Hepatic transcriptional responses to copper in the three-spined stickleback are affected by their pollution exposure history

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Abstract

Some fish populations inhabiting contaminated environments show evidence of increased chemical tolerance, however the mechanisms contributing to this tolerance, and whether this is heritable, are poorly understood. We investigated the responses of two populations of wild three-spined stickleback (*Gasterosteus aculeatus*) with different histories of contaminant exposure to an oestrogen and copper, two widespread aquatic pollutants. Male stickleback originating from two sites, the River Aire, with a history of complex pollution discharges, and Siblyback Lake, with a history of metal contamination, were depurated and then exposed to copper (46 µg/L) and the synthetic oestrogen ethinyloestradiol (22 ng/L). The hepatic transcriptomic response was compared between the two populations and to a reference population with no known history of exposure (Houghton Springs, Dorset). Gene responses included those typical for both copper and oestrogen, with no discernable difference in response to oestrogen between populations. There was, however, some difference in the magnitude of response to copper between populations. Siblyback fish showed an elevated baseline transcription of metallothionein and a lower level of metallothionein induction following copper exposure, compared to those from the River Aire. Similarly, a further experiment with an F1 generation of Siblyback fish bred in the laboratory found evidence for elevated metallothionein transcription in unexposed fish, together with an altered transcriptional response to 125 µg/L copper, compared with F1 fish originating from the clean reference population exposed to the same copper concentration. These data suggest that the stickleback from Siblyback Lake have a differential response to copper, which is inherited to the F1 generation in laboratory conditions, and for which the underlying mechanism may include an elevation of baseline metallothionein transcription. The genetic and/or epigenetic mechanisms contributing to this inherited alteration of metallothionein transcription have yet to be established.

Keywords

Metals, Pollution, Adaptation, freshwater, teleost

1. Introduction

Metals and oestrogenic chemicals are widespread contaminants of freshwater systems worldwide. Anthropogenic sources of metal pollution include mining and industrial activity, and oestrogenic pollution derives from industrial, agricultural and domestic sources. Toxic effects of chronic metal exposure in wild fish populations include impaired metabolic activity, growth, immunity and genetic diversity (Bourret et al. 2008; Couture & Kumar 2003; Levesque et al. 2003; Pierron et al. 2009; Rajotte & Couture 2002). Exposure to environmental oestrogens has been reported to induce intersex in some fish species (Jobling et al. 1998; van Aerle et al. 2001) whilst roach (*Rutilus rutilus*) inhabiting some of the more contaminated sections of UK rivers may have reduced reproductive success and genetic diversity (Hamilton et al. 2014; Harris et al. 2011; Jobling et al. 2002). Viable populations of fish, however, do exist in some heavily polluted environments, including those contaminated with metals and/or oestrogens (Hamilton et al. 2014; Uren Webster et al. 2013).

Selective pressures favouring increased tolerance to pollution are likely to drive adaptive change in populations inhabiting contaminated environments. One of the best characterised examples of this are populations of North American Atlantic killifish (*Fundulus heteroclitus*), which have adapted to estuarine environments heavily contaminated with aromatic hydrocarbons. These killifish showed reduced sensitivity to aromatic hydrocarbon exposure, primarily due to a lack of induction of cytochrome P4501a (CYP1A) through suppression of the aryl hydrocarbon receptor (AHR) signalling pathway (Whitehead et al. 2010; Wirgin & Waldman 2004), and this mechanism of tolerance was inherited to F1, and in some cases F2, generations (Whitehead et al. 2012). Evidence of a genetic basis of this tolerance to aromatic hydrocarbons includes functional differences in the CYP1A promoter (Williams & Oleksiak 2011) and altered SNP frequency in AHRs (Reitzel et al. 2014), although this tolerance may not be entirely due to genetic selection, and is also likely to vary with the contaminant (Clark et al. 2013).

Altered response to oestrogen exposure has also been demonstrated in several populations of killifish in New Bedford Harbour and Newark Bay with a history of exposure to chemicals affecting oestrogen signalling, including PCBs. Depurated male killifish from polluted environments showed reduced transcription of oestrogen dependent genes including vitellogenins, chorion proteins and aromatase following exposure to 17β-oestradiol (E2) compared to those from a clean, reference population (Bugel et al. 2014; Greytak et al. 2010). Their F1 larvae showed attenuation of the ERα transcriptional response, but not the other oestrogen-response biomarkers (Greytak et al. 2010). This suggests that chronic environmental exposure drives a reduced sensitivity

to oestrogen, through modulation of oestrogen receptor signalling, but this response can vary with life stage (Bugel et al. 2014; Greytak et al. 2010). Studies on roach populations originating from river stretches in the UK heavily contaminated by oestrogenic chemicals have found the opposite, with a sensitisation of oestrogen responsiveness (Lange et al. 2009). Genetic and epigenetic mechanisms contributing to these responses are suspected but have not yet been established.

Potential adaptation to chronic metal exposure has been reported in various wild fish populations. For example, brown trout (*Salmo trutta*) from the River Hayle in Cornwall, UK, have shown tolerance of high concentrations of a mixture of metals that are lethal to naïve trout. These trout displayed relatively little evidence of overt toxicity, despite accumulating high tissue concentrations of metals, and showed evidence of several mechanisms of metal tolerance including up-regulation of metal-handing pathways and ion homeostasis (Uren Webster et al. 2013). Genetic analysis revealed differences between populations, both within this river and compared to clean rivers nearby, which were predicted to coincide with increases in local mining activity, suggesting that local adaption to metal contamination had contributed to a reduction in gene flow between these populations (Paris et al. 2015). Studies on yellow perch (*Perca flavescens*) populations inhabiting lakes in North America contaminated through industrial and mining activity have shown tolerance of high concentrations of metals including copper and cadmium, and the potential mechanisms of tolerance were reported to include elevated metallothionein and oxidative stress responses (Defo et al. 2015; Giguère et al. 2005; Pierron et al. 2009). There is also some evidence of selection in these perch populations driven by metal contamination (Bélanger-Deschênes et al. 2013; Bourret et al. 2008).

Evidence of considerable toxicity was also found in yellow perch chronically exposed to metals, including impaired metabolism and poor condition (Couture & Kumar 2003; Levesque et al. 2003; Pierron et al. 2009; Rajotte & Couture 2002), compared to less obvious signs of toxicity in the Hayle brown trout (Uren Webster et al. 2013). This may reflect a greater adaptive change in the trout following longer historical contamination in the river Hayle (~1000 years) compared to the ~100 years of contamination experienced by fish in the North American lakes. A genetic contribution to metal tolerance in fish therefore seems likely, but the precise mechanisms of this adaptive change and the potential relative contributions of phenotypic plasticity or other mechanisms are yet to be established. In addition, little is known about the response of depurated individuals to metal exposure or whether metal tolerance is inherited in F1 or subsequent generations raised in clean environments.

Three-spined stickleback (*Gasterosteus aculeatus*) inhabit many water systems worldwide, are relatively tolerant of stressors and can undergo rapid speciation in response to environmental change (McKinnon & Rundle 2002). We have previously used global transcript profiling to establish the transcriptional signatures of response to copper and 17alpha-ethinylestradiol (EE2) in stickleback originating from a reference site (Houghton Springs, Dorset, UK), receiving borehole water with no known history of metal or oestrogenic contamination (Katsiadaki et al. 2010; Santos et al. 2010). Here, we examined the hypothesis that historical exposure to these classes of pollutants modifies the tolerance of exposed populations, altering their response to further exposure. We investigated the transcriptional responses to EE2 and copper in two populations of stickleback that originate from water systems with a history of contamination by a wide range of chemicals including oestrogens (River Aire, Leeds, UK) and metals (Siblyback Lake, Cornwall, UK), followed by periods of improved water quality. We found responses typical for both copper and oestrogen exposure in both populations, as identified previously for stickleback at Houghton Springs (Katsiadaki et al. 2010; Santos et al. 2010), but with differences in the magnitude of response to copper between populations, and differences in the baseline transcription of copper-responsive genes, including metallothionein. A further experiment with an F1 generation of Siblyback fish bred and maintained in a clean environment, and subsequently exposed to copper, showed evidence of an elevated baseline transcription of metallothionein, together with an altered transcriptional and behavioural response, compared with F1 fish originating from the reference (Houghton Springs) population.

2. Materials and Methods

All experiments were performed using reagents from Sigma-Aldrich (Dorest, UK), unless otherwise stated.

2.1. F0 exposure

2.1.1 Site selection

Stickleback fish were collected from two sites chosen based on their history of oestrogen and metal pollution. The River Aire flows through a heavily industrialised area in Yorkshire, UK, and has a long history of heavy pollution. Considerable stretches of this river downstream of wastewater treatment outflows were reported to contain high concentrations of oestrogenic chemicals, particularly alkylphenol polyethoxylates (Harries et al.

1997), although improvements in wastewater treatment have since reduced considerably the degree of oestrogenic activity in this river (Sheahan et al. 2002). High prevalence and severity of feminisation in wild fish populations have been reported in this river (Jobling et al. 2002; Jobling et al. 1998; van Aerle et al. 2001). Resident stickleback populations are also known to have undergone significant population bottlenecks, although this has been shown not to have impacted on male reproductive competitiveness (Santos et al. 2013).

The Siblyback reservoir, constructed in the 1960s, is situated on Bodmin Moor in Cornwall, in an area with a history of intense tin and copper mining that began in the Bronze Age, intensified during the 18th century and later declined during the latter 19th century. Several tributaries of the River Fowey are feeder streams to the reservoir, and are also known to have been directly used for metal streaming (extraction and washing) (Pirrie et al. 2002). The population of stickleback in Siblyback reservoir, originating from these feeder streams, are therefore likely to have been historically exposed to high levels of metal contamination for hundreds of years. Recent water chemistry data (kindly supplied by the Environment Agency) shows that the copper concentrations in the reservoir have been low (generally <2.5 µg/L) for at least the last 15 years, suggesting multiple generations (over 15 generations considering that typically this species have a new generation each year) of this population have been living in cleaner conditions.

2.1.2 Maintenance, exposure and experiment

Mixed populations of juvenile three-spined sticklebacks were collected from Siblyback Reservoir and the River Aire and housed in aquaria at the University of Exeter for a minimum of 4 months, to allow depuration and acclimation to laboratory conditions. Stock tanks were supplied with de-chlorinated tap water, aerated and maintained at the ambient temperature (12-17 °C), pH 7.5, total hardness of 23.8 mg/L. Air saturation was maintained above 90%, and ammonia, nitrate and nitrite levels were maintained within an appropriate range. Fish were maintained under a constant photoperiod (12 hours light/dark cycle), and fed to satiation with bloodworm (Tropical Marine Centre, Hertfordshire, UK). During the depuration and acclimation period, fish from the river Aire suffered a higher mortality rate, due to white spot disease, compared to fish originating from Siblyback, resulting in lower density in the holding tanks. This contributed to a significantly higher body size in the remaining Aire fish prior to the start of the exposure experiment.

For the exposure experiment, fish (average 4.39 cm; 0.81 g) were randomly allocated into groups of 20 individuals and maintained in 40 L glass tanks for one week prior to the onset of exposure. Fish were exposed to three concentrations of copper (3.2, 32, 128 µg/L; prepared using copper sulphate (Fisher, UK)), three

concentrations of EE2 (1, 10, 32 ng/L) and three mixtures (3.2 µg Cu/L and 32 ng EE2/L; 128 µg Cu/L and 1 ng EE2/L; 128 µg Cu/L and 32 ng EE2/L) in duplicate tanks. These nominal exposure concentrations were selected to match those used previously by Santos et al. (2010) and Katsiadaki et al. (2010) to enable comparisons between the responses measured in the current and previous studies. A dilution water control treatment was also included in duplicate. At the start of the chemical exposure, tanks were spiked to achieve the required nominal exposure concentrations. Fish were then exposed for four days under flow-through conditions with a water replacement time of 0.6 volumes every 24 h. The tank water was sampled on days 0, 2 and 4 for determination of dissolved Cu and EE2 concentrations.

At the end of the exposure period, fish were humanely sacrificed by an overdose of benzocaine (0.5 g/L) followed by destruction of the brain in accordance with UK Home Office regulations. Wet weights and fork lengths were determined and used to calculate condition index ($k = (\text{weight (g)} \times 100) / (\text{fork length (cm)}^3)$). The livers were dissected, weighed, and immediately frozen in liquid nitrogen and stored at -80°C for subsequent transcriptomic analyses. The gonads were dissected, weighed, and used to verify the sex of each individual. Hepatosomatic index (HSI), nephrosomatic index (NSI) and gonadosomatic index (GSI) were calculated as a percentage of total body weight for each tissue.

Statistical analysis of morphometric measurements were conducted using SigmaStat (version 12.0). All data met assumptions of normality and equal variance, and were analysed using single factor one-way analysis of variance (ANOVA), followed by Holm-Sidak post hoc test using a pairwise comparison method. Differences between groups were considered to be statistically significant when $p < 0.05$.

2.1.3 Water Chemistry

Water samples (0.5 L) were collected at days 0, 2 and 4 into acid washed plastic bottles and measurement of copper and EE2 determined as before (Katsiadaki et al. 2010; Santos et al. 2010). Briefly, for measurements of copper, water samples were filtered using a nucleopore 0.4 µm filter, and acidified by adding 100 µl of nitric acid (Aristar grade) and 60 µl of hydrogen peroxide and UV-digested over 12 hours. The samples were stored at 4°C in the dark until determination of Cu concentrations. Total dissolved copper (TDCu) was determined by differential pulse anodic stripping voltametry at a hanging mercury drop electrode (DPASV-HMDE), Metrohm Computrace 767 Multimode electrode system. For measurement of EE2, water samples were filtered using a nucleopore 0.4 µm filter and extracted using C18 solid phase cartridge. Extracts were dried under nitrogen gas, reconstituted in 1ml radioimmunoassay buffer (0.5M phosphate buffer containing 0.2% bovine serum albumin,

0.8% sodium chloride, 0.03% EDTA and 0.01% sodium azide) and stored at −20°C. EE2 concentrations were determined using a radioimmunoassay, using a standard of radiolabelled EE2 at a concentration of 500 µg/ml in ethanol, and an EE2 antiserum, as described previously(Katsiadaki et al. 2010).

2.1.4 Transcriptomic analyses

Analysis of gene transcription was conducted using the stickleback 'PGPS2' cDNA microarray (Array Express Accession A-MAXD-23) comprising 14,496 probes representing 5038 individual stickleback genes as described previously (Brown et al. 2008; Geoghegan et al. 2008; Katsiadaki et al. 2010; Santos et al. 2010; Williams et al. 2009). Individual samples labelled with Cy5 were hybridized to the microarray in competition with a common Cy3-labeled reference pool synthesised from pooled male treated and control samples from both populations. Samples from 5 individual fish from each treatment group were analysed, for a total of 120 samples. MIAME-compliant data and protocols are archived at ArrayExpress under Accession E-MTAB-4907. Mean values were used for all probes annotated to each stickleback gene after filtering. Gene transcription analysis was conducted within Genespring (GX 7.3; Agilent Technologies, Berkshire, UK).

A T-test with Benjamini & Hochberg(Benjamini & Hochberg 1995) multiple testing correction for a false discovery rate (FDR) <0.05 was used to compare Aire and Siblyback control groups. Similar T-tests comparing individual treatment concentrations with control groups identified few differentially transcribed transcripts, therefore for each population, corrected T-tests were performed comparing all fish exposed to 0 µg/L copper against all exposed to 128 µg/L copper, and all fish exposed to 0 ng/L EE2 against all exposed to 32 ng/L EE2. Annotation enrichment analysis was conducted in DAVID(Huang et al. 2008) using lists of differentially transcribed genes in comparison with a background list consisting of all detected genes.

In stickleback, the size of the kidney increases dramatically in mature males due to spiggin production, a protein produced as a result of androgen stimulation and used by males to build nests. Kidney size is therefore a reliable indicator of maturity status in males (Katsiadaki et al. 2010). In the Aire population, there was high variability in gene expression and we hypothesised that this was related to differences between males with large and small kidneys, reflecting different maturity status, therefore additional statistical tests were performed to determine the influence of kidney size (as a proxy for maturation status) on transcription, comparing samples with high (>10 mg) and low (<10 mg) kidney weights.

Following analysis of response to 128 µg/L copper and 32 ng/L EE2 for the Siblyback and Aire populations, Gene Set Enrichment Analysis (GSEA)(Subramanian et al. 2005) was used to investigate whether responses to

copper and EE2 in each population were similar to those detected in previous experiments performed with male sticklebacks from a reference site (Houghton Spring) with no known history of pollutant exposure, that were exposed to the same nominal concentrations (Katsiadaki et al. 2010; Santos et al. 2010).

2.2 F1 exposure

2.2.1 Maintenance, exposure and sampling

Adult stickleback from two wild populations (Houghton Springs, Dorset and Siblyback Reservoir, Cornwall) were collected, transported to the University of Exeter, maintained in mixed sex stock tanks and allowed to breed naturally after a period of depuration of four months. Fry from each population were housed in stock tanks supplied with de-chlorinated tap water, aerated and maintained at 15-16 °C, pH 7.5, with a total hardness of 23.8 mg/L. All other husbandry conditions were as described for the F0 exposure. As in the F0 experiment, the parental Houghton Springs stickleback suffered an outbreak of white spot disease while the Siblyback fish remained healthy. As a result, fewer F1 Aire fish survived until maturity, and these were maintained at lower stocking density and were larger in size than those originating from Siblyback reservoir.

Sexually mature male sticklebacks (average 4.46 cm; 0.85 g) from the F1 generation of each population were exposed to copper via a flow-through system for a period of 4 days. The exposure was conducted in 35 L tanks, supplied with one full tank water replacement each day. For the Siblyback population, fish were exposed to five concentrations of copper (3.2, 10, 32, 64 and 128 µg/L; prepared using copper sulphate (Fisher, Fair Lawn, USA)) and a dilution water control, and each treatment group was comprised of two replicate tanks, each containing six fish. These concentrations were selected to match those used for the F0 exposure, with additional intermediate concentrations. For the Houghton Springs population, due to the limited number of fish available the exposure was conducted using only the highest treatment concentration (128 µg/L) and a dilution water control. Water samples were collected from each tank on day 2 of the exposure period and total copper concentrations were determined using ICP-MS by an accredited laboratory (South West Water, Exeter Laboratories). Qualitative observations of activity and feeding behaviour were recorded over the course of the exposure period.

Fish were humanely sacrificed on day four of the exposure period by a lethal dose of benzocaine (0.5 g/L) followed by destruction of the brain, in accordance with UK Home Office regulations. For each individual fish, wet weight and fork length were recorded and the condition factor was calculated. Sex of individuals was

confirmed by observation of the gonads. Livers and kidneys were dissected and weighed, and the HSI and NSI were determined. Liver samples were snap frozen in liquid nitrogen and stored at -80°C prior to transcript profiling. Blood was collected from the caudal vein using a heparinised collection tube and kept on ice for assessment of DNA damage using the Comet assay.

