Identification of hypoxia-specific biomarkers in salmonids using RNA-sequencing and validation using high-throughput qPCR

Running title: Hypoxia biomarkers in salmonids

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Abstract

Identifying early gene expression responses to hypoxia (i.e., low dissolved oxygen) as a tool to assess the degree of exposure to this stressor is crucial for salmonids, because they are increasingly exposed to hypoxic stress due to anthropogenic habitat change, e.g., global warming, excessive nutrient loading, and persistent algal blooms. Our goal was to discover and validate gill gene expression biomarkers specific to the hypoxia response in salmonids across

multi-stressor conditions. Gill tissue was collected from 24 freshwater juvenile Chinook salmon (*Oncorhynchus tshawytscha*), held in normoxia [dissolved oxygen (DO) > 8 mg L^{-1}] and hypoxia (DO < 4□5 mg L^{-1}) in 10 and 18°C temperatures for up to six days. RNA-sequencing (RNA-seq) was then used to discover 240 differentially expressed genes between hypoxic and normoxic conditions, and not affected by temperature. The most significantly differentially expressed genes had functional roles in the cell cycle and suppression of cell proliferation associated with hypoxic conditions. The most significant genes (n = 30), based on p-values were selected for real-time qPCR assay development. These assays demonstrated a strong correlation ($r = 0.88$; $p < 0.001$) between the expression values from RNA-seq and the fold changes from qPCR. Further, qPCR of the 30 candidate hypoxia biomarkers was applied to an additional 322 Chinook salmon exposed to hypoxia and normoxia conditions to reveal the top biomarkers to define hypoxic stress. Multivariate analyses revealed that smolt stage, water salinity, and morbidity status were relevant factors to consider with the expression of these genes in relation to hypoxic stress. These hypoxia candidate genes will be put into application screening Chinook salmon to determine the identity of stressors impacting the fish.

Keywords: gene expression, hypoxia biomarkers, oxygen, microfluidics qPCR, RNA-seq,

Introduction

Aquatic ecosystems are increasingly being affected by a complex mixture of anthropogenic stressors (Hering et al. 2015). In recent years, the prevalence and intensity of aquatic hypoxic episodes have increased worldwide, resulting in aquatic environments with large variations in

dissolved oxygen (Leveelahti et al. 2011; Robertson e t al. 2014). Both natural and anthropogenic drivers can be responsible for dissolved oxygen (DO) depletion in aquatic ecosystems. Natural hydrodynamic conditions, such as the oxidation of organic matter, and the release of gases (e.g., methane or carbon dioxide) may make certain aquatic ecosystems prone to oxygen depletion. However, climate change can also reduce the solubility of oxygen in water due to increase of thermal stratification and enhance microbial activity, all which would cause DO depletion (Weinke and Biddanda, 2018). Moreover, agriculture, industry, and urbanization can lead to nutrient loading (i.e., eutrophication) and consequent persistent algal blooms in aquatic ecosystems, which will also affect both the supply and uptake of DO (Friedrich et al. 2014). The combined effects of warming and eutrophication in aquatic ecosystems may cause dramatic declines in DO, which can lead to hypoxic conditions in aquatic environments (DO < 4□5 mg L^{-1}).

Hypoxia can adversely impact a wide variety of biochemical and physiological processes in fish, such as cell cycle, fish growth, and aerobic metabolism (Zhang et al. 2009; Douxfils et al. 2012). Hypoxia can also negatively impact normal biological functions, resulting in suppressed development, reduced activity, feeding and growth, disturbed endocrine function, and impaired reproductive performance (Callier et al. 2013; Wang et al. 2016; Abdel-Tawwab et al. 2019). Under extreme hypoxic conditions, mass mortality of wild populations can occur (Douxfils et al. 2012; Long et al. 2015; Closs et al. 2016).

Salmon can be negatively impacted by hypoxia, through impacts on swim performance, growth and development, which may ultimately affect fitness and survival. Typically, fish respond to hypoxia through a number of behavioural, morphological, physiological and molecular changes. Behaviourally, fish can actively avoid hypoxic areas or reduce energy

demands by decreasing swimming activity. Morphologically, fish can adapt to have increased respiratory surface area on the gills. Physiological and molecular adaptations can include enhanced production of respiratory proteins (e.g. hemoglobin, myoglobin and neuroglobin) to increase oxygen carrying capacity, up-regulation of genes encoding enzymes within the glycolytic pathway, and down-regulation of genes involved in aerobic energy production and other energy-consuming processes (Tiedke et al. 2015). Differential regulation of genes involved in general metabolism, catabolism, and the ubiquitin-proteasome pathway have also been demonstrated in fish responses to low DO (Ju et al. 2007; Zhang et al. 2012; Olsvik et al. 2013).

