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Identification of a gene set to evaluate the potential effects of loud sounds from seismic surveys on the ears of fishes: a study with *Salmo salar*

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Functional genomic studies were carried out on the inner ear of Atlantic salmon *Salmo salar* following exposure to a seismic airgun. Microarray analyses revealed 79 unique transcripts (passing background threshold), with 42 reproducibly up-regulated and 37 reproducibly down-regulated in exposed *v*. control fish. Regarding the potential effects on cellular energetics and cellular respiration, altered transcripts included those with roles in oxygen transport, the glycolytic pathway, the Krebs cycle and the electron transport chain. Of these, a number of transcripts encoding haemoglobins that are important in oxygen transport were up-regulated and among the most highly expressed. Up-regulation of transcripts encoding nicotinamide riboside kinase 2, which is also important in energy production and linked to nerve cell damage, points to evidence of neuronal damage in the ear following noise exposure. Transcripts related to protein modification or degradation also indicated potential damaging effects of sound on ear tissues. Notable in this regard were transcripts associated with the proteasome–ubiquitin pathway, which is involved in protein degradation, with the transcript encoding ubiquitin family domain-containing protein 1 displaying the highest response to exposure. The differential expression of transcripts observed for some immune responses could potentially be linked to the rupture of cell membranes. Meanwhile, the altered expression of transcripts for cytoskeletal proteins that contribute to the structural integrity of the inner ear could point to repair or regeneration of ear tissues including auditory hair cells. Regarding potential effects on hormones and vitamins, the protein carrier for thyroxine and retinol (vitamin A), namely transthyretin, was altered at the transcript expression level and it has been suggested from studies in mammalian systems that retinoic acid may play a role in the regeneration of damaged hair cells. The microarray experiment identified the transcript encoding growth hormone I as up-regulated by loud sound, supporting previous evidence linking growth hormone to hair cell regeneration in fishes. Quantitative (q) reverse transcription (RT) polymerase chain reaction (qRT-PCR) analyses confirmed dysregulation of some microarray-identified transcripts and in some cases revealed a high level of biological variability in the exposed group. These results support the potential utility of molecular biomarkers to evaluate the effect of seismic surveys on fishes with studies on the ears being placed in a priority category for development of exposure–response relationships. Knowledge of such relationships is necessary for addressing the question of potential size of injury zones.

Key words: biomarker; ear; fish; microarray; seismic; sound.

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INTRODUCTION

The marine environment is subject to a broad range of anthropogenic noise, some major sources being seismic surveys for oil and gas exploration, commercial shipping, sonar and pile driving (Richardson *et al.*, 1995; National Research Council, 2003; Hastings, 2008; Bailey *et al.*, 2010; Nieukirk *et al.*, 2012). Over the past century, the levels of human-generated sound introduced into the marine environment have been increasing (Ross, 2005; Hatch & Wright, 2007; André *et al.*, 2011*a*; Chapman & Price, 2011), leading to a growing concern regarding potential effects on the health of aquatic life (Popper, 2003; Popper *et al.*, 2004; Weilgart, 2007; Slabbekoorn *et al.*, 2010). Concerns include the potential for effects on animal behaviour through masking of auditory sounds, actual physical damage to various tissues or production of neuroendocrine-mediated stress (Kastelein *et al.*, 2007; Payne *et al.*, 2007; Wysocki *et al.*, 2007; Popper & Hastings, 2009; Filiciotto *et al.*, 2013). The extent of biological damage caused by noise exposure depends on the characteristics of the sound including the frequency, intensity and duration (Popper & Hastings, 2009).

It has been suggested that intense noise from airguns used in seismic surveys for exploration of oil and gas deposits represent the most abundant impulsive sounds introduced into the ocean by human activity (Hatch & Wright, 2007). Seismic surveys involve the deployment of intense sound-producing airguns from a survey vessel almost invariably in multiple arrays with airguns being shot simultaneously, such that an array can produce thousands of shots and cover hundreds of square kilometres over a 24 h period (Payne *et al.*, 2007). Seismic airguns produce loud sounds with peak sound levels of individual airguns as high as 260 decibels relative to 1 micropascal (dB *re* 1 μPa) at a range of 1 m from the source (Hatch & Wright, 2007). By comparison, 186 dB *re* 1 μPa is about the sound level that a human listener will feel pain (Popper, 2003). Sounds generated by seismic airguns are generally in the low frequency range that can allow the signal to travel for thousands of kilometres from its source (Nieukirk *et al.*, 2012). Most of the energy released from seismic airguns is at a frequency of 10–100 Hz, but can reach levels up to 1000 Hz (Hatch & Wright, 2007), which is within the auditory range of most aquatic species (Hastings & Popper, 2005). Gun discharges are typically every 8–10 s, and by way of note, seismic surveys in offshore Labrador over the past 3 years covered *c*. 42 000 km of track lines (www.cnlopb.nl.ca).

There is concern that intense sounds could affect the auditory receptor cells (*e.g.* hair cells) or structures (*e.g.* saccule and lagena) involved in fish hearing and a few field studies have been carried out (McCauley *et al.*, 2003; Popper *et al.*, 2005; Hastings *et al.*, 2008; Song *et al.*, 2008). Comparisons between studies can be complicated by differences in species and life stage as well as in the characteristics of receiving sounds that can be influenced by the source of sound, distance from the sound source, water depth, temperature and bottom topography (Lawson, 2009; Popper & Hastings, 2009).

Since 2000, development of high-throughput functional genomics research tools has revolutionized research in areas such as toxicology, aquaculture, fish health and ecology. In particular, microarrays are important genomics research tools as they can allow the rapid identification of molecular pathways altered in response to environmental stressors by revealing the relative expression of thousands of genes simultaneously. Functional genomics research strategies have been used to determine how environmental stressors, such as pathogens, temperature stress and environmental contaminants

affect molecular pathways and biological processes in fishes (Rise *et al.* 2004; Finne *et al.*, 2007; Hori *et al.*, 2010, 2012; Rise *et al.*, 2010; Hall *et al.*, 2011). If loud sound is considered to be an environmental stressor, it can be hypothesized that valuable information on molecular mechanisms involved in acoustic stress could be provided.

Microarray technology has been used to address the effect of noise on the ears of mammals and birds (Lomax *et al.*, 2001; Taggart *et al.*, 2001; Cho *et al.*, 2004; Morris *et al.*, 2005; Kirkegaard *et al.*, 2006; Sun *et al.*, 2008; Gratton *et al.*, 2011) as well as zebrafish *Danio rerio* (Hamilton 1822) (Schuck *et al.*, 2011). Using microarray technology and quantitative (q) reverse transcription (RT) polymerase chain reaction (qRT-PCR), Schuck *et al.* (2011) demonstrated differential regulation of transcripts encoding proteins known to be important in biological processes such as proliferation and differentiation in the *D. rerio* inner ear following acoustic over-exposure to a pure tone. A global gene expression approach has not been used to investigate the potential effect of seismic-related sound exposure on molecular changes in the inner ear of fishes.

Atlantic salmon *Salmo salar* L. 1758 is a good model for functional genomics research on the potential sub-lethal effects of seismic stimuli on fishes, since microarrays and other genomic resources are available for this species (Rise *et al.*, 2007). It is also of interest that a considerable amount of seismic activity is being carried out in offshore Newfoundland and Labrador, in migratory and feeding areas for *S. salar* moving into rivers in eastern North America (Reddin & Shearer, 1987). In the current study, salmonid cDNA microarrays and qRT-PCR were used to investigate the effects of seismic sounds on the inner ear of *S. salar*. The microarray platform used in this study was the 16 K version 2.1 cDNA microarray developed by the consortium for Genomic Research on All Salmonids Project (cGRASP) (von Schalburg *et al.*, 2005). The 16 K cGRASP microarray has been used to study salmonid responses to stressors such as pathogens (Morrison *et al.*, 2006; Workenhe *et al.*, 2009), environmental contaminants (Finne *et al.*, 2007) and heat stress (Lewis *et al.*, 2010). The objective of this study was to employ the 16 K cGRASP microarray to (1) explore for genes in the inner ear of *S. salar* responsive to loud sounds from an airgun, (2) establish a basis for eventual selection of important genes that could be used to define exposure–response relationships for assessing potential damage to fish ears and (3) establish a basis for eventual development of sensitive biomarker responses for use during seismic surveys.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Juvenile *S. salar* smolts were obtained from an aquaculture facility and held in a 730 l insulated polyethylene aquarium (length 1·09 m x width 0·97 m x height 0·69 m) supplied with air and continuous flow-through of non-filtered sea water, with a flow rate of *c.* 4 l min^{-1} at ambient temperature, at the Northwest Atlantic Fisheries Centre, St John's, NL, Canada. Due to on-going renovations at the time of the study, fish were held under a constant daylight regime. Fish were fed commercial 2 mm pellets (EWOS; www.ewos.com) three times per week and were *c.* 1 year old at the time of exposure.

EXPOSURES

Thirty-three fish (16 for control and 17 for exposed groups) were transferred from the 730 l holding aquarium and divided into two 1 m^3 cages with 7 mm × 7 mm Vexar mesh placed inside a 15 000 l fibreglass aquarium (length 4·73 m × width 2·60 m × height 1·22 m), supplied with air and continuous flow-through of non-filtered sea water, with a flow rate of *c.* 24 l min^{-1} at ambient temperature (0·2° C). The mean ± S.E. fork length (L_F) for both control and exposed groups was 26 ± 1 cm and the mean ± S.E. mass was 150 ± 8 g and 146 ± 8 g for the control and exposed groups. Fish were acclimated for 2 weeks to their new surroundings and then fasted for 2 days prior to exposure. Air supply, seawater flow, temperature and dissolved oxygen (100% saturation) remained the same during the acclimation period (prior to exposure) and exposure period. Sixteen control fish from one of the 1 m^3 cages were sampled prior to seismic activity. Immediately following sampling of control fish, 17 fish in the remaining 1 m^3 cage were placed 2 m from a sleeve airgun with a 0·164 l (10 cubic inches) chamber volume (Texas Instruments; www.ti.com). Fish were subjected to 50 exposures, one exposure every 10 s, at an average sound pressure level (SPL) of *c.* 204 dB peak-to-peak relative to 1 μPa, considered to be an approximate worst-case scenario within a few hundred metres of a survey vessel according to extrapolation from general models on sound propagation (Richardson *et al.*, 1995). The received levels were measured with a calibrated Reson Model TC 4014 hydrophone (www.teledyne-reson.com) (sensitivity −186·4 ± 3 dB *re* 1 V μPa^{-1}) with a built-in 26 dB preamplifier, which was placed directly in front of the cage. A 1 s hydrophone output amplitude time series, which is representative of the airgun blast portion of the (near) periodic signal having a period of 10 s, along with the associated energy density spectrum (EDS), is shown in Fig. 1. The average EDS was 140·4 dB *re* 1 μPa^2 Hz^{-1}. The average particle velocity level (L_v) of 136·0 dB *re* 1 nm s^{-1} was calculated assuming planar wave propagation using the pressure gradient between two calibrated Reson Model TC 4014 hydrophones placed directly in front of the cage and 0·5 m apart. $L_v = 20 \log_{10} (v_1\ v_0^{-1})$ dB *re* 1 nm s^{-1}; $v_1 = p\ (\rho_0\ c)^{-1}$, where p is the pressure difference between two hydrophones measured as a function of peak sound pressure level (*i.e.* SPL peak), ρ_0 is the density of sea water (1035 kg m^{-3}) and c is the speed of sound in water (1522 m s^{-1}); v_0 = the standard reference particle velocity (1 nm s^{-1}). Hydrophone signals were fed through underwater cables to 50 kHz first-order Butterworth low-pass anti-aliasing filters that were connected to a 16-bit, 100 kHz simultaneous sampling, analog to digital, four-channel converter (National Instruments USB9215A; www.ni.com) and digitized using National Instruments NI-DAQmx 8.3 and VI logger software. A Panasonic Toughbook 30 MIL-STD-810 F laptop (www.panasonic.com) was used for all the waveform generation, data collection and storage.

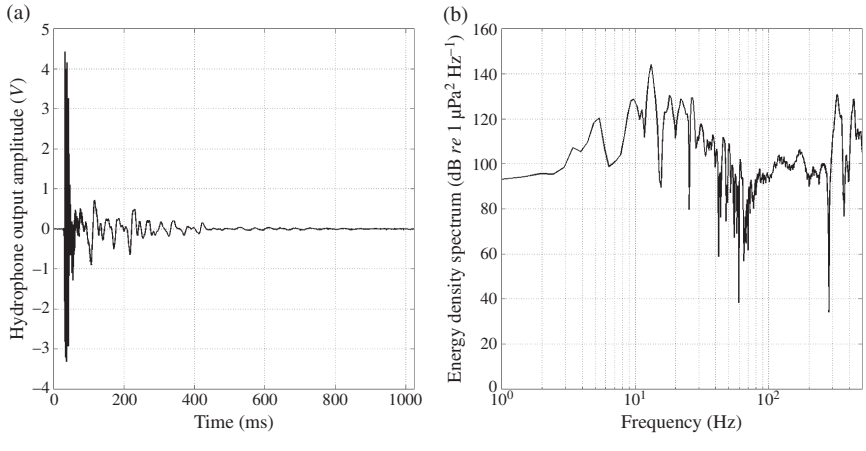

FIG. 1. (a) Representative time series of amplitude of a single airgun blast. In total, there were 50 blasts with *c.* 10 s between blasts. (b) Energy density spectrum representative of a single airgun blast of the signal shown in (a).

Seventeen exposed fish were sampled 16 h following exposure. In previous studies of the effect of various stressors (*e.g.* immune stimulation and heat shock) on fish gene expression, significant up-regulation of stressor-responsive biomarker genes was observed at time points between 3 and 24 h post-stimulation (Rise *et al.*, 2008; Feng *et al.*, 2009; Hori *et al.*, 2010, 2012). Therefore, 16 h later, seismic exposure was thought to be an appropriate sampling time point for the detection of loud noise-responsive ear transcripts in the current study.

BEHAVIOURAL OBSERVATIONS

Observations were made by five individuals (present at the time) on reaction of animals to airgun exposures. Observations were made on changes in swimming direction and speed, as well as time of reaction with respect to airgun blasts (*i.e.* immediate or delayed response). Observations were also made on net-avoidance of animals by two individuals during capture prior to sampling. It is important to note that these were visual observations only.

SAMPLING

Fish were collected by dip-net and euthanized by severing the spinal cord. The right and left inner ear of each individual fish was removed, placed immediately in RNase-free 2 ml tubes and flash frozen in liquid nitrogen. Tissues were held at −80° C until isolation of RNA. Airgun exposures and sampling of fish were carried out in accordance with an Animal Care Protocol (NAFC 2007–02) issued by the Northwest Atlantic Fisheries Centre's Animal Care Committee.

RNA ISOLATION

Total RNA was extracted from individual frozen ears using TRIzol Reagent (Invitrogen; www.lifetechnologies.com) following the manufacturer's instructions with modifications. Samples (*c.* 38 mg) were disrupted using disposable RNase-free pellet pestles in 1·5 ml microtubes and homogenized using QIAshredders (Qiagen Inc.; www.qiagen.com) according to the manufacturer's instructions. Individual total RNA samples (5–17 μg, based on NanoDrop UV spectrophotometry) were treated with RNase-free DNase Set (Qiagen) at room temperature for 10 min to remove any residual genomic DNA and then column purified using the RNeasy MinElute cleanup kit (Qiagen) following the manufacturer's instructions. Each cleaned RNA sample was eluted from an RNeasy MinElute column with 12 μl nuclease-free water (Invitrogen). RNA quality and quantity were assessed by 1·5% agarose gel electrophoresis and NanoDrop UV spectrophotometry. All samples used in the preparation of Poly(A) + RNA pools were from high-quality total RNA (OD 260 nm and 280 nm > 1·8; sharp 18S and 28S ribosomal RNA bands).

POLY(A)$^+$ RNA PREPARATION

DNase-treated, column-purified total RNA pools (48·0 μg) for control and exposed groups were prepared from the right and left ears (four pools in total) of 12 control and 12 exposed individuals, each providing 4·0 μg of column-purified total RNA. Twelve of the 16 control samples and 12 of the 17 exposed samples were selected for the pools based on those individuals providing a sufficient quantity of purified total RNA. Poly(A)$^+$ RNA [messenger (m)RNA] was isolated from the total RNA pools using MicroPoly (A) purist small-scale mRNA purification kit (Ambion Inc.; www.lifetechnologies.com) following the manufacturer's instructions. Purified mRNA pools were assessed for quality and quantity by 1·5% agarose gel electrophoresis and NanoDrop UV spectrophotometry. Poly(A)$^+$ RNA yield ranged from 1·7 to 1·9% of total RNA.

MICROARRAY HYBRIDIZATION

The microarray data from this study have been deposited in NCBI's Gene Expression Omnibus (GEO) database and are accessible through GEO series accession number GSE33257 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE33257).