2.2.2. Comet Assay

Measurement of single- and double-stranded DNA damage in blood cells was performed using the alkaline comet assay at the end of the exposure. Briefly, 1 µl of whole blood was diluted 2000x in cold PBS and centrifuged at low speed. The red blood cell pellet was then mixed with 180 µl of 1% low melting point agarose solution, warmed to 37 °C, then spread on slides coated with 1% high melting point agarose. The slides were cooled to 4 °C for 10 min, then placed in a lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 µM Tris, 10% DMSO, 1% Triton X-100, pH 10) for 1 h at 4 °C. Cells were then denatured in an alkaline electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH 13) for 40 min before an electric current (24 V, 300 mA) was applied for 30 min. Cells were washed in neutralising buffer (0.4 M Tris, pH 7.5) before staining with 20 µl of 20 mg/L ethidium bromide solution). Each slide was viewed with a fluorescence microscope (420–490 excitation filter; 520 nm emission filter) and the % tail DNA from each sample was quantified for 100 cells (Kinetic COMET software). For each individual fish the comet assay was performed in duplicate, and an average value of DNA damage was calculated.

2.2.3. RT-qPCR analysis

RT-qPCR was used to quantify the hepatic transcription of known copper-regulated genes, selected based on their response to copper in a previous study using stickleback males from the same reference site (Houghton Springs, Dorset)(Santos et al. 2010) and in the F0 exposure. The target genes chosen included, metallothionein (*mt*) and transcripts encoding four enzymes from the cholesterol biosynthesis pathway (isopentenyl-diphosphate-delta-isomerase; *idi1*, 3-hydroxy-3-methylglutaryl-CoA synthase; *hmgcs*, 3-hydroxy-3-methylglutaryl-CoA reductase; *hmgcr*,24-dehydrocholesterol reductase; *dhcr24*). Ribosomal protein 8 (*rpl8*) was used as a control gene, and the assays were optimised as described previously (Santos et al. 2010). RNA was extracted using TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions. cDNA was synthesised from 2 µg of total RNA treated with RQ1 DNase (Promega, Southampton, UK) using random hexamers (MWG-Biotech) and M-MLV reverse transcriptase (Promega), according to the manufacturer's

instructions. RT-qPCR was performed using 1:2 diluted cDNA in triplicate, using SYBR green chemistry, with an iCycler iQ Real-time Detection System (Bio-Rad Laboratories, Hercules, CA). A negative control was also run in triplicate on each plate to verify the absence of cDNA contamination. Efficiency-corrected relative transcription levels for each transcript were determined by normalising to the control transcript, *rpl8*.

Statistical analysis was conducted in SigmaStat (version 12.0). Transcriptional data that did not meet normal distribution criteria were log transformed before statistical analysis. All data was analysed using single factor one way analysis of variance (ANOVA), followed by the all pairwise Holm-Sidak post hoc test. All values presented are mean ± SEM. Data were considered to be significant when $P < 0.05$.

3. Results

3.1 F0 exposure

3.1.1. Morphometric parameters

Following dissection, male fish were identified and all analyses were conducted on males only to reduce possible variation associated with sex. The mean length, mass and condition index, for Aire and Siblyback fish respectively, were 4.55 ± 0.05 cm and 4.24 ± 0.03 cm; 0.93 ± 0.04 g and 0.66 ± 0.02 g; and 0.95 ± 0.01 and 0.84 ± 0.01. The mean HSI, GSI and NSI, for the Aire and Siblyback fish respectively, were 2.95 ± 0.08 and 1.7 ± 0.05; 0.58 ± 0.03 and 1.02 ± 0.05; and 1.25 ± 0.09 and 0.85 ± 0.04. Within each population there were no significant differences between treatment groups in length, weight, condition factor, HSI, NSI or GSI. During the course of the experiment, no obvious differences in swimming or feeding behaviour were observed in any exposure group.

3.1.2. Water chemistry

The results of the water chemistry are shown in Table S1. For the copper exposure, the mean measured concentrations were lower than the nominal in the intermediate and high treatment groups (ranging from 24-38 %), likely due to degradation or binding of the chemicals to organic matter in the water or to the surface of the tanks during the exposures. However, importantly, the measured concentrations were similar for the exposures conducted on both the Siblyback and Aire populations (49.1 and 43.5 µg/L, respectively, for 128 µg/L copper), ensuring that comparisons between the responses of each population are valid. Similarly, for the EE2 exposure,

measured concentrations were lower than the nominals for the highest treatment group (32 ng/L), but were similar between populations (23.8 and 21.0 ng/L for Siblyback and Aire, respectively). The average measured exposure concentrations will be referred to throughout the rest of the manuscript.

3.1.3. Transcriptomic analysis

3.1.3.1 Hepatic responses to copper and EE2 treatment

For the Siblyback stickleback, a total of 15 genes were differentially regulated following exposure to 22 ng/L EE2 (Table 1), and they included genes known to be oestrogen-responsive (e.g. oestrogen receptor 1 and zona pellucida proteins 3 and 4). Following exposure to 46 µg/L copper, 1071 genes were differentially transcribed (Table 2a and Supporting file 2). These included typical copper-responsive genes including metallothionein and proteins involved in cellular stress response. Annotation enrichment analysis of these gene lists showed induction of transcription-related genes and repression of those encoding immune response, secreted, plasma and ribosomal proteins (Supporting file 3).

For the Aire population, there were a total of 22 significantly differentially transcribed genes (FDR<0.05) in fish exposed to 46 µg/L copper, 10 of which were also regulated in the Siblyback population (Table 2b). For EE2, no individual genes were found to be significantly differentially transcribed, potentially due to the high variability between individuals that were associated with the differences in sexual maturity (indicated by the differences in kidney size). To explore this further, we analysed differences associated with kidney weight in Aire male fish exposed to 22 ng EE2/L. There was a significant negative correlation of *zp3* transcription with kidney weight (R^2=0.82; P<0.05), and several other transcripts found to be oestrogen-responsive in the Siblyback fish were differentially transcribed between Aire males with smaller (<10mg) kidneys and larger (>10mg) kidneys (Table S4), indicating a confounding effect of kidney size in the Aire population for identifying oestrogen-responsive transcripts.

3.1.3.2 Comparing the Aire and the Siblyback populations

The loss of Aire fish due to white spot disease caused a differential stocking density, resulting in different body sizes and sexual maturation rates between populations. This may be expected to cause differences in baseline hepatic transcription, associated with size or maturity, between unexposed control fish from the different

populations. Despite this confounding effect, there were also clear differences in the baseline transcription of copper-responsive genes identified in this, and previous, studies. Transcription of a total of 157 genes was significantly different (FDR < 0.05) between the control groups from Aire and Siblyback populations, including metallothionein and those involved in response to cellular stress and protein folding (Table S5). Functional analysis identified a number of Gene Ontology (GO) terms and pathways that were significantly over-represented (FDR < 0.05) amongst the differentially transcribed genes. These are shown in Supporting file 3. Terms related to the endoplasmic reticulum was strongly over-represented amongst the genes that were more highly transcribed in the Aire population, while terms associated with energy metabolism were most significantly enriched for genes that were more highly transcribed in the Siblyback fish.

3.1.3.2 Comparing responses to copper and EE2 between the previously published Houghton Springs population and the Aire and Siblyback populations

Genes found to be differentially transcribed in our previous studies for a reference population (Houghton Springs) following exposure of sticklebacks to 128 µg/L copper(Santos et al. 2010) and 32 ng/L EE2 (Katsiadaki et al. 2010) are shown in Table S3. Gene set enrichment analysis (GSEA) demonstrated a significant similarity between these previous responses to 128 µg/L copper and those for the Aire fish exposed to 46 µg/L copper (FDR=0.032), compared with controls. In contrast, there was no significant similarity for the Siblyback copper-exposed fish (FDR=0.173). Transcriptional responses of the populations from the River Aire and Siblyback to 22 ng EE2/L were both similar to those measured in sticklebacks from Houghton Springs exposed to 32 ng EE2/L. This similarity was highly significant for the Siblyback stickleback (FDR=0.001), but less so for the Aire fish (FDR=0.07) that showed transcriptional variability related to kidney weight (thus sexual maturity).

3.2 F1 exposure

3.2.1. Morphometric parameters and behaviour

All fish used for the analysis in this experiment were males as confirmed by macroscopic evaluation of gonads. The Aire fish were significantly larger than the Siblyback fish, potentially also affecting baseline transcription between unexposed fish. Within each population there were no significant differences between treatment groups in length, weight, condition factor, HSI or NSI following the exposure. During the course of the exposure, we

observed a considerable reduction in activity and feeding, together with darker colouration, in the Houghton Springs stickleback exposed to 128 µg copper/L compared to the controls, but no differences were observed for the Siblyback fish.

3.2.2 Water chemistry

Measured concentrations of copper in each of the treatment groups are shown in Table S2. Concentrations measured in the highest treatment concentration (128 µg copper/L) were 96 % and 98 % of the nominal value for the Siblyback and Houghton Spring's populations, respectively. For the lower treatment concentrations, the measured concentrations were between 5-7 µg/L higher than the nominal values, and the background level of copper measured in the controls was between 6-9 µg/L. This is higher than expected, but is within the normal range expected for de-chlorinated tap water. However, background copper was identical for all treatment groups from both populations and therefore unlikely to affect the comparisons of response to copper compared to the control group within or between populations. The average measured exposure concentrations will be referred to throughout the rest of the manuscript.