Every year, billions of juvenile salmon pass through estuaries on the North American Pacific coast, where anthropogenic impacts and climate change may increase exposure to hypoxic waters (Birtwell and Kruzynski 1989). Given the intensive migrations these fish must endure to reach optimal feeding grounds in the ocean, and their need to evade predators along the way (Quinn, 2005), if exposure to low DO results in reduced swimming performance, as demonstrated by Bjornn and Reiser (1991), it could have dire indirect consequences on survival. Eutrophic lakes can also become hypoxic (Conley et al., 2009), which may be particularly problematic for fry and for adult salmon utilizing these habitats, e.g., Sockeye salmon *Oncorhynchus nerka* in Cultus Lake, BC (Putt et al. 2019), and Okanagan system lakes (Hyatt et al. 2003). Low DO can also have adverse effects in combination with other stressors (Del Rio et al. 2019), and under extreme conditions, low DO can be lethal to salmonids (Carter, 2005).

Measuring DO concentrations in aquatic ecosystems is one aspect of monitoring hypoxic stress. However, frequent DO measurements over large regions and long periods of time are impractical, unless sampling moorings are continuously used. Furthermore, such environmental measurements do not directly address whether hypoxic stress was experienced by a fish. We

know that low DO conditions have serious consequences for farmed salmon (e.g. Burt et al. 2012; Solstorm et al. 2018), but it is not clear whether migratory fish inhabiting or moving through such conditions can behaviourally moderate their exposure or whether they are also negatively affected. The application of biomarkers to detect the specific stress response to DO, and to other stressors for that matter, could thus be an important approach (Zhang et al. 2012; Akbarzadeh et al. 2018), and be a more integrative technique (Froehlich et al. 2015; Houde et al. 2019a). Biomarkers can be defined as measurable biochemical, cellular, and/or physiological changes in an organism caused by perturbations in the environmental conditions, such as hypoxia. The ideal biomarker should be specific to the stressor of interest, be easy to assay, and be relatively unaffected by sampling procedures (Zhang et al. 2012).

A major advantage of the biomarker approach is the ability to detect sub-lethal impacts at low levels of stressor intensity. Moreover, the inferred consequences of sub-lethal exposure can be made more robust because the biomarkers can also provide detailed physiological information of an organism (Froehlich et al. 2015). Molecular ecologists have become interested in studying early genetic responses of various organisms to stressors such as hypoxia (Nikinmaa and Rees, 2005; Boswell et al. 2009). Several approaches have been used to identify hypoxia responsive genes in fishes and their altered expression levels, including microarrays, real-time qPCR, and proteomics (Rashid et al. 2017**)**. RNA-seq has been more recently used to identify the transcriptional responses of fish to hypoxia at the whole transcriptome level without a reference genome (Olsvic et al. 2013; Long et al. 2015; Zhang et al. 2016).

As most genes and proteins can be involved in several physiological pathways, it may not be realistic to obtain such a specific predictor with a single biomarker. Miller et al. (2017) demonstrated that the detection of a viral disease state in salmon could be accomplished by the

co-regulation of as few as seven biomarkers. This approach has led to the discovery of several novel viruses in salmon (Mordecai et al. 2020) and the elucidation of the developmental pathway of a natural disease outbreak, from a viral carrier state to disease causing death (Di Cicco et al. 2018). Recently, salmonid biomarker panels have been developed and validated for the detection of thermal and salinity stress (Akbarzadeh et al. 2018; Houde et al. 2019a), but the identification of a panel specific to hypoxic stress activated in gill tissue has proven to be more difficult. This may be due to a lack of available transcriptional studies to mine for candidate hypoxia biomarkers in salmon gill, whereas such studies were available for thermal and salinity stress. For hypoxia, only two genes (i.e., *hypoxia inducible factor 1 alpha* [*hif1a*] and *cytochrome C oxidase subunit 6B1* [*cox6b1*]) of the 24 candidate hypoxia genes applied to the gill tissue of juvenile ocean-type Chinook salmon (*O. tshawytscha*) exposed to combinations of salinity, temperature, and DO manipulations were primarily influenced by DO. However, these two genes alone were not sufficient to separate salmon exposed to normoxia from those exposed to hypoxia across differing salinities and temperatures.

Our objective here was to discover and validate a biomarker panel of at least eight genes specific to the hypoxia response across several conditions. To this end, we applied RNA-seq (Ozsolak and Milos 2011, Wang et al. 2009) with best practises for analysis (Conesa et al. 2016) to discover additional candidate genes for the response to hypoxia in salmonids. In total, gill tissue from 24 freshwater (FW) Chinook salmon juveniles were sequenced after exposure to normoxia or hypoxia within 10 and 18°C temperatures (n = 6 fish per condition). These fish were sourced from a previous exposure experiment (Houde et al. 2019a). TaqMan assays were then designed to the top 30 candidate hypoxia genes (based on p-values) that were consistent across

temperatures. Biomarkers were validