion of immature blood cells present in blood smears taken from fish (data not shown). The blood sample taken 4 days since the previous one (day 14) showed minimal change in the packed cell volume

compared to the previous sample taken. Based on this finding we suggest that repeat bleeding of the same individual should not occur at a frequency less than 4 days apart. Work carried out by Montero et al. (1995) found that in juvenile Gilthead sea bream regeneration of the red blood cell count started to increase 2-4 days post bleeding and returned to the basal value after 8 days. The duration of the bleeding procedure in this study was at 0.5, 1, 2, 4, 8, 16 and 32 days, and it was only when there was a break of 4 consecutive days or more that recovery to the basal level was observed. In this study, groups of animals were repeatedly bled but data was not recorded or analysed individually. Although the experimental procedure revealed some similarities with the present study, we analysed the data individually in rainbow trout.

This is the first attempt to monitor gene expression in fish from the same individual over time. The only record to date was a study by Raida et al., (2011) where rainbow trout were infected with Yersinia ruckeri in individual tanks and one blood sample was collected just before infection. It is possible to carry out a non-lethal collection of gill fragment from anaesthetised fish (McCormick, 1993). However, gills are less informative than blood cells for certain biological functions and repeated biopsies could be harmful to the animals. Cannulation has also been used in the past, but as stated above the procedure requires surgery and gives restricted animal movement due to protruding apparatus, often requiring the fish to be held in individual tanks (Eliason et al., 2007; Sohlberg et al., 1996; Nichols and Weisbart, 1983) To date, the vast majority of fish experiments are based on the culling of a large number of animals combined with the lethal sampling of tissues. Using a non-lethal sampling method combined with single-animal study has the potential to lower inter-individual variability and to increase statistical power.

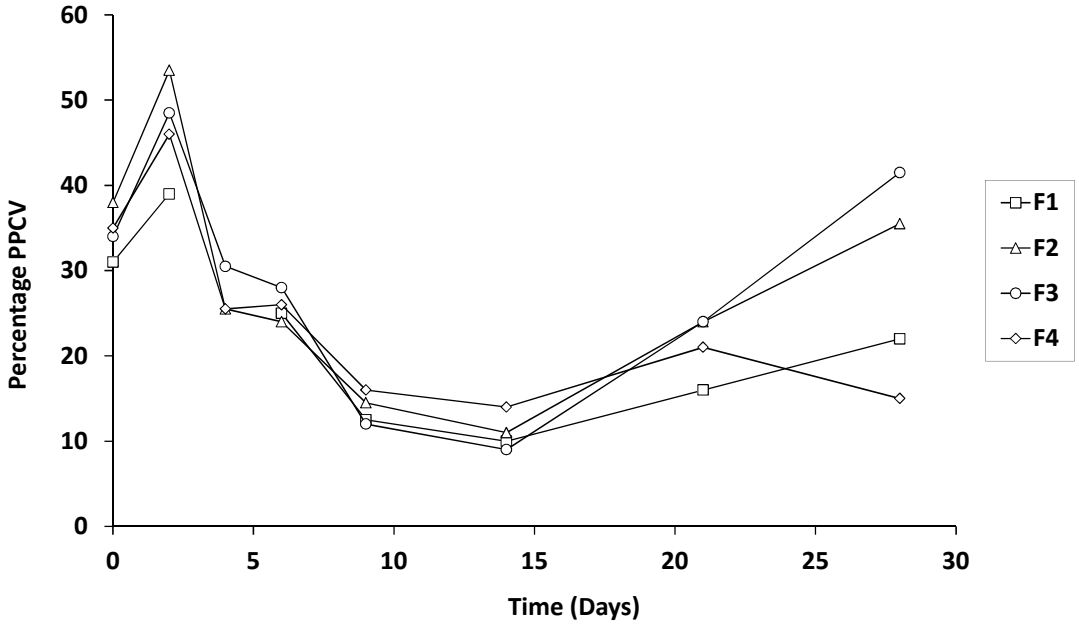

Figure 1: Percentage of blood packed cell volume (PPCV) measured at sampling days 0, 2, 4, 6, 9, 14, 21 and 28. In rainbow trout. Individuals were PIT-tagged and PPCV was individually measured at different time points. The data represents individual values from 4 fish identified as F1, F2, F3 and F4.

QPCR melt curve analyses and visualisation of QPCR products on gels confirmed presence of single product in reactions. The Elongation Factor alpha (ELFα) assay amplifies both genomic DNA and cDNA, with primers in exon regions on either side of a splicing site. Analysis of melt curves for ELFα QPCR confirmed the presence of a single peak corresponding to cDNA, indicating absence of sub-detection levels of contaminating genomic DNA.

The expression levels of the three stress genes analysed showed a negative association with the changes in haematocrit. Higher levels of stress gene expression were observed with falling haematocrit and a reduction in stress gene expression was seen as haematocrit stabilised and recovered from day 9 onwards. The spike in haematocrit levels at day 2 is also reflected in a dip in stress gene expression. However, this dip occurs at day 4, which may reflect a delay in return to temporary homeostasis following the hypothesised compensatory reduction in plasma volume. The reduction and stabilisation of stress gene expression from day 9 supports the conclusion drawn from the haematocrit values that 4 day intervals for bleeding result in lower chronic stress in fish.