3.2.3 Comet assay

The percentage of tail DNA, representing degree of single- and double-stranded DNA damage, measured using the comet assay, is shown in Figure 1a. In the Siblyback fish there was a strong positive correlation between treatment concentration and degree of DNA damage (R^2 =0.57, P = 4.7e-10). There was a significant increase in DNA damage in the blood cells of Siblyback fish exposed to both 70 and 124 µg copper/L compared to both the water control and lowest treatment groups. In the Houghton Springs population, that were only exposed to the highest copper concentration (126 µg/L), there was a significant increase in DNA damage compared to fish in the water control group. There was an apparent higher rate of DNA damage in the Houghton Springs stickleback compared with the Siblyback fish in response to the highest copper concentration, but this was not statistically significant (P= 0.11).

3.2.4 Transcript profiling

Transcript profiling using RT-qPCR for the target genes selected is shown in Figure 1b-f. For metallothionein (*mt2*), there was a significant increase in transcription in Siblyback fish exposed to 37, 70 and 124 µg copper/L compared with the control group, and also a strong correlation between treatment concentration and *mt2*

transcription (R^2=0.20 , P<0.001). There was also a significant increase in transcription of *mt2* in Houghton Springs fish exposed to 126 µg copper/L compared to the control group (P <0.001). There was also significantly higher baseline metallothionein transcription in the Siblyback controls compared with the Houghton Springs controls (P=0.027). For the four cholesterol biosynthesis enzymes (*idds, hmgcs, hmgcr, dhcr24*), there was a clear, but non-monotonic concentration effect of copper on their transcript profiles in the Siblyback fish that was very similar for each gene. The lowest treatment concentration (10.6 µg/L) induced increased transcription compared to the control (significant for *hmgcs* and *dhcr24*), while concentrations of 37, 70 and 124 µg/L (*idds, hmgcs*), or 124 µg/L only (*hmgcr, dhcr24*) significantly reduced transcription compared to the control group. For the Houghton Springs population, the single treatment concentration of 126 µg/L caused a significant decrease in transcription of all four cholesterol biosynthesis genes compared to the control group. Comparing the control groups between the two populations, significantly higher baseline transcription was observed for both *idds* and *hmgcs* in the Siblyback population compared to the Houghton Springs population and the same trends towards higher transcription were observed for *hmgcr* and *dhcr24*.

4. Discussion

Conserved response to oestrogen

The transcription profiles of stickleback males exposed to EE2 from both the Aire and Siblyback populations showed a significant degree of concordance using Gene Set Enrichment Analysis (GSEA) to that previously characterised by Katsiadaki et al. (Katsiadaki et al. 2010), demonstrating a conserved oestrogenic response in stickleback from three independent populations. For the Siblyback fish exposed to 22 ng EE2/L the majority of differentially-transcribed genes identified have been previously shown to be oestrogen-responsive, including oestrogen receptor and zona pellucida proteins 3 & 4, and also other oestrogen responsive genes (*mobkl2c, tln1, aldh5a1, tsc2*) previously identified in stickleback(Katsiadaki et al. 2010), confirming their value as good markers of oestrogen exposure for this species. In the Aire fish, kidney size (which is associated with sexual maturity in males(Katsiadaki et al. 2002)) had a strong influence on typical oestrogen responsive genes, increasing variability in the data and preventing us from performing a comprehensive comparison of response to EE2 between the two populations.

Comparative gene responses to copper

The response to copper exposure in both the Siblyback and Aire populations showed some similarities to that previously described for the stickleback from the clean Houghton Springs site(Santos et al. 2010), as well as other studies on fish. For the Siblyback fish, the transcriptional response included genes broadly related to induction of a cellular stress response, consistent with previously described response to copper exposure. Up-regulated transcripts included the metal binding protein, metallothionein, and a number of well-characterised stress-response molecules such as heat shock proteins and other molecular chaperones, DNA damage-inducible transcripts and components of the antioxidant system. Functional enrichment of up-regulated transcripts was dominated by terms related to 'regulation of transcription', likely reflecting a specific increase in the transcription of regulatory factors of stress-responsive pathways. The associated genes included several elongation factors and a number of transcriptional regulators of cell signalling pathways involved in cell proliferation and apoptosis, including tumour necrosis factor (TNF) and epidermal growth factor (EGF) signalling, which have previously been linked with hepatic cellular stress response to acute chemical exposure (Song et al. 2009; Uren Webster & Santos 2015). Notable in the list of down-regulated transcripts were several enzymes involved in cholesterol biosynthesis (*dhcr7, idi2*) which is consistent with the previously described down-regulation of this pathway in stickleback exposed to copper (Santos et al. 2010) and also in other species (Huster et al. 2007). Functional analysis of the gene responses revealed strong enrichment of terms associated with 'Translation' and 'Ribosome', with reduced transcription of genes encoding over 40 ribosomal proteins. This is consistent with a global down-regulation of translation, and is likely to be a protective mechanism to limit protein mis-folding and/or conserve energy which again has previously been reported following exposure to environmental stress including toxic metals (Planelló et al. 2007; Spriggs et al. 2008). 'Immune response' was also functionally enriched and, in particular, transcripts encoding a number of components of the complement system were down-regulated (*c1s, c2, c4a, c6, c8a, c8b, c8g, c9, cfh*). Links between copper and the immune system have been widely reported, with both excess and deficiency being linked to disruption of immune function (Cunningham-Rundles et al. 2005; Hood & Skaar 2012). Impaired immunity and down-regulation of components of the immune system have also been reported in wild fish chronically exposed to metals (Pierron et al. 2011; Uren Webster et al. 2013). For the Aire fish, considerably fewer transcripts were significantly differentially transcribed, likely reflecting the greater degree of intra-individual variation in this population. However, several known markers of copper exposure, including metallothioneins, were regulated and GSEA analysis showed similarity to the dataset generated previously (Santos et al. 2010). This evidence demonstrates a qualitatively similar response to copper in three independent populations of stickleback.

There was also evidence of notable differences in the magnitude of transcriptional response between the Siblyback and Aire populations, exposed to similar measured concentrations of copper. Of the 10 transcripts that were differentially transcribed in both populations, all were regulated in the same direction, and the majority of these (8) were more responsive in the Aire fish compared with fish from Siblyback. This suggests that despite the greater number of significantly differentially regulated genes, the amplitude of the response to copper in the Siblyback population was lower than that for the Aire fish, suggesting lower sensitivity to copper for Siblyback fish. Amongst these genes were two transcripts encoding metallothionein, which were significantly up-regulated by 2.4/2.0 and 1.7/1.5 fold in the Aire and Siblyback populations, respectively. Metallothionein also had a significantly higher baseline transcription level in the Siblyback population compared to the Aire stickleback population (by 2.5-fold). By binding free metal ions and limiting oxidative damage, metallothionein plays a central role in cellular copper detoxification and is consistently induced following copper exposure in fish (Craig et al. 2009; Dang et al. 2009; Hogstrand & Haux 1991; Santos et al. 2010; Wood 2112). In the Siblyback population, this lower degree of metallothionein up-regulation in response to copper, coupled with higher baseline transcription level, suggests the possibility of a priming effect and reduced need for metallothionein induction on exposure. Elevated levels of metallothionein transcription and expression have been reported for various populations of wild fish exposed to chronic metal pollution, and this is known to be a key mechanism of metal tolerance (Hansen et al. 2006; Uren Webster et al. 2013). The data presented here for sticklebacks indicate a persistence of elevated baseline metallothionein transcription in the Siblyback population despite the recent reduction in copper levels in this area for at least the last 15 years, suggesting this may be an adaptive and inheritable response to historical metal exposure. Evidence of different baseline transcription levels of other metal-handling genes was limited, although in the Siblyback fish there was higher transcription of a number of genes associated with iron binding and storage, (including ferritin heavy subunits and haem-binding subunits). Iron handling pathways are known to have some transferability to the homeostasis of other metals (Craig et al. 2009; Kwong et al. 2011) and similar up-regulated responses were found previously in a metal-tolerant population of brown trout (Uren Webster et al. 2013).

The differential size of fish from the two populations, resulting from an outbreak of white spot disease prior to the exposure experiment and subsequent difference in stocking densities, is likely to have influenced baseline hepatic transcription. Functional enrichment analysis revealed that the most over-represented pathways in the

list of genes that showed differential baseline transcription between the Siblyback and Aire fish were related to energy metabolism, protein folding and lipid synthesis, which may well reflect their differential body size.

Despite this, there are some similarities between the enriched processes and known copper-responsive genes and processes. In particular, there was strong enrichment of the endoplasmic reticulum (ER), including differential baseline transcription of many genes involved in the unfolded protein response (UPR), and lipid metabolism. The UPR is an essential stress response mechanism which prevents secretion of misfolded proteins, preserving cell function and survival (Malhotra & Kaufman 2007; Schröder & Kaufman 2005), and has been previously linked to copper exposure (Song et al. 2015), and generation of oxidative stress (Malhotra & Kaufman 2007).

Evidence of altered response to copper maintained in the F1 generation

Given the results of the F0 generation studies, which were suggestive of a persistent primed response to copper in stickleback fish originating from Siblyback Lake despite the recent improvement in water quality, we conducted a follow-up experiment to investigate if the effects of copper seen in the stickleback males originating from Siblyback Lake were inherited in the next generation for fish kept in laboratory conditions. To investigate this question, we collected new populations of fish from the Siblyback Lake and from a historically clean site (Houghton Springs), depurated them in the laboratory and allowed them to breed naturally. We tested F1 males from both populations to investigate if differential responses to copper occurred.

Exposure to a measured average concentration of 125 µg/L copper caused a significant increase in DNA damage in blood cells in both populations. There was a tendency for a higher rate of DNA damage in the Houghton Springs population compared with the Siblyback population (although this was not statistically significant; P=0.11). Furthermore, stickleback originating from Houghton Springs showed a pronounced behavioural response to exposure to 125 µg/L copper, that was entirely absent in the Siblyback fish exposed to an equivalent concentration. The reduced feeding and swimming behaviour observed, as well as the darker colouration, is a typical stress response associated with acute chemical toxicity (Scott & Sloman 2004). This suggests that this high concentration of copper was less acutely toxic to the stickleback originating from Siblyback Lake.

Transcription of metallothionein and cholesterol biosynthesis genes were similar in their patterns of response to copper as for those observed in the F0 populations. Baseline transcription of metallothionein (in the unexposed control groups) was significantly higher in the Siblyback fish compared with the Houghton Springs population (by 2-fold). This is similar to the difference between the F0 populations of Siblyback and Aire stickleback,

where Siblyback baseline *mt2* transcription was 2.5 fold higher compared to that in the Aire population. Following exposure to 125 µg/L copper, there was an increase in *mt2* transcription by 7-fold in the Siblyback population and 10.3-fold in the Houghton Springs fish. Again, the smaller relative induction of metallothionein in the Siblyback stickleback in the F1 study was similar to that observed in the F0 Siblyback fish, compared to those from the river Aire. Increased metallothionein transcription in response to metal exposure is a well-established inducible stress response. It is the most consistently responsive gene in acute copper exposures and its up-regulation is also a key mechanism of metal tolerance in chronically exposed wild populations (Hansen et al. 2006; Uren Webster et al. 2013). Therefore, we have found evidence of increased baseline levels of metallothionein transcription, not only in the F0 Siblyback stickleback in the absence of immediate contamination, but also inherited in F1 fish bred and maintained in the laboratory. This suggests altered baseline metallothionein transcription may constitute a mechanism of metal tolerance in this historically exposed population, complementary to its role in acute stress response.

For the cholesterol biosynthesis genes there was also evidence of differential baseline transcription between the two populations; the Siblyback fish had significantly higher transcription for *idds* and *hmgcs*, and similar trends towards elevated *hmgcr* and *dhcr24* transcription were also observed. Following exposure to 125 µg copper/L, there was an apparent up-regulation followed by a significant down-regulation of all four cholesterol biosynthesis genes, in both populations. This confirms the inhibitory effects of a high concentration (46 µg/L) of copper on the cholesterol biosynthesis pathway found in the F0 population and previously in stickleback (Santos et al. 2010). We also found clear evidence of a non-monotonic dose-response relationship when the Siblyback population was exposed to copper. This transcription pattern was similar for all four cholesterol biosynthesis genes analysed, and was characterised by up-regulation in the lowest treatment concentration (10.6 µg/L measured), little change in the group exposed to 16 µg/L and down-regulation in the higher treatment groups (37, 70 and 124 µg/L). The consistency in the pattern between the various enzymes that are part of the cholesterol biosynthesis pathway aligns with the known co-regulation of these genes by SREBP-2 (Sharpe & Brown 2013), and of copper disrupting the function of this transcription factor. It is thought that copper does not disrupt regulation of sterol regulatory element binding protein 2 (SREBP-2) maturation and nuclear migration, but interferes with SREBP-2 binding to sterol regulatory elements (SREs) or subsequent activation of transcription (Huster et al. 2007). A stimulatory effect of low concentrations of copper on the cholesterol biosynthesis pathway has not been previously reported, although similar trends in transcription were apparent for these genes in stickleback originating from Houghton springs (Santos et al. 2010). Low concentrations of

copper have been reported to cause stimulatory effects on a number of cellular metabolic processes, while higher concentrations cause inhibition, potentially reflecting the beneficial effects of low concentrations of this micronutrient, and/or a compensatory response to low-level toxicity (Bundy et al. 2008). Similarly to a primed response of metallothionein, it is possible that the observed elevation in baseline transcription of these cholesterol biosynthesis genes in the Siblyback population reflects a legacy of compensatory up-regulation at low copper concentrations, although at higher copper concentrations the transcription of cholesterol biosynthesis genes was inhibited.

Conclusions

Variation in other environmental factors and genetic background between the Aire and Siblyback populations may well have contributed to the different responses to copper observed, and indicate the need for some caution in comparing responses across populations and between studies. There was no apparent difference in response to oestrogen between the two population, however, we have found evidence to suggest that stickleback from Siblyback reservoir have developed an altered response to copper likely due to historical metal exposure, and that this has persisted not only following a more recent reduction in environmental copper exposure encompassing multiple generations, but was also maintained in F1 fish bred in the laboratory. Metallothionein is likely to play a role in this possible increase in copper tolerance of fish originating from the Siblyback Lake. The mechanisms for the observed difference in baseline metallothionein transcription are yet to be established, and could include both genetic and/or epigenetic changes, including alterations of the regulation, function or copy number of this gene. Further studies are required to address these hypotheses, and to clarify the mechanisms by which fish populations develop tolerance to toxic metals in the wild.

Acknowledgments

This work was funded by the UK NERC postgenomic and proteomic programme grant NE/C507661/1 and by a Fisheries Society of the British Isles research grant to EMS. Birmingham functional genomics facilities were funded by BBSRC grant 6/JIF13209. We thank R.E. Godfrey, S. Jondhale, A. Jones, and L. Klovrza for technical assistance, J.K. Chipman for help and support, and the Environment Agency for provision of water chemistry data.

Tables

Table 1. Transcripts differentially regulated in response to EE2 exposure.

A. Transcripts differentially regulated following exposure to 24 ng/l EE2 in Siblyback male sticklebacks

Gene ID	Name	Symbol	EE2/Ctrl	FDR
ENSGACG00000012381	Zona pellucida sperm-binding protein 3	*zp3*	2.52	6.26E-05
ENSGACG00000002960	Succinate-semialdehyde dehydrogenase, mitochondrial	*aldh5a1*	2.378	0.000112
ENSGACG00000011891	Mps one binder kinase activator-like 2C	*mobkl2c*	2.218	0.00103
ENSGACG00000007240	Probable prolyl-tRNA synthetase, mitochondrial	*pars2*	1.983	0.000112
ENSGACG00000008711	Estrogen receptor alpha	*nr4a2*	1.762	0.0227
ENSGACG00000018952	Tuberin	*tsc2*	1.723	0.00986
ENSGACG00000005956	Choline dehydrogenase, mitochondrial	*chdh*	1.718	0.0227
ENSGACG00000015930	Prostaglandin E2 receptor EP4 subtype	*ptger4*	1.718	0.00653
Gac.7287	EST		1.67	0.000161
ENSGACG00000012495	Elongation factor Tu GTP-binding domain-containing protein 1	*eftud1*	1.643	0.00063
ENSGACG00000003367	NK-tumor recognition protein	*nktr*	1.507	0.000112
ENSGACG00000013548	Ras-related protein Rab-35	*rab35*	1.475	0.0321
ENSGACG00000018584	Talin-1	*tln1*	1.446	0.00986
ENSGACG00000011851	Zona pellucida sperm-binding protein 4	*zp4*	1.443	0.00171
ENSGACG00000018876	Transmembrane protein 85	*tmem85*	1.383	0.00128

B. Transcripts differentially regulated following exposure to 21 ng/l EE2 in Aire male sticklebacks

None statistically significant at FDR<0.05

Table 2. Transcripts differentially regulated in response to copper exposure

A. Selected Transcripts differentially regulated following exposure to 46 µg/l Cu in the liver of Siblyback male sticklebacks

Note: A full list is shown in Supporting File 2. Asterisks & shading indicate overlap between populations.

Gene ID	Name	Symbol	Cu/Ctrl	FDR
ENSGACG00000003652	Glucose-6-phosphatase	*g6pc*	4.025	2.60E-06
ENSGACG00000002379	DNA-damage-inducible transcript 4 protein	*ddit4*	3.159	0.0103
Ga_S1TG_12G03	EST *		2.342	0.00144
ENSGACG00000000514	Thioredoxin-interacting protein	*txnip*	2.252	0.000123
ENSGACG00000011066	Protein phosphatase 1 regulatory subunit 3C *	*ppp1r3c*	2.213	0.000495
ENSGACG00000010966	Nucleoredoxin	*nxn*	1.741	0.00535
ENSGACG00000017856	Beta-crystallin B1	*crybb1*	1.733	0.0361
ENSGACG00000020884	S-phase kinase-associated protein 1	*skp1a*	1.732	0.000321
Ga_MT_1	Metallothionein *		1.708	0.00349
ENSGACG00000019469	Heme oxygenase	*hmox*	1.682	0.011
ENSGACG00000019868	DnaJ (Hsp40) homolog, subfamily A, member 3B	*dnaja3b*	1.68	0.000594
Gac.20319	Metallothionein *		1.515	0.00464
ENSGACG00000007648	Tetratricopeptide repeat protein 8 *	*ttc8*	1.351	0.000962
ENSGACG00000001320	RAB5-interacting protein *	*c20orf24*	0.912	0.0311
ENSGACG00000003052	Calreticulin Precursor *	*calr*	0.847	0.00704
ENSGACG00000005083	Alpha-2-macroglobulin-like 1	*a2ml1*	0.769	0.00168
ENSGACG00000007033	Protein disulfide-isomerase A3 *	*pdia3*	0.661	0.00103
ENSGACG00000002478	Methionine-R-sulfoxide reductase B2 *	*msrb2*	0.599	0.00746
ENSGACG00000017565	Nuclear receptor coactivator 2 *	*ncoa2*	0.499	3.20E-05
ENSGACG00000012104	Deiodinase, iodothyronine, type I	*dio1*	0.234	2.60E-06
ENSGACG00000009463	Cysteine sulfinic acid decarboxylase	*csad*	0.212	2.60E-06
ENSGACG00000012003	Thyroid hormone-inducible hepatic protein	*thrsp*	0.159	1.72E-05