Salmonid **complementary (c)**DNA microarrays (cGRASP16K salmonid cDNA arrays; batch number GG003, slides 011–014) were purchased from B.F. Koop (cGRASP, University of Victoria, BC, Canada; http://web.uvic.ca/grasp/microarray/array.html/). Microarray fabrication and quality control have been previously described (von Schalburg *et al.*, 2005). To assist in gridding, 16 K cGRASP microarrays contain five 280 bp green fluorescent protein (GFP) cDNA spots printed in each of the 48 sub-grids. A GFP spike was used in this study due to uncertainty of the number of transcripts from inner ear tissue that would hybridize to the arrays. A GFP100 expression clone was provided by B. Koop (cGRASP, University of Victoria, BC, Canada) and a 280 bp GFP product was prepared and purified from this clone (Hall *et al.*, 2011). Fifty nanograms of purified GFP product was labelled with 0·25 mM Cy5-labelled deoxycytidine phosphate (dCTP; Perkin Elmer; www.perkinelmer.com) using the random primed DNA labelling kit following the manufacturer's instructions (Roche Diagnostics Canada; www.rochecanada.com). The Cy5-labelled GFP product was column purified using a QIAquick PCR purification kit (Qiagen) following the manufacturer's instructions and was later used in the cDNA hybridization step.

Microarray experimental design involved comparison of *S. salar* right inner ear pooled mRNA templates from seismic exposed and control fish. Microarray experimental designs involving pooled RNA samples and technical replicates with dye-swaps (similar to the design used in the current study) have previously been shown to be effective for the identification of candidate molecular biomarkers of fish cell or tissue responses to stressors including pathogens and decreased temperature (Rise *et al.*, 2004; Workenhe *et al.*, 2009; Hall *et al.*, 2011). Preparation of mRNA pools and assessment of quality and quantity of mRNA pools is described above. As the microarray experiment used pooled mRNA samples, it did not provide information on biological variability of expression of informative genes. Individual fish total RNA samples contributing to the pools were archived at −80° C and used as templates in the qRT-PCR validation of a selection of microarray-identified genes. The qRT-PCR data revealed biological variability of expression levels for transcripts validated in this manner. *Salmo salar* left inner ears were used in a complementary study for development of reciprocal suppression subtractive hybridization (SSH) cDNA libraries.

Stock solutions of 10% sodium dodecyl sulphate (SDS; Ambion) and 20× sodium citrate sodium chloride (SSC; Ambion) used in microarray slide preparation and post-hybridization washes were diluted to working concentrations using UltraPure DNase–RNase-free distilled water (Invitrogen). Microarrays were prepared for hybridization by washing at room temperature 2 × 5 min in 0·1% SDS, 2 min × 5 min in UltraPure DNase–RNase-free distilled water (Invitrogen) and 1 × 3 min in UltraPure DNase–RNase-free distilled water (Invitrogen), with gentle agitation in 50 ml sterile conical tubes. This was followed by a 3 min dip in 95° C UltraPure DNase–RNase-free distilled water (Invitrogen) and drying by centrifugation (800 *g*, 5 min, in loosely capped 50 ml sterile conical tubes) at room temperature. Arrays were placed in a slide box and stored at 48° C in a hybridization oven until ready for use.

Microarray hybridizations were performed using the 3DNA Array 900 detection kit and instructions (Genisphere Inc.; www.genisphere.com). Briefly, 50 ng mRNA from the right inner ear of exposed and control fish pools ($n = 12$ individuals per pool; four reactions for each treatment) were reverse transcribed using 100 units Superscript II (Invitrogen) and oligo-d(T) primers with unique 5′ sequence overhangs for the Cy3 and Cy5 labelling reactions at 42° C for 2·5 h. RT primer with 3DNA capture sequence specific for Cy3 capture reagent was added to two of the exposed ear mRNA pools and two of the control ear mRNA pools. RT primer with 3DNA capture sequence specific for Cy5 capture reagent was added to the remaining two mRNA samples from exposed and control ears. Two cDNA targets were prepared for hybridization by mixing the Cy3-labelled exposed ear cDNA with the Cy5-labelled control ear cDNA (technical replicates). Two dye-swaps were prepared by mixing the Cy5-labelled seismic exposed ear cDNA with the Cy3-labelled control ear cDNA. The cDNA targets were mixed with 2 µl of LNA dT blocker, 3 µl of GFP spike and 35 µl of 2× formamide-based hybridization buffer (25% formamide, 4× SSC, 0·5% SDS, 2× Denhardt's solution) (Genisphere) and hybridized to the pre-warmed arrays by application of 22 mm × 60 mm HybriSlip coverslips (Grace Biolabs; www.gracebio.com), in microarray hybridization chambers (Corning; www.corning.com) in a 50° C water bath for 16 h in the dark.

Cover slips were floated off of the arrays in 49° C, 2× SSC, 0·2% SDS. The arrays were then washed once in 49° C, 2x SSC, 0·2% SDS for 15 min, once in room temperature 2 x SSC for

15 min and finally once in room temperature 0·2 x SSC for 15 min before drying by centrifugation (800 *g* for 5 min) at room temperature. The Cy3 and Cy5 fluorescent molecules (3DNA Capture Reagent, Genisphere) were subsequently hybridized to the bound cDNA on the arrays in 2x formamide-based buffer (Genisphere) in a 50° C water bath for 4 h in the dark. Cover slips were then floated off, and arrays were washed and dried as described previously.

DATA EXTRACTION AND ANALYSES

Fluorescent images of hybridized arrays were acquired immediately using a ScanArray G_X PLUS Microarray Scanner (Perkin Elmer) at 10 µm resolution using ScanArray Express software (Perkin Elmer). The Cy3 and Cy5 cyanine fluors were excited at 543 and 633 nm, respectively, using 90% laser power and photomultiplier tube (PMT) settings of PMT 75 for Cy5 and PMT 78 for Cy3. Fluorescent intensity data (*i.e.* numerical data) were extracted from TIFF image data using ImaGene 5.5 software (BioDiscovery; www.biodiscovery.com).

The extracted data were imported into GeneSpring GX version 7.3.1 (Silicon Genetics, Agilent Technologies; www.agilient.com) for data transformation (background correction and setting background-corrected values <0·01–0·01), normalization (Lowess) and analysis (formation and comparison of fold change gene lists through generation of scatterplots and Venn diagrams). Gene lists were prepared from transcripts with > 1·75-fold difference in expression in background-corrected, Lowess-normalized (BCLN) signal between the two treatments for each microarray and Venn diagrams were used to identify reproducibly informative transcripts on at least three of the four microarrays in the study (including at least one dye-swap). Gene lists (containing information about BCLN signal intensities in each channel, quality flags and Cy5:Cy3) were compiled in Excel (www.microsoft.com) for each slide. Fluorescence signal thresholds were set for each slide by calculating the mean of the median local background values plus 3 S.D. for all spots in the dominant channel using raw data generated from ImaGene. In the up-regulated in exposed compared to control gene list, exposed was considered to be the dominant channel (*i.e.* the channel with the higher transcript expression); in the down-regulated in exposed compared to control gene list, control was considered to be the dominant channel. Genes differentially regulated > 1·75-fold in at least three slides of the study (including at least one dye-swap) with a BCLN signal above threshold on all four slides were selected as high-trust data.

Expressed sequence tags (ESTs) obtained from GenBank accession numbers of reproducibly informative transcripts were submitted to the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) server for annotation using BLASTx (against the non-redundant protein sequence database, nr) and BLASTn (against the non-redundant nucleotide sequence database nt). ESTs having significant (E-value $< 10^{-5}$) named BLASTx or BLASTn hits (*e.g.* best BLAST hit with an associated protein or gene name, excluding hypothetical or predicted names) against the GenBank nr or nt databases, as well as unknowns (*i.e.* no significant BLAST hit), are described for reproducibly informative transcripts. BLAST statistics were compiled in May 2010 and reflect the state of the GenBank sequence databases at that time. Complete gene ontology (GO) functional annotations including GO terms and GO descriptions (for biological process, molecular function and cellular component categories) of the most significant named BLAST hit or related sequences (*e.g.* putative human orthologues), were obtained from Protein Knowledgebase UniProt/Swiss-Prot (www.uniprot.org) in May 2010.

QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION

Validation of selected transcripts from the microarray results was conducted using qRT-PCR. Eight transcripts of interest (TOI) > 1·75-fold differentially expressed in exposed and control ears (encoding growth hormone I, nicotinamide riboside kinase 2, C-type lectin receptor A, retinol binding protein I cellular, caspase-8 precursor, haemoglobin subunit alpha-4, C14orf159 protein and proteasome subunit beta type-4 precursor) were subjected to qRT-PCR using two PCR primers (forward and reverse) per TOI, SYBR Green I dye chemistry and a 7500 fast real-time PCR system (Applied Biosystems; www.appliedbiosystems.com). The primer pair chosen for each TOI was determined to have a single peak in the dissociation curve and no

primer dimer product in the no-template control. Amplicons were electrophoresed on 1·5% agarose gels and compared to a 1 kb plus ladder (Invitrogen) to ensure correct fragment size. Standard curves were generated using a five-point 1:4 dilution series starting with cDNA corresponding to 10 ng of input total RNA prepared from pools for both seismic and control groups, except for the transcript encoding C14orf159 protein, where the efficiency was calculated using a four-point 1:4 dilution series starting with cDNA representing 10 ng of input RNA. Amplification efficiency (Pfaffl, 2001) was calculated for both exposed and control groups, with between 85 and 109% efficiency for all primer pairs for relative quantification by the 7500 fast real-time PCR system (Applied Biosystems).

All individual RNA samples contributing to pools for exposed and control ear were quantified by qRT-PCR. The transcript encoding 60S ribosomal L6 RNA was selected as a normalizer gene, as the mean fold change (MFC) was within 1·2-fold on all four microarrays and its expression was stable for all individuals tested by qRT-PCR [the average threshold cycle (Ct) value (24·9) for the normalizer gene was the same for both the exposed and control groups with an overall range in Ct values of 24·2–25·8]. Total RNA was prepared as previously described using TRIzol reagent and methods (Invitrogen). For each individual, first-strand cDNA was synthesized in a 20 µl reaction from 1 µg of DNase treated, column-cleaned, total RNA, 250 ng random hexamer primers (Invitrogen), using 200 units of Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Invitrogen), 10 mM dithiothreitol (DTT; Invitrogen), in the manufacturer's first strand buffer (1× final concentration, Invitrogen) following the manufacturer's instructions. The RT was carried out at 25° C for 10 min, 37° C for 50 min and then 70° C for 15 min. PCR amplification for each individual was performed in duplicate with the Applied Biosystems 7500 fast real-time PCR system in a 13 µl volume reaction using 2 µl cDNA (corresponding to 10 ng of input total RNA), 50 nM each of forward and reverse primer and 1× Fast SYBR green master mix (Applied Biosystems) and expression levels were normalised to the transcript encoding 60S ribosomal L6 RNA. The real-time analysis programme consisted of one cycle of 95° C for 20 s, followed by 40 cycles of 95° C for 3 s, 60° C for 30 s, with fluorescence detection at the end of each 60° C step.

Ct values were determined using the 7500 software 2.0 relative quantification study application (Applied Biosystems). The relative quantity (RQ) of each TOI was determined using the comparative Ct method for relative quantification (Livak & Schmittgen, 2001), using the individual sample with the lowest expression (highest normalised Ct value) as the calibrator. The data from each TOI were analysed as a multi-plate study. cDNA from a reference RNA sample was included (for the normaliser) in duplicate on each plate to assess inter-plate variability. Variation in the Ct values for the linker sample between plates was $< 0{\cdot}5$ cycles. The RQ of each TOI was calculated using the mean amplification efficiencies reported for exposed and control groups. Overall MFC was calculated as average RQ exposed group per average RQ control group for genes up-regulated by seismic sound, and as average RQ control group per average RQ exposed group for genes down-regulated by seismic sound.

Sigma-Stat 3.10 (Systat Software Inc.; www.sigmaplot.com) was used to determine significant difference in gene expression (with a P-value threshold of 0·05) in the ear from exposed compared to control fish. TOIs, qRT-PCR primer sequences and amplification efficiencies are shown in Supporting Information Table S1.

RESULTS

There was no statistically significant difference ($P > 0{\cdot}05$) in masses and L_F between control and exposed groups (t-test). Visual observations made during exposure of animals revealed an initial startle response for approximately the first three airgun discharges, followed by little activity for the remainder of the exposure (*c*. 10 min; 50 airgun discharges). Also, a difference in net-avoidance and swimming speed between control and seismic-exposed groups was observed during sampling. Fish from the control group demonstrated little swimming activity and were therefore very easy to capture, whereas fish from the seismic exposed group demonstrated rapid and erratic

swimming activity (compared to controls) during attempted capture. While these were visual observations only, without supporting video files or quantitative measures, the observations are important and therefore worth noting.

In the microarray study, 42 different transcripts were reproducibly up-regulated and 37 different transcripts were reproducibly down-regulated >1·75-fold in the inner ear of *S. salar* following exposure to noise from the airgun (Table I). GenBank accession number, best named BLASTx or BLASTn hit (most negative *E*-value $< 10^{-5}$), the degree of similarity (length and per cent identity over aligned region) between the translation of each salmonid cDNA's EST and its best named BLASTx or BLASTn hit, MFC and complete GO information are compiled in Tables S2 and S3 (Supporting Information). The presence of multiple entries of genes in a given informative transcript list (*e.g.* the transcript encoding haemoglobin subunit beta in Table I) provides an internal validation of microarray results.

ENERGY AND METABOLISM

Many of the microarray-identified inner ear transcripts responsive to noise exposure are involved in iron homeostasis and energy metabolism. Reproducibly informative transcripts having functions related to iron homeostasis that were up-regulated in response to seismic noise exposure included those encoding haemoglobin subunit alpha, haemoglobin subunit alpha-4, haemoglobin subunit beta, haemoglobin subunit beta-1 and ferritin, middle subunit (Table I). There were no transcripts with these functions reproducibly down-regulated in response to noise exposure. qRT-PCR confirmed induction of the transcript encoding haemoglobin subunit alpha-4 in exposed compared to control fish (Table II).

Several reproducibly informative transcripts important in energy metabolism were responsive to seismic sound exposure. Transcripts directly involved in the Krebs cycle that were up-regulated include those encoding succinyl-CoA ligase subunit alpha, mitochondrial precursor, nicotinamide adenine dinucleotide phosphate (NADP)-dependent malic enzyme and malate dehydrogenase cytoplasmic (Table I). The transcript encoding succinyl-CoA ligase beta-chain mitochondrial precursor was down-regulated by seismic sound (Table I). Transcripts important in the electron transport chain were also differentially regulated in seismic noise exposed ear. Transcripts encoding cytochrome c oxidase VIc 2 and nicotinamide adenine dinucleotide (NADH) dehydrogenase 1 beta subcomplex subunit 1 were up-regulated by noise exposure, whereas transcripts encoding cytochrome c oxidase subunit 5B mitochondrial precursor and cytochrome c oxidase polypeptide VIIb mitochondrial precursor were down-regulated (Table I). The transcript encoding nicotinamide riboside kinase 2, also involved in energy metabolism, was up-regulated in the microarray study (Table I) with validation by qRT-PCR (Table II).

PROTEIN POST-TRANSLATIONAL MODIFICATION OR DEGRADATION

The ear transcript with the highest MFC response to noise exposure encodes ubiquitin family domain-containing protein 1 (Table S2). While there were no GO terms assigned to this protein, it is reported to be important in the regulation of protein metabolism and function in humans (Fenner *et al.*, 2009). Other reproducibly informative transcripts

TABLE I. Proteins encoded by transcripts greater than 1·75-fold differentially regulated in seismic-exposed *Salmo salar* ear relative to control *S. salar* ear on at least three slides of study*

Up-regulated transcripts	Down-regulated transcripts
DNA/RNA binding, transcription, translation	
Eukaryotic translation initiation factor 5A-1	Splicing factor, arginine/serine-rich 2
Heterogeneous nuclear ribonucleoprotein A0	TAR DNA-binding protein 43
	39S ribosomal protein L36
	Transposase
	5′-nucleotidase, cytosolic 3
	Activated RNA polymerase II transcriptional coactivator p15
Energy, metabolism	
Succinyl-CoA ligase subunit alpha, mitochondrial precursor	Cytochrome c oxidase subunit 5B, mitochondrial precursor
Nicotinamide riboside kinase 2_2	L-xylulose reductase
Cytochrome c oxidase polypeptide VIc-2	Carbonyl reductase 1
NADP-dependent malic enzyme	Succinyl-CoA ligase beta-chain mitochondrial precursor
Malate dehydrogenase, cytoplasmic	Cytochrome c oxidase polypeptide VIIb, mitochondrial precursor
Apolipoprotein A-IV precursor	
Adrenodoxin, mitochondrial	
NADH dehydrogenase 1 beta subcomplex subunit 1	
Signalling, synaptic transmission	
Growth hormone I	Signal recognition particle 19 kDa protein
ADP-ribosylation factor 1	Calmodulin 2
Retinol-binding protein I, cellular	Purpurin precursor
Transthyretin	Retinoid x receptor beta a
Collagen alpha-2I chain precursor	
ADAM metallopeptidase domain 10	
Cytoskeleton structure, dynamics	
Coronin-1A	Ezrin–radixin–moesin–binding phosphoprotein 50
Vasodilator-stimulated phosphoprotein	Collagen alpha 2 type IV chain
	Motile sperm domain-containing protein 2
Cell cycle, cell death, axonogenesis	
Histone H1x	B-cell receptor-associated protein 31
Caspase-8 precursor	BCCIP homologue
Deoxycytidylate deaminase	Protein SET
Catenin beta-1	S100-B
	Nuclear migration protein nudC
Protein post-translational modification, protein degradation, protein folding	
Ubiquitin family domain-containing protein 1	Proteasome subunit beta type-1-A
Ubiquitin carboxyl-terminal hydrolase isozyme L1	Proteasome subunit beta type-4 precursor