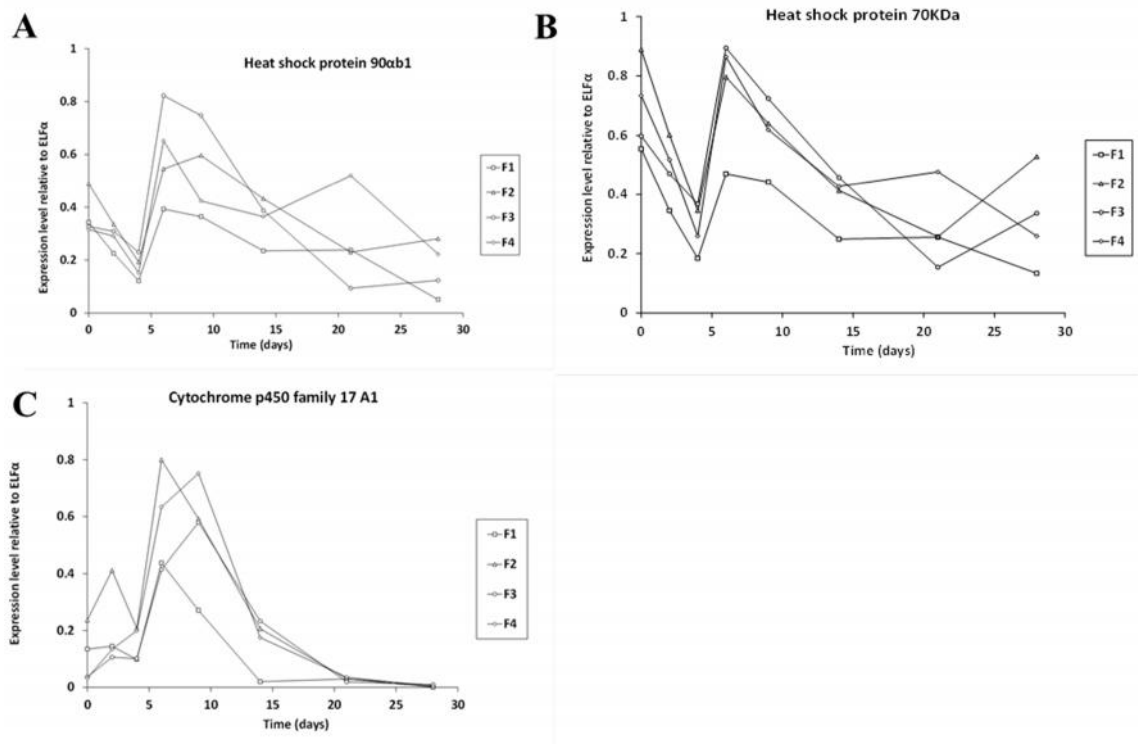

Figure 2. Stress protein expression levels over time in the blood cells of individual fish. A. Heat shock protein 90αb1. B. Heat shock protein 70KDa. C. Cytochrome p450 family 17 A1. The data represents individual values from 4 fish identified as F1, F2, F3 and F4.

Stress genes are induced by multiple different stressors (Roberts et al., 2010). The potential stressor in this case may be hypoxia resulting from falling haematocrit and oxygen

transport to the different tissues. There is little or no information on the expression of cyp17a1 in fish in relation to hypoxia (or other stressors), but it is known to be involved in the production of a number of hormones that are known to be affected by hypoxia in fish (Richards et al., 2009; Fuzzen et al., 2011). What is even more interesting is the finding of cyp17a1 expression in blood cells, and this requires further investigation. Induction of HSP70 has been previously shown in hypoxia conditions (Delaney & Klesius, 2004; Lückstädt et al., 2004), while HSP90 is closely associated with Hypoxia Inducible factor α (Isaacs et al., 2002), though reports on its induction are limited. It should be noted that the isoform of HSP 90Kda analysed here is not that generally considered inducible by stressors but the constitutively expressed β form (Chen et al., 2005; Ojima et al., 2005; Manchado et al., 2008). However inducible expression of HSP 90KDa β has been shown recently in Channa striatus in relation to hypoxia (Mohapatra et al, 2013) and in Atlantic salmon in relation to fasting and feeding (Garcia de la Serrana & Johnston, 2013), indicating that it may have as yet undefined roles in physiology, cell cycling or other processes. Other causes of stress resultant from regular bleeding may also include osmotic stress and hypotension. In addition, tress experienced during animal capture can have a negative effect upon blood coagulation following the bleeding procedure (Travares-Dias & Oliveira 2009; Casillas & Smith 1977), thus it is preferable to opt for an in-tank anaesthesia method rather than subjecting fish to the stress of net capture prior to withdrawing blood.

The present method allows archival of blood samples quickly for future RNA extraction. Thirty (30) µl blood cells produce enough RNA for 6 RT reactions allowing for over 100 gene assays by QPCR. This model can be applied to a wide range of areas of research such as infectiology, toxicology, physiology, reproduction, and genetics. The reduction in the number of animals used by the non-lethal model compared to the traditional lethal model is expected to be approximately 90%.

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CONFLICT OF INTERESTS

The authors have declared no conflict of interests.

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