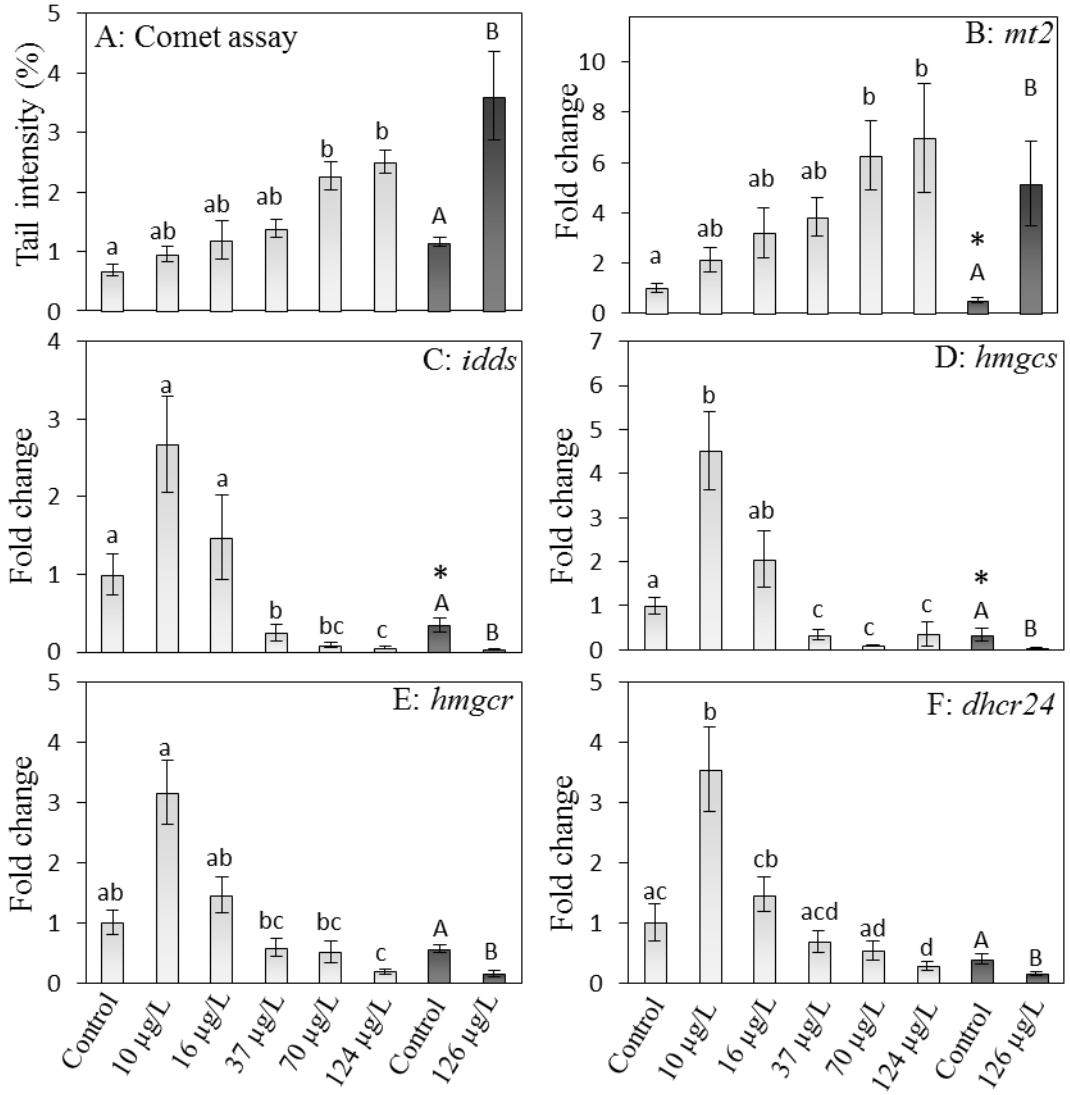
B. Transcripts differentially regulated following exposure to 46 ug/l Cu in the liver of Aire male sticklebacks

Gene ID	Name	Symbol	Cu/Ctrl	FDR
ENSGACG00000011066	Protein phosphatase 1 regulatory subunit 3C *	*ppp1r3c*	2.324	0.00494
Gac.20319	Metallothionein *		2.239	0.00222
ENSGACG00000007648	Tetratricopeptide repeat protein 8 *	*ttc8*	2.069	0.0226
Ga_MT_1	Metallothionein *		2.05	0.00245
Ga_S1TG_12G03	EST *		1.575	0.00425
ENSGACG00000003973	Glutamine synthetase	*glul*	1.504	0.0173
ENSGACG00000003445	Zinc finger protein 704	*znf704*	1.251	0.00836
ENSGACG00000016883	AP-1 complex subunit mu-1	*ap1m1*	0.846	0.0436
ENSGACG00000001320	RAB5-interacting protein *	*c20orf24*	0.82	0.014
ENSGACG00000017565	Nuclear receptor coactivator 2 *	*ncoa2*	0.716	0.034
ENSGACG00000013828	Glucosidase 2 subunit beta Precursor	*prkcsh*	0.692	0.0204
Gac.20103	EST similar to insulin-like growth factor 2 precursor		0.651	0.00425
ENSGACG00000005083	Alpha-2-macroglobulin-like 1	*a2ml1*	0.638	0.00425
ENSGACG00000020193	Subunit of the oligosaccharyltransferase complex	*stt3a*	0.583	0.0455
ENSGACG00000002478	Methionine-R-sulfoxide reductase B2, mitochondrial *	*msrb2*	0.54	0.00836
ENSGACG00000003052	Calreticulin Precursor *	*calr*	0.52	0.00425
ENSGACG00000014423	Protein canopy homolog 1	*cnpy1*	0.445	0.00435
ENSGACG00000007033	Protein disulfide-isomerase A3 *	*pdia3*	0.419	0.00136
ENSGACG00000010067	Heat shock protein 90, beta	*hsp90b1*	0.409	0.0247
ENSGACG00000017459	Microtubule-associated protein 1A	*map1a*	0.406	0.00222
ENSGACG00000016898	Calreticulin Precursor	*calr*	0.36	0.00136
ENSGACG00000002512	Protein disulfide-isomerase A4	*pdia4*	0.272	0.00222

Figure legends

Figure 1. Effects of copper exposure on F1 Siblyback and Houghton Spring fish: A- DNA damage measured using the Comet assay and B-F- transcript profiling conducted using RT-qPCR. Light-shaded bars represent the Siblyback population and dark coloured bars represent the population originating from Houghton Springs. Common letters indicate no significant difference (P>0.05) between groups from the same population. Asterisks indicate a significant difference (P<0.05) between control groups in each population.

Figure 1.



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SUPPORTING INFORMATION

Hepatic transcriptional responses to copper in the three-spined stickleback are affected by their pollution exposure history

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This supporting Information contains:

Supporting File 2. Transcripts significantly (FDR<0.05) differentially regulated between Siblyback male sticklebacks exposed to a measured concentration of 49 µg/L Cu and the controls

Supporting File 3. Annotation term enrichment analysis for transcripts differentially regulated in Siblyback sticklebacks exposed to 49 µg/L Cu, and between Siblyback and Aire control groups.

Table S1. Average measured water concentrations of copper and EE2 in the F0 exposure.

	Cu				EE2		
	0 µg/L	**3.2 µg/L**	**32 µg/L**	**128 µg/L**	**1 ng/L**	**10 ng/L**	**32 ng/L**
Aire	2.87	2.83	12.14	43.51	1.37	7.83	21.03
Siblyback	2.50	2.86	7.80	49.10	1.47	9.16	23.75

Table S2. Average measured water concentrations of copper in the F1 exposure.

Site	Treatment	Concentration (µg/L)
Siblyback Lake	control	7.88
	3.2 µg/L	10.55
	10 µg/L	16.45
	32 µg/L	37.45
	64 µg/L	70.2
	128 µg/L	123.5
Houghton Springs	HS control	7.94
	Hs 128 µg/L	126

Table S3. Transcriptional changes of Houghton Springs stickleback in response to 128 µg Cu/L or 32 ng EE2/L from Santos *et al.* 2010 and Katsiadaki *et al.* 2010., used for GSEA analysis.

Repressed after Cu exposure		
Gene ID	Name	Symbol
Gac.20329	Unnamed protein product	
ENSGACG00000020775	Lathosterol oxidase	*sc5dl*
ENSGACG00000016984	C-4 methylsterol oxidase	*sc4mol*
ENSGACG00000014264	7-dehydrocholesterol reductase	*dhcr7*
ENSGACG00000013539	Transmembrane protein 97	*tmem97*
ENSGACG00000011874	Farnesyl pyrophosphate synthetase	*fdps*
ENSGACG00000009052	Acetyl-CoA acetyltransferase, cytosolic	*acat2*
ENSGACG00000005228	Alpha-2 macroglobulin like-protein	*a2ml1*
ENSGACG00000003052	Calreticulin Precursor	*calr*
ENSGACG00000001814	Isopentenyl-diphosphate Delta-isomerase 2	*idi2*
ENSGACG00000001258	3-hydroxy-3-methylglutaryl-coenzyme A reductase	*hmgcr*
ENSGACG00000000860	Peptidylprolyl isomerase A, like	*ppial*