TABLE I. Continued

Up-regulated transcripts	Down-regulated transcripts
Midasin homolog	Serpin H1 precursor
40 kDa peptidyl-prolyl cis-trans isomerase	S-phase kinase-associated protein 1A
	Low molecular weight phosphotyrosine protein phosphatase
	Proteasome subunit beta type-5 precursor
Immune responsive	
Ig kappa chain V-IV region B17 precursor	4 F2 cell-surface antigen heavy chain
C type lectin receptor A	H-2 class II histocompatibility antigen gamma chain
IGL1E2 gene for immunoglobulin light chain variable region	
Iron homeostasis	
Haemoglobin subunit beta-1	
Haemoglobin subunit $beta_3$	
Haemoglobin subunit $alpha\text{-}4_2$	
Haemoglobin subunit $alpha_2$	
Other	
UPF0527 membrane protein	C17orf37 homolog
Coiled-coil domain containing protein 109A	C14orf159 protein
UPF0466 protein C22orf32 homolog, mitochondrial	
SH2/SH3 adaptor Crk	
UbiA prenyltransferase domain containing 1	
Unknown	
$Unknown_3$	$Unknown_4$

*Transcript names are from the most significant (lowest *E*-value) named BLASTx or BLASTn hit and reflect the state of the GenBank non-redundant (nr) amino acid and nucleotide databases in May 2010. Subscripts indicate the number of different microarray features with identical best named BLASTx or BLASTn hits appearing in this list or the number of different unknown features (*i.e.* with no significant BLAST hits). The complete gene list, including EST accession numbers, BLAST statistics (gene or protein name with associated GenBank accession number, species affiliation, per cent identity over the aligned region and associated *E*-value), mean fold change data for all four slides in the study and functional annotation information [*e.g.* gene ontology (GO) terms], is available in online Tables S2 and S3.

found to be differentially regulated in response to seismic noise that are important in protein post-translational modification, protein degradation or protein folding are listed in Table I. Quantitative RT-PCR did not validate down-regulation of the transcript encoding proteasome beta type-4 precursor (Table II); however, the down-regulation of three transcripts encoding for proteasome beta subunits revealed in the microarray experiment (Table I) provides confirmation that proteasome beta subunit-like transcript suppression is involved in the *S. salar* ear response to seismic exposure.

CELL CYCLE AND CELL DEATH

Differentially regulated transcripts with gene ontologies related to cell death (apoptosis) encode proteins including up-regulation of caspase-8 precursor and catenin beta-1 as well as down-regulation of B-cell receptor-associated protein 31 (Table I).

TABLE II. Summary of microarray and quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analyses of microarray-identified transcripts

Expressed sequence tag	Name of best BLAST hit	Microarray*		qRT-PCR†	
		MFC	±S.E.	MFC	*P*
CA045755	Growth hormone I	+3·4	±0·8	+7·78	0·24
CB492469	Nicotinamide riboside kinase 2‡	+2·9	±0·3	+1·89	0·02
CA056108	C-type lectin receptor A	+2·4	±0·3	+2·20	0·14
CA058654	Retinol-binding protein I, cellular	+2·3	±0·2	+1·24	0·89
CA060239	Caspase-8 precursor	+2·1	±0·2	+1·01	0·67
CB515375	Haemoglobin subunit alpha-4‡	+2·0	±0·1	+3·76	0·01
CA057346	C14orf159 protein	−2·2	±0·3	−1·35	0·04
CB512698	Proteasome subunit beta type-4 precursor	−2·3	±0·2	−1·07	0·34

*Mean ± S.E. of exposed: control (+) and control: exposed (−) mean fold changes (MFC) for microarray data. In the microarray experiment, $n = 4$ replicates with two technical replicates and two dye swaps using pools of 12 individuals for exposed *Salmo salar* ear and 12 individuals for control *S. salar* ear with equal amount of mRNA per individual contributing to the pools. As microarray analyses were performed comparing the same sample pools, S.E. for microarray data reflects technical rather than biological variability.
†Mean RQ ratios (MFC) and *P*-values of exposed: control (+) and control: exposed (−) for qRT-PCR data. qRT-PCR analyses were performed on $n = 12$ biological replicates from exposed and control groups, with two technical replicate reactions for each biological replicate. qRT-PCR data conveys information on biological variability as individuals (*i.e.* biological replicates) were incorporated into the qRT-PCR experimental design.
‡For multiple same-named transcripts from different microarray features, one representative microarray feature was selected for qRT-PCR and inclusion on this list. The complete list for all reproducibly informative features is available in Supporting Information Tables S2 and S3.

qRT-PCR, however, did not reveal any significant difference in expression of the transcript encoding caspase-8 precursor (Table II). Associated with neuronal cell death and regeneration is S100-B and nuclear migration protein, shown in the microarray experiment to be down-regulated in response to seismic exposure (Table I). Other microarray-identified transcripts important in cellular turnover as well as regulation of transcription/translation and nucleic acid binding are listed in Table I.

IMMUNITY

Only a few reproducibly informative transcripts identified in the microarray study have functional annotations suggesting important roles in immune responses. The microarray-identified transcript encoding immune-relevant C-type lectin receptor A was up-regulated in response to seismic noise exposure (Table I). qRT-PCR validation of the transcript encoding C-type lectin receptor A revealed an overall induction in response to seismic exposure (Table II); however, due to the high variability in the seismic exposed group, the qRT-PCR showed no significant difference in the expression of the transcript encoding C-type lectin receptor A between control and seismic groups ($P > 0{\cdot}05$).

SIGNALLING, SYNAPTIC TRANSMISSION

Several differentially regulated transcripts identified in this study have important roles related to signalling. For example, signalling-relevant transcripts identified

by the microarray experiment as being up-regulated by seismic noise include those encoding growth hormone I, retinol-binding protein I and transthyretin (retinol transport) (Table I). Microarray-identified transcripts down-regulated by seismic noise and involved in signalling encode proteins including calmodulin 2, purpurin precursor (retinoid binding) and retinoid x receptor (Table I). qRT-PCR validation of the transcript encoding growth hormone I confirmed overall up-regulation in response to seismic noise exposure (Table II); however, there was high biological variability in expression of the transcript encoding growth hormone I (as seen with C-type lectin receptor A) in the seismic-exposed group, resulting in there being no significant difference between control and seismic groups ($P > 0{\cdot}05$). qRT-PCR validation of the transcript encoding retinol binding protein I revealed a slight overall induction in seismic-exposed ears compared to control ears ($P > 0{\cdot}05$) (Table II).

STRUCTURAL GENES

A transcript encoding a hypothetical protein similar to motile sperm domain-containing protein 2, important in cell motility, was down-regulated in response to seismic noise exposure and represented the most abundant (*i.e.* highest mean fluorescent signal; data available through NCBI's GEO database, GEO series accession number GSE33257) down-regulated transcript in the microarray study (Table I). Other microarray-identified transcripts that were differentially regulated with important roles in cytoskeletal structure and dynamics encode proteins including coronin 1A, vasodilator-stimulated phosphoprotein, ezrin–radixin–moesin-binding phosphoprotein 50 (all actin binding) and collagen alpha 2 type IV chain (extracellular matrix structural constituent) (Table I).

OTHER AND UNKNOWN

The seismic noise-responsive transcript encoding C14orf159 protein has no well-defined function in energy metabolism; however, it is known to be important in the mitochondrion (GO Cellular Component term 'mitochondrion'; Supporting Information Table S3). Microarray results show down-regulation of the transcript encoding C14orf159 protein [Table I, which was validated by qRT-PCR (Table II)]. Other microarray-identified transcripts with no well-known function or not having any significant BLAST hit (unknown) are listed in Table I.

DISCUSSION

The visual observations made with respect to swimming behaviour upon exposure were similar to responses noted in other studies with fishes exposed to noise (McCauley *et al.*, 2000; Smith *et al.*, 2004; Buscaino *et al.*, 2010). An increase in swimming activity indicates an increase in overall metabolic rate, which implies a higher demand for oxygen. A prolonged increase in metabolic activity associated with faster swimming speeds could affect other biological activities, such as feeding, migration and reproduction (Buscaino *et al.*, 2010) as well as immune responses (Barton, 2002).

DNA microarrays have been used to identify genes responsible for deafness in human cochlear and vestibular tissues (Abe *et al.*, 2003) as well as genes responsive to intense

noise exposure in *Rattus norvegicus* cochlea (Lomax *et al.*, 2001; Cho *et al.*, 2004; Kirkegaard *et al.*, 2006), *Chinchilla chinchilla* cochlea (Taggart *et al.*, 2001) and more recently in the inner ear of *D. rerio* following exposure for 36 h to a 100 Hz tone (Schuck *et al.*, 2011). The purpose of this study was to investigate the potential for change in gene expression in the inner ear of *S. salar* upon exposure to intense noise generated from a seismic airgun. The microarray study revealed 42 different transcripts reproducibly up-regulated and 37 different transcripts reproducibly down-regulated in response to noise. Gene lists provided insights into the effects of noise-induced stress on *S. salar* inner ear and revealed alteration in the expression of transcripts with functional annotations important in metabolism, iron homeostasis, stress response [including cell cycle and cell death (apoptosis), post-translational protein modification and immune response], signalling and cytoskeletal structure (Table I). The remainder of the discussion is focused on these biological processes.

ENERGY AND METABOLISM

Differential expressions of several transcripts encoding proteins important in cellular respiration, including those with roles in oxygen transport, are reported in Table I. The microarray study showed up-regulation of transcripts encoding different subunits of haemoglobin in *S. salar* ear in response to seismic noise exposure: haemoglobin subunit beta-1 (2·6-fold), haemoglobin subunit beta (2·5-fold), haemoglobin subunit alpha-4 (2·3-fold) and haemoglobin subunit alpha (2·1-fold) (Supporting Information Table S2). These were represented by a total of eight informative microarray features; different microarray features with identical best named BLAST hits represent single genes or closely related paralogues. qRT-PCR validated the increase in the transcript encoding haemoglobin alpha-4 showing an overall increase of 3·8-fold ($P < 0{\cdot}01$) in seismic-exposed ear compared to control ear (Table II). Higher levels of haemoglobin in ear following exposure to intense noise may be due to tissue rupture or haemorrhage, which has been observed in other studies with fishes exposed to intense sound (Hastings & Popper, 2005; Casper *et al.*, 2012; Halvorsen *et al.*, 2012), including damage to hair cells (Enger, 1981; Hastings *et al.*, 1996; McCauley *et al.*, 2003; Smith *et al.*, 2006; Schuck & Smith, 2009; André *et al.*, 2011*b*; Smith *et al.*, 2011). Increased haemoglobin may also suggest a higher demand for oxygen in the seismic-exposed fish compared to control fish, due to an increase in metabolic activity (Franklin *et al.*, 1993; Eliason *et al.*, 2013). An increase in blood flow and capillary dilation in the cochlea of albino guinea pig *Cavia porcellus* was reported to be the key mechanism for meeting cellular metabolic demand in response to intense noise exposure (Dai & Shi, 2011).

The Krebs cycle is a central pathway of metabolism and also functions in a biosynthetic capacity, including synthesis of haeme through succinyl-coenzyme A (CoA) (McCammon *et al.*, 2003). Several transcripts representing genes involved in energy metabolism with important roles in the Krebs cycle or electron transport chain were differentially regulated in response to seismic noise exposure in the *S. salar* ear (Table I). Changes in metabolism have been reported in relation to noise-induced stress. In a previous microarray study, time-dependent (measured up to 6 h) noise-induced changes in expression of transcripts encoding proteins important in the Krebs cycle and the electron transport chain were reported following intense noise exposure in the *C. chinchilla* cochlea (Taggart *et al.*, 2001). Cytochrome c oxidase, which is highly expressed in teleost hair cells (Saidel & Crowder, 1997), has been shown using histochemical

techniques to decrease in the cochlea of albino *C. porcellus* in response to acoustic trauma (Hsu *et al.*, 1998). Hsu *et al.* (1998) suggested that a decrease in cytochrome c oxidase activity implies that metabolic damage may play a role in noise-induced hearing loss. The microarray results presented here show altered regulation of transcripts encoding different subunits of cytochrome c oxidase in *S. salar* ear, further confirming the potential for metabolic disturbance in response to intense noise exposure. Of particular interest is the transcript encoding succinyl-CoA ligase beta subunit, which was shown to be down-regulated in response to noise exposure (Table I). A mutation in the gene encoding the beta subunit of succinyl-CoA ligase has been reported to be responsible for a specific mitochondrial brain disease in humans, characterized by severe hearing impairment (Ostergaard *et al.*, 2007).

Metabolic and gene regulatory pathways that control cell life and death require nicotinamide adenine dinucleotide (NAD+). A recently discovered third vitamin precursor of NAD + in eukaryotes, nicotinamide riboside (NR), is converted to NAD + in a novel salvage pathway, the nicotinamide riboside kinase pathway (Bieganowski & Brenner, 2004; Tempel *et al.*, 2007). Nicotinamide riboside kinase 2 was induced >20-fold and other NAD-synthesizing enzymes increased two to eight-fold 14 days after injury in mouse neuronal cell culture (Sasaki *et al.*, 2006). Sasaki *et al.* (2006) suggest that pathways that synthesize NAD + are activated after neuronal injury to enable increased NAD + production for downstream functions associated with the neuronal response to injury. In the current microarray study, two transcripts encoding nicotinamide riboside kinase 2 were shown to be reproducibly up-regulated in response to seismic noise exposure (Table I; Supporting Information Table S2). qRT-PCR confirmed up-regulation of this transcript (1·9-fold; $P < 0{\cdot}05$) (Table II). Up-regulation of the transcript encoding nicotinamide riboside kinase 2 in response to noise exposure points to potential neuronal protection in *S. salar* ear through increased production of NAD+. Nicotinamide riboside kinase 2 may be an important biomarker for seismic noise exposure in salmonids and other fish species.

PROTEIN POST-TRANSLATIONAL MODIFICATION OR DEGRADATION

The ubiquitin–proteasome pathway (UPP) functions in the process of protein turnover by the combined action of ubiquitin-activating enzyme (E1), one of many ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3), a large multicatalytic protease complex called the 26S proteasome, and accessory factors (Wilkinson, 1997; Lecker *et al.*, 2006; Hanna & Finley, 2007; Alberts *et al.*, 2008). Induction of *ube3b*, a gene encoding a member of the E3 ubiquitin ligase family, has been previously reported in injured tissues of chick *Gallus gallus domesticus* cochlea immediately following noise exposure (Lomax *et al.*, 2000). Lomax *et al.* (2001) infer that *ube3b* may play a protective role in either the classic stress response or in the stress response invoked by oxidative damage, as it is homologous to a nematode *Caenorhabditis elegans* oxidative stress-responsive gene. Some E3 ubiquitin ligases are associated with multiprotein complexes including the Skp1-Cullin-F-box (SCF) protein complex. SCF family ubiquitin ligases play an important role in co-ordinating glucose metabolism with proliferation, as well as responding to cellular energy status (Benanti, 2012). Microarray results presented here reveal a two-fold down-regulation of the transcript encoding Skp1A (S-phase kinase-associated protein 1A) (Table I; Supporting Information

Table S3), a highly conserved protein (BLAST shows that orthologues from salmonids and mammals are 100% identical over 149 aligned amino acid residues) (Supporting Information Table S3). Skp1, also called Organ of Corti protein 2 (OCP2), is an abundant inner ear protein in mammalian cochlea (Chen *et al.*, 1995; Thalmann, 2001; Thalmann *et al.*, 2003). Nelson *et al.* (2007) have shown Skp1 to be a potential genetic modifier in age-related hearing loss in mice *Mus musculus* and suggest that Skp1 probably plays a role in auditory function. Additional transcripts encoding proteins important in the UPP that were shown by microarray to be down-regulated in response to intense noise exposure in *S. salar* ear include proteasome subunit beta type-1A (three-fold), proteasome subunit beta type-4 precursor (2·3-fold) and proteasome subunit beta type-5 precursor (1·9-fold) (Table I; Supporting Information Table S3).

The transcript encoding ubiquitin carboxyl terminal hydrolase isozyme L1 (UCH L1) was shown by microarray to be up-regulated in response to noise exposure in *S. salar* (2·3-fold) (Table I; Supporting Information Table S2). Ubiquitin carboxyl terminal hydrolases (UCH) are thought to be essential for deubiquitination activity by releasing ubiquitin from its substrates (Wilkinson, 1997). UCH L1, highly expressed in neuronal cells in mammals (Wilkinson *et al.*, 1989, 1992; Saigoh *et al.*, 1999), has been shown to play a crucial role in synaptic transmission (Chen *et al.*, 2010) and neurodegeneration (Saigoh *et al.*, 1999) in *M. musculus*. Therefore, UCH L1 may be up-regulated in *S. salar* ear to serve as protection against nerve damage due to intense noise exposure.

Ubiquitin family domain-containing protein 1-encoding transcript was the most highly up-regulated transcript (5·0-fold) (Table I; Supporting Information Table S2) in response to noise-induced stress in *S. salar* ear. While there are no GO terms assigned to this protein, it is reported to be involved in protein modification (Fenner *et al.*, 2009). As well, the gene encoding 40 kDa peptidyl-prolyl *cis–trans* isomerase, important in post-translational protein modification, was also up-regulated at the mRNA level in *S. salar* ear in response to seismic noise exposure (1·8-fold) (Table I; Supporting Information Table S2) and has previously been reported to be up-regulated at the mRNA level in a time-dependent manner in response to intense noise exposure in *C. chinchilla* cochlea (Taggart *et al.*, 2001). Down-regulation of transcripts important in the UPP, along with increased expression of a transcript important in deubiquination (UCH) points to dysregulation of regulated protein degradation machinery *via* the UPP in response to noise-induced stress in *S. salar* ear.

CELL CYCLE AND CELL DEATH

Regulated cell death (*i.e.* apoptosis) is important during development and maintenance of tissues. The involvement of apoptosis in noise-induced hearing loss is reviewed in Op de Beeck *et al.* (2011). Apoptotic activity has been reported in noise-damaged *C. porcellus* cochlea (Hu *et al.*, 2000, 2002) as well as noise-damaged *C. chinchilla* cochlea (Nicotera *et al.*, 2003). An increase in apoptotic activity was also observed in goldfish *Carassius auratus* (L. 1758) ear tissues 0–2 days following noise exposure, coinciding with the period of greatest hair cell loss (Smith *et al.*, 2006). Altered expression of genes involved in regulation of apoptosis has also been reported in mammalian ear tissues (Kirkegaard *et al.*, 2006), including *C. porcellus* cochlea (Yamashita *et al.*, 2008), as well as *D. rerio* inner ear (Shuck *et al.*, 2011) following noise exposure. Microarray-identified transcripts encoding proteins involved in apoptosis that are differentially regulated in *S. salar* ear following seismic noise exposure

include caspase-8 precursor (2·1-fold up-regulated) and B-cell receptor-associated protein 31 (2·7-fold down-regulated) (Table I; Supporting Information Tables S2 and S3). qRT-PCR results, however, did not confirm up-regulation of caspase 8 precursor-encoding transcript (Table II). There may be other *caspase-8* paralogues influencing the microarray results; the upcoming availability of an *S. salar* reference genome (Davidson *et al.*, 2010) will facilitate discovery of additional paralogues (if present) and the development of paralogue-specific qRT-PCR assays in the future.

IMMUNITY

Unlike apoptosis, non-programmed cell death (*i.e.* necrosis) is induced by injury and is characterized by cellular membrane rupture, eliciting inflammatory immune responses (Alberts *et al.*, 2008). Changes in genes related to immune function have been reported following acoustic trauma in *R. norvegicus* cochlea (Cho *et al.*, 2004; Kirkegaard *et al.*, 2006) and *D. rerio* inner ear (Shuck *et al.*, 2011). The microarray study showed a 2·4-fold induction of the transcript encoding C-type lectin receptor A in the seismic-exposed ear compared with the control ear (Table I; Table S2), and qRT-PCR confirmed the up-regulation of this transcript (Table II). C-type lectins sense products from dying cells and induce inflammatory signals that alter immune system responses (Cambi & Figdor, 2009). As seen with some other noise-induced transcripts reported in this study (*e.g.* growth hormone I encoding transcript), expression of C-type lectin receptor A-encoding transcript is highly variable among individuals from the seismic-exposed group. This high biological variability of expression suggests that there are differences in the sensitivity of individual *S. salar* ears to stress induced by noise exposure. Other transcripts with immune-relevant functional annotations that reproducibly responded to seismic noise exposure in *S. salar* inner ear are listed in Table I. The up-regulation of two immunoglobulin-related transcripts and down-regulation of the transcript encoding H-2 class II histocompatibility antigen gamma chain (involved in MHC class II antigen processing) (Table I) suggest that seismic-exposed *S. salar* ears have altered immune activity. Microarrays were previously used to show that the transcript encoding immunoglobulin heavy variable 2−2 was up-regulated, and several other immune-relevant genes (including *mhc2a*) responded at the transcript expression level, in *D. rerio* ears after acoustic overexposure (Schuck *et al.*, 2011). The dysregulation of immune-relevant genes in ear following loud sound exposure appears to be conserved between *S. salar* and *D. rerio* and warrants further investigation.

SIGNALLING, SYNAPTIC TRANSMISSION

The neuroendocrine system reacts to physiological challenges (*i.e.* stresses) to restore homeostasis in organs and cells throughout the body (Dorshkind & Horseman, 2001). Multiple studies have confirmed that hormones secreted by the neuroendocrine system (*e.g.* growth hormone and insulin-like growth factor-1) play an important role in the stress response and immune function (Pickering *et al.*, 1991; Kelley *et al.*, 2007). The microarray results here revealed a 3·4-fold increase in growth hormone I-encoding transcript in *S. salar* ear in response to noise exposure (Table I; Supporting Information Table S2) with an increase of 7·8-fold demonstrated by qRT-PCR (Table II). Again, there was high biological variability in the qRT-PCR data for the seismic-exposed

group. This high biological variability in biomarker transcript expression may be a reflection of individual variability in response to stress, and differences in timing of transcript induction between individuals (*i.e.* some animals may have passed peak expression of growth hormone I-encoding transcript or may not have reached peak expression at time of sampling). Shuck *et al.* (2011) demonstrated a 64-fold increase in growth hormone I-encoding transcript in *D. rerio* inner ear 2 days after noise exposure, which coincided with the period of greatest cell proliferation reported in their previous study (Shuck & Smith, 2009). Shuck *et al.* (2011) also showed that growth hormone I-encoding transcript decreased to 5·5-fold higher in noise-exposed *D. rerio* ear compared to controls 4 days after exposure and indicated that growth hormone played an important role in recovery of *D. rerio* inner ear following acoustic trauma. Shuck *et al.* (2011) injected growth hormone in non-sound exposed *D. rerio* and reported an increase in cell proliferation, suggesting that growth hormone is able to stimulate cell proliferation. Furthermore, Sun *et al.* (2011) reported that growth hormone can promote hair cell regeneration in *D. rerio* inner ear following acoustic trauma by stimulating proliferation and suppressing apoptosis. By contrast, a two-fold decrease in growth hormone gene (*gh1*) expression has been detected in *R. norvegicus* cochlea 2·5 h following noise exposure (Cho *et al.*, 2004). It is possible that differences in growth hormone response to intense noise exposure in the *R. norvegicus* cochlea (Cho *et al.*, 2004), compared to *S. salar* and *D. rerio* inner ear growth hormone-encoding transcript expression responses to intense noise exposure, are due to time sampled after exposure, although other factors such as duration, intensity and frequency of sound, as well as differences in the mammalian auditory system *v.* fish auditory systems, may play a role.