Induced after Cu exposure		
Gene ID	Name	Symbol
Gac.20319	Metallothionein	*mt1*
Ga_CDA_026E11	EST	
ENSGACG00000019954	UDP glucuronosyltransferase 2 polypeptide A3	*zgc:175099*
ENSGACG00000011464	U4/U6 small nuclear ribonucleoprotein Prp31	*prpf31*
ENSGACG00000016373	Transferrin receptor protein 1	*tfrc*
ENSGACG00000007648	Tetratricopeptide repeat protein 8	*ttc8*
ENSGACG00000015892	Ribosome biogenesis protein NSA2 homolog	*tinp1*
ENSGACG00000017481	Nucleolar GTP-binding protein 1	*gtpbp4*
ENSGACG00000018175	Cyclin-G1	*ccng1*
ENSGACG00000003491	Catalase	*cat*
ENSGACG00000015778	Betaine-homocysteine S-methyltransferase 1	*bhmt*
ENSGACG00000006029	Betaine-homocysteine S-methyltransferase 1	*bhmt*
ENSGACG00000007836	Actin, beta-like 2	*actbl2*
ENSGACG00000003641	Adenosylhomocysteinase	*ahcy*
ENSGACG00000000326	Actin, beta-like 2	*actbl2*
ENSGACG00000004710	4-aminobutyrate aminotransferase, mitochondrial Precursor	*abat*

Induced after EE2 exposure		
Gene ID	Name	Symbol
ENSGACG00000002767	60S ribosomal protein L7	*rpl7*
ENSGACG00000012495	Elongation factor Tu GTP-binding domain-containing protein 1	*eftud1*
ENSGACG00000011880	Glycogen phosphorylase, liver form	*pygl*
ENSGACG00000011891	Mps one binder kinase activator-like 2C	*mobkl2c*
ENSGACG00000009200	Mx protein	*q804s8_fugru*
ENSGACG00000007695	NADPH--cytochrome P450 reductase	*por*
ENSGACG00000003367	NK-tumor recognition protein	*nktr*
ENSGACG00000007240	Probable prolyl-tRNA synthetase, mitochondrial Precursor	*pars2*
ENSGACG00000002512	Protein disulfide-isomerase A4 Precursor	*pdia4*
ENSGACG00000005904	Transglutaminase 5-like	*tgm5l*
ENSGACG00000002960	Succinate-semialdehyde dehydrogenase, mitochondrial Precursor	*aldh5a1*
ENSGACG00000018584	Talin-1	*tln1*
ENSGACG00000018876	Transmembrane protein 85	*tmem85*
ENSGACG00000009490	Vitellogenin C	*vtg3*
ENSGACG00000012381	Zona pellucida sperm-binding protein 3 Precursor	*zp3*
ENSGACG00000011851	Zona pellucida sperm-binding protein 4 Precursor	*zp4*
Gac.2235	EST	
Gac.5890	EST	

Table S4. Transcripts differentially regulated between Aire stickleback males with low (<10mg) and high (>10mg) kidney weight

Gene ID	Name	Symbol	Low/High	FDR
ENSGACG00000012381	Zona pellucida sperm-binding protein 3	*zp3*	3.176	0.000265
ENSGACG00000002960	Succinate-semialdehyde dehydrogenase, mitochondrial	*aldh5a1*	2.787	0.000265
ENSGACG00000007240	Probable prolyl-tRNA synthetase, mitochondrial	*pars2*	2.422	0.000597
ENSGACG00000011891	Mps one binder kinase activator-like 2C	*mobkl2c*	1.829	0.00104
ENSGACG00000005956	Choline dehydrogenase, mitochondrial	*chdh*	1.533	0.019
ENSGACG00000003509	Uncharacterised protein		0.833	0.0207
ENSGACG00000005045	Zinc finger RNA-binding protein	*zfr*	0.827	0.0207
ENSGACG00000020874	Solute carrier family 22 member 4	*slc22a4*	0.791	0.00772
ENSGACG00000019268	Peroxisomal acyl-coenzyme A oxidase 1	*acox1*	0.772	0.00772
ENSGACG00000009299	Multidrug resistance protein 3	*abcb4*	0.76	0.0226
Gac.12497	EST		0.749	0.0171
ENSGACG00000006219	Solute carrier family 27 (fatty acid transporter), member 2	*slc27a2*	0.662	0.00318
ENSGACG00000011783	Solute carrier family 13 member 3	*slc13a3*	0.609	0.00108
Gac.8086	EST		0.593	0.0226
ENSGACG00000007411	Sulfotransferase family 1, cytosolic sulfotransferase 6	*sult1st6*	0.536	0.00525

Table S5. Transcripts differentially regulated between the Aire and Siblyback control groups

Gene ID	Name	Symbol	Siblyback/Aire	FDR
ENSGACG00000010817	Inactive L-threonine 3-dehydrogenase, mitochondrial Precursor	TDH	3.50	0.02
ENSGACG00000014852	Complement C1q-like protein 2; Flags: Precursor	si:rp71-1g18.7	2.83	0.03
ENSGACG00000006786	Target of Myb protein 1	TOM1 (2 of 2)	2.81	0.01
ENSGACG00000019606	Malate dehydrogenase, cytoplasmic	MDH1 (1 of 2)	2.75	0.02
ENSGACG00000007534	Aspartate aminotransferase, cytoplasmic	GOT1 (2 of 2)	2.67	0.02
ENSGACG00000015857	Fructose-1,6-bisphosphatase 1	FBP1	2.58	0.01
ENSGACG00000006155	5'-nucleotidase domain-containing protein 1	NT5DC1	2.55	0.03
ENSGACG00000003641	Adenosylhomocysteinase	AHCY	2.51	0.01
ENSGACG00000017044	Annexin A5	ANXA5 (1 of 2)	2.51	0.03
Gac.20319	Metallothionein		2.50	0.03
ENSGACG00000012866	Alpha-1,6-mannosyl-glycoprotein 2-beta-N-acetylglucosaminyltransferase	MGAT2	2.46	0.03
ENSGACG00000017099	Ubiquitin carboxyl-terminal hydrolase isozyme L1	UCHL1	2.44	0.03
ENSGACG00000001534	Hydroxyacid-oxoacid transhydrogenase, mitochondrial Precursor	ADHFE1	2.36	0.01
ENSGACG00000002813	Glutamate dehydrogenase 1, mitochondrial Precursor	GLUD1	2.35	0.02
Ga_MT_1	Metallothionein		2.34	0.05
ENSGACG00000017982	Fructose-bisphosphate aldolase B	ALDOB	2.24	0.01
ENSGACG00000010219	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	2.16	0.01
ENSGACG00000013529	protein phosphatase 1, catalytic subunit, alpha-like	wu:fi22e08	2.16	0.01
ENSGACG00000020857	FAD-dependent oxidoreductase domain-containing protein 1	FOXRED1	2.16	0.03
ENSGACG00000015778	Betaine--homocysteine S-methyltransferase 1	BHMT	2.12	0.02
ENSGACG00000007648	Tetratricopeptide repeat protein 8	TTC8	2.11	0.04
ENSGACG00000011008	Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial Precursor	ALDH4A1	2.10	0.04
ENSGACG00000002519	Alanine aminotransferase 1	GPT (1 of 2)	2.08	0.02
ENSGACG00000010933	Beta-microseminoprotein		2.07	0.01
ENSGACG00000017283	uncoupling protein 4	ucp4	2.04	0.03
ENSGACG00000017272	Alanine aminotransferase 1	GPT (2 of 2)	2.01	0.01
ENSGACG00000013959	Fumarylacetoacetase	FAH	2.01	0.03
ENSGACG00000006183	GTP cyclohydrolase 1 feedback regulatory protein	GCHFR	2.00	0.01
ENSGACG00000013666	Serine--pyruvate aminotransferase	AGXT (2 of 2)	1.99	0.01
ENSGACG00000013918	Beta globin.	Q804E5_GASAC	1.98	0.02
ENSGACG00000009946	heme-binding protein 2	A8KBA7_DANRE	1.98	0.05
Gac.6392			1.97	0.04
ENSGACG00000020619	Fructose-bisphosphate aldolase C	ALDOC (1 of 2)	1.95	0.02
ENSGACG00000007323	histamine n-methyltransferase		1.95	0.04
Ga_NmlG_034A1			1.94	0.04
ENSGACG00000004520	Ankyrin repeat domain-containing protein 5	ANKRD5	1.94	0.02
ENSGACG00000007727	Fructose-bisphosphate aldolase A	ALDOA (1 of 2)	1.92	0.01
ENSGACG00000001099	Plexin-B2 Precursor	PLXNB2 (3 of 3)	1.91	0.03
Gac.7287	Hypothetical protein LOC574426		1.90	0.05
Gac.5739			1.90	0.04
ENSGACG00000005965	Tripartite motif-containing protein 47	TRIM47 (14 of 15)	1.85	0.02