Growth hormone induction in *S. salar* ear in response to seismic noise exposure may act to restore homeostasis in the inner ear in response to stress, possibly through stimulation of the immune system (Yada, 2007). Thyroid hormones can also act as immunostimulatory mediators and are important during response to environmental and physiological stress (Dorshkind & Horseman, 2001). Transthyretin is a carrier for both thyroxine (a thyroid hormone), as well as for retinol through binding to the retinol-binding protein, and it has been demonstrated to play an important role in nerve regeneration and sensorimotor function in *M. musculus* (Fleming *et al.*, 2007). Microarray-identified transcripts encoding transthyretin and retinol binding protein I were up-regulated (2·1-fold and 2·3-fold, respectively) (Table I; Supporting Information Table S2) in seismic-exposed *S. salar* ear, while retinoid x receptor beta a-encoding transcript was down-regulated (1.8-fold) (Table II; Supporting Information Table S3). Cellular retinol-binding protein type I and retinoid x receptors are important in retinoic acid biosynthesis, which is reported in the developing sensory epithelium of *R. norvegicus* inner ear (Ylikoski *et al.*, 1994). Retinoic acid may play a role in damage-induced hair cell regeneration in developing and mature inner ear organs as well as in the developing auditory organ (Ylikoski *et al.*, 1994) and is implicated in playing a role in anti-apoptosis in mammalian systems in response to intense noise exposure in outer hair cells (Ahn *et al.*, 2005). Alteration of expression of transcripts important in retinoic acid biosynthesis and thyroid hormone transport in inner ear of *S. salar* may point to protective mechanisms *via* immunostimulation or anti-apoptosis, as well as a role in neuroregeneration.

Calmodulin, a major intracellular calcium receptor, maintains intracellular calcium homeostasis in many tissues including hair cells (Furness *et al.*, 2002; Zuo *et al.*, 2008). Calmodulin serves as a marker for inner ear ganglion neurons and hair cells

and is involved in several biological processes including inflammation and apoptosis (Schuck *et al.*, 2011). Furthermore, a study using *Rana catesbeiana* saccular hair cells has shown that calmodulin participates in mechanoelectrical transduction (Cyr *et al.*, 2002). In the current study, microarray-identified calmodulin 2-encoding transcript was 2·1-fold down-regulated (Table I; Supporting Information Table S3) in *S. salar* ear in response to seismic noise exposure. Acoustic overstimulation, however, has been shown to cause an increase in the transcript encoding calmodulin 3 in *C. chinchilla* inner ear (Taggart *et al.*, 2001) and an increase in calmodulin protein expression in *C. porcellus* cochlear hair cells (Zuo *et al.*, 2008). Direct comparisons of transcript expression between this study on *S. salar* inner ear and previous studies on mammalian ear are difficult since the mammalian studies targeted specific organs or tissues within the ear, whereas the *S. salar* study presented here includes the whole inner ear. In addition, two subregions of *R. norvegicus* cochlea showed differential expression of several transcripts, including the transcript encoding calmodulin (Cho *et al.*, 2001). In *S. salar*, it is hypothesized that the down-regulation of the transcript encoding calmodulin 2 may be part of a mechanism aimed at limiting damage in the fish ear after loud sound exposure.

STRUCTURAL GENES

Cytoskeletal proteins contribute to the structural integrity and specialized function of the inner ear (Frolenkov *et al.*, 2004; Vollrath *et al.*, 2007); however, acoustic overstimulation can result in reorganization and other changes in cytoskeletal proteins (Avinash *et al.*, 1993; Hu & Henderson, 1997). Altered expression of transcripts encoding proteins with cytoskeletal roles have been previously observed in *D. rerio* inner ear (Schuck *et al.*, 2011), *R. norvegicus* cochlea (Chen, 2006), *C. chinchilla* cochlea (Taggart *et al.*, 2001), *G. g. domesticus* ear tissues (Cotanche *et al.*, 1994; Adler *et al.*, 1995; Gong *et al.*, 1996; Lomax *et al.*, 2000) and *C. porcellus* cochlear hair cells (Zuo *et al.*, 2008) following noise exposure. While the studies conducted on mammalian and avian ear tissues demonstrate primarily elevated levels of cytoskeletal transcripts following exposure, the cytoskeleton-relevant TOI in the *D. rerio* study were significantly down-regulated following noise exposure. In the current study, microarray-identified reproducibly informative transcripts encoding proteins important in structural scaffolding were differentially regulated in response to seismic noise exposure in the *S. salar* ear. *Salmo salar* ear transcripts up-regulated by seismic noise included those encoding coronin-1A (2·7-fold) and vasodilator-stimulated phosphoprotein (1·9-fold) (both important in actin-binding) (Table I; Supporting Information Table S2), while transcripts reproducibly down-regulated included those encoding ezrin-radixin-moesin-binding phosphoprotein 50 (2·3-fold) (involved in crosslinking actin filaments with plasma membranes), collagen alpha 2 type IV chain (two-fold) (part of the extracellular matrix) and motile sperm domain-containing protein 2 (two fold) (a motor protein important in cell motility, and representing the most abundant down-regulated transcript identified in the microarray study) (Table I; Supporting Information Table S3). Cytoskeletal proteins are known to be important in normal hair cell development and function in mammals (Petit & Richardson, 2009), and an increase in cytoskeletal proteins in *C. porcellus* cochlear hair cells following noise conditioning has been observed (Zuo *et al.*, 2008). Dysregulation of cytoskeletal-relevant transcripts in *S. salar* ear following noise exposure may point to

hair cell regeneration following damage or possibly an adaptive mechanism to protect hair cells against further exposure to intense noise (*i.e.* conditioning).

OTHER AND UNKNOWN

Transcripts that are not currently classified or have no known function are also summarized in Table I and also represent transcripts potentially responsive to noise-induced stress in *S. salar* inner ear. The microarray-identified transcript encoding C14orf159 protein is down-regulated (2·2-fold) (Table I; Supporting Information Table S3) in response to seismic noise exposure in *S. salar* ear and validated by qRT-PCR (−1·4-fold, $P < 0{\cdot}05$) (Table II). C14orf159 is highly conserved; BLASTx against GenBank's non-redundant (nr) amino acid sequence database shows that *S. salar* partial *c14orf159* cDNA sequence (GenBank accession number CA057346) is 73% identical to *D. rerio c14orf159* (AAI55802, 82/112 identities, *E*-value 3e-41) and 61% identical to human *c14orf159* (BAG53908, 70/113 identities, *E*-value 9e-33) at the hypothetical amino acid level. C14orf159 protein has no well-defined function, although it is thought to be important in the mitochondrion (GO Cellular Component term 'mitochondrion'; Supporting Information Table S3). Mitochondria are specialized organelles that produce most of the cell's energy (Alberts *et al.*, 2008). Changes in mitochondrial respiration (Hu *et al.*, 2008; Crawley & Keithley, 2011; Park *et al.*, 2012) and activation of a mitochondrial cell death pathway (Wang *et al.*, 2007) have been reported in mammalian hair cells following sound trauma. Additionally, the transcript encoding C14orf159 is up-regulated in *S. salar* gill affected by amoebic gill disease immediately following exposure to virulent amoebae (Morrison *et al.*, 2006), and the human *c14orf159* gene is known to bind ERα in 17β-oestradiol-treated human breast cancer cells (Creekmore *et al.*, 2007). While current functional information on *c14orf159* is scant, its transcript may prove to be an important biomarker of noise-induced stress in *S. salar* ear.

Microarray technology provides an avenue for investigating changes in the expression of large numbers of genes, thereby offering a potential for identifying candidate molecular markers of cell and organ damage, as well as potential for gaining insight into fundamental mechanisms of cellular stress. The microarray platform used in this study, a 16 000-gene (16 K) cDNA array with probes stringently selected from large salmonid EST databases (von Schalburg *et al.*, 2005), was an effective tool for this functional genomics research on the effects of exposure to loud sounds from a seismic airgun on gene expression in the inner ear of *S. salar*. Loud sound-responsive *S. salar* ear transcripts identified in this study belonged to functional categories including (1) energy and metabolism, (2) protein post-translational modification and degradation, (3) cell cycle and cell death, (4) immunity, (5) signalling, synaptic transmission and (6) structural genes. In addition, a number of microarray-identified transcripts in this study are not currently classified or have no known function. Overall, results support the hypothesis that effects on the ears of fishes are important endpoints to investigate in considerations by management, academic and industrial interests regarding the potential effects on fishes of seismic surveys, which may be carried out in a particular area over a number of weeks. Further, in any attempt to establish an exposure and response relationship for injury to fishes, with a view toward defining the dimensions of effect–no effect zones for seismic surveys, it seems reasonable to suggest that assessing the potential for effects on the ears would fall in a priority category.

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Supporting Information

Supporting Information may be found in the online version of this paper:

Table S1. Primers used in QPCR.

Table S2. Transcripts greater than 1·75-fold up-regulated in seismic exposed *Salmo salar* ear relative to control *Salmo salar* ear on at least three slides of study.

Table S3. Transcripts greater than 1·75-fold down-regulated in seismic exposed *Salmo salar* ear relative to control *Salmo salar* ear on at least three slides of study.

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