ENSGACG00000017414	7-alpha-hydroxycholest-4-en-3-one 12-alpha-hydroxylase	CYP8B1	1.84	0.03
ENSGACG00000020231	Williams-Beuren syndrome chromosomal region 14 protein	MLXIPL	1.84	0.03
ENSGACG00000006667	Transcription factor Sp1	SP1	1.81	0.04
ENSGACG00000000973	Malate dehydrogenase, cytoplasmic	MDH1 (2 of 2)	1.80	0.02
ENSGACG00000018725	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	1.80	0.01
Gac.6583	c5orf4 protein		1.79	0.01
ENSGACG00000012993	Transmembrane protein 91	TMEM91	1.77	0.05
ENSGACG00000013895	Hemoglobin subunit epsilon	HBE1	1.77	0.04
ENSGACG00000020047	tyrosine hydroxylase 2	NP_001027874.1	1.77	0.04
ENSGACG00000014573	Ribose-phosphate pyrophosphokinase 2	PRPS2	1.77	0.02
ENSGACG00000012482	Electron transfer flavoprotein subunit beta	ETFB	1.75	0.01
ENSGACG00000006498	Monocarboxylate transporter 10	SLC16A10	1.74	0.01
ENSGACG00000005192	2-oxoisovalerate dehydrogenase subunit beta, mitochondrial Precursor	BCKDHB	1.73	0.02
ENSGACG00000018299	Keratin, type I cytoskeletal 13	KRT13	1.73	0.02
ENSGACG00000000824	Probable urocanate hydratase	UROC1	1.71	0.03
Ga_NmlY_10D10			1.69	0.03
Gac.5942	necdin-like 2		1.68	0.01
Gac.735			1.67	0.01
ENSGACG00000008815	Nucleolar protein 56	NOP56	1.67	0.01
Gac.8760	cdgsh iron sulfur domain-containing protein 1		1.67	0.03
ENSGACG00000005520	Upstream-binding protein 1	UBP1	1.66	0.05
ENSGACG00000014527	Fructose-1,6-bisphosphatase isozyme 2	FBP2	1.65	0.02
ENSGACG00000015460	Nicotinamide mononucleotide adenylyltransferase 3	NMNAT3	1.64	0.02
ENSGACG00000013449	Novel protein similar to vertebrate glyceronephosphate O-acyltransferase	B8JM22_DANRE	1.64	0.04
ENSGACG00000007695	NADPH--cytochrome P450 reductase	POR	1.63	0.02
ENSGACG00000001522	Myosin heavy chain M001034	Q2HWQ5_FUGRU	1.62	0.02
ENSGACG00000020054	Probable imidazolonepropionase	AMDHD1	1.58	0.05
ENSGACG00000001242	Ankyrin repeat, SAM and basic leucine zipper domain-containing protein 1	ASZ1 (1 of 2)	1.58	0.03
ENSGACG00000006087	Malate dehydrogenase, mitochondrial Precursor	MDH2	1.56	0.02
ENSGACG00000004178	Fructose-bisphosphate aldolase A	ALDOA (2 of 2)	1.56	0.01
ENSGACG00000020941	ATP synthase F0 subunit 6	NP_443416.1	1.56	0.02
ENSGACG00000017443	Caspase-6 Precursor	CASP6	1.53	0.01
ENSGACG00000010028	60S ribosomal export protein NMD3	NMD3	1.53	0.05
ENSGACG00000015892	Ribosome biogenesis protein NSA2 homolog	TINP1	1.52	0.05
ENSGACG00000018053	UPF0498 protein KIAA1191	KIAA1191 (2 of 2)	1.52	0.02
ENSGACG00000015287	atg3 autophagy related 3 homolog		1.50	0.02
ENSGACG00000005864	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	GAPDHS	1.49	0.03
ENSGACG00000001640	Cytochrome P450 2J2	CYP2J2 (3 of 6)	1.49	0.03
ENSGACG00000017403	Vacuolar protein sorting-associated protein 33B	VPS33B	1.47	0.05
ENSGACG00000016095	Alpha-2-HS-glycoprotein Precursor	AHSG	1.47	0.02
ENSGACG00000020842	RNA-binding protein 41	RBM41	1.45	0.02
ENSGACG00000018310	Keratin, type I cytoskeletal 13	KRT13	1.44	0.04
ENSGACG00000019607	UTP--glucose-1-phosphate uridylyltransferase	UGP2 (2 of 2)	1.42	0.05
Gac.16606			1.41	0.02
ENSGACG00000018201	Protein KIAA1045	KIAA1045	1.39	0.02

ENSGACG00000013450	Proline-rich AKT1 substrate 1	AKT1S1	1.39	0.01
ENSGACG00000018559	Integral membrane protein 2A	ITM2A	1.39	0.02
ENSGACG00000003516	Eukaryotic translation initiation factor 3 subunit L	EIF3EIP	1.39	0.04
ENSGACG00000004336	39S ribosomal protein L15, mitochondrial Precursor	MRPL15	1.39	0.05
Gac.13621			1.39	0.03
ENSGACG00000014690	Natterin-3; Flags: Precursor		1.37	0.05
Gac.6014			1.35	0.02
ENSGACG00000006838	Selenoprotein P Precursor	Sepp1	1.34	0.04
ENSGACG00000015484	Ferritin heavy polypeptide-like 17	FTHL17	1.32	0.03
ENSGACG00000001133	Macrophage asialoglycoprotein-binding protein 1	A2CEH6_DANRE	1.28	0.04
ENSGACG00000018627	Importin subunit alpha-8	KPNA7	1.27	0.03
ENSGACG00000008584	Vitronectin Precursor	VTN (1 of 2)	1.24	0.01
ENSGACG00000004528	Histidine ammonia-lyase	HAL	1.19	0.05
ENSGACG00000007026	Probable ATP-dependent RNA helicase DDX5	DDX5 (1 of 2)	0.81	0.02
ENSGACG00000000529	Uncharacterized protein C1orf77	C1orf77	0.80	0.02
ENSGACG00000017908	Complement component C6 Precursor	C6	0.76	0.03
ENSGACG00000014416	Phosphatidylcholine transfer protein	PCTP	0.71	0.01
Gac.13403			0.70	0.02
ENSGACG00000005452	Guanine nucleotide-binding protein G	GNB1 (1 of 2)	0.69	0.03
Gac.13040			0.68	0.03
ENSGACG00000016323	Complement component C8 beta chain Precursor	C8B	0.67	0.05
ENSGACG00000002588	multiple coagulation factor deficiency 2		0.67	0.04
ENSGACG00000009473	UPF0546 membrane protein C1orf91	C1orf91	0.66	0.03
ENSGACG00000019459	Nucleosome assembly protein 1-like 3	NAP1L3	0.64	0.04
ENSGACG00000008607	Signal transducer and activator of transcription 3	STAT3	0.64	0.04
ENSGACG00000018891	Aldose reductase	AKR1B1	0.62	0.02
ENSGACG00000006631			0.62	0.05
ENSGACG00000000830	Protein transport protein Sec61 subunit alpha isoform 1	SEC61A1 (2 of 2)	0.61	0.03
ENSGACG00000015685	Protein transport protein Sec31A	SEC31A	0.60	0.03
ENSGACG00000013828	Glucosidase 2 subunit beta Precursor	PRKCSH	0.59	0.01
ENSGACG00000017028	fk506-binding protein 1a		0.59	0.04
ENSGACG00000020678	Peroxiredoxin-4	PRDX4	0.59	0.05
ENSGACG00000016984	C-4 methylsterol oxidase	SC4MOL	0.57	0.02
ENSGACG00000019282	Beta-2-microglobulin Precursor	B2M (1 of 2)	0.57	0.03
ENSGACG00000005487	Aldehyde dehydrogenase family 3 member B2	ALDH3B2	0.56	0.03
ENSGACG00000007851	Transmembrane protein 150	B8A6A2_DANRE	0.55	0.01
ENSGACG00000002397	delta-9-desaturase 1	NP_001072045.1	0.54	0.04
ENSGACG00000003027	E3 ubiquitin-protein ligase RNF123	RNF123 (1 of 2)	0.54	0.03
Gac.20312	unnamed protein product		0.53	0.05
ENSGACG00000020401	transmembrane 4 l6 family member 4	NP_001098587.1	0.53	0.03
ENSGACG00000004729	Reticulocalbin-3 Precursor	RCN3	0.50	0.05
ENSGACG00000019523	Novel protein	B8JHX2_DANRE	0.50	0.05
ENSGACG00000002457	NADH-cytochrome b5 reductase 2	CYB5R2	0.49	0.01
Ga_NmlY_15B03			0.49	0.02
ENSGACG00000020563	Dolichyl-phosphate beta-glucosyltransferase	ALG5	0.49	0.03
ENSGACG00000009052	Acetyl-CoA acetyltransferase, cytosolic	ACAT2	0.44	0.02
ENSGACG00000003052	Calreticulin Precursor	CALR	0.41	0.02
ENSGACG00000010067	Heat shock protein 90 kDa beta member 1	HSP90B1	0.39	0.03

ENSGACG00000020193	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3A	STT3A	0.38	0.01
ENSGACG00000007238	Peptidyl-prolyl cis-trans isomerase B Precursor	PPIB	0.38	0.01
ENSGACG00000007033	Protein disulfide-isomerase A3 Precursor	PDIA3	0.37	0.01
ENSGACG00000002365	GTP-binding protein SAR1a	SAR1A	0.37	0.01
ENSGACG00000000672	nod3 protein		0.36	0.03
ENSGACG00000009188	Interferon-induced GTP-binding protein Mx1	MX1	0.36	0.03
ENSGACG00000014423	Protein canopy homolog 1	CNPY1	0.35	0.01
ENSGACG00000001814	Isopentenyl-diphosphate Delta-isomerase 2	IDI2	0.35	0.03
Ga_NmlY_02A08			0.35	0.01
Ga_NmlY_12A08			0.35	0.02
Gac.14758	EST;unnamed protein product CAG03272		0.33	0.02
Gac.5890			0.33	0.03
Ga_S1TG_25C10			0.32	0.03
ENSGACG00000013539	Transmembrane protein 97	TMEM97	0.31	0.01
ENSGACG00000016898	Calreticulin Precursor	CALR	0.31	0.01
ENSGACG00000014264	7-dehydrocholesterol reductase	DHCR7	0.30	0.02
ENSGACG00000017459	Microtubule-associated protein 1A	MAP1A	0.28	0.01
ENSGACG00000002512	Protein disulfide-isomerase A4 Precursor	PDIA4	0.27	0.02
ENSGACG00000004468	Tuftelin	TUFT1 (1 of 2)	0.25	0.02
ENSGACG00000009200	Mx protein	Q804S8_FUGRU	0.24	0.04
ENSGACG00000001319	Interferon-induced protein 44	IFI44	0.20	0.05
Gac.6711	Tuftelin		0.17	0.02
ENSGACG00000016488	Elongation of very long chain fatty acids protein 6	ELOVL6	0.14	0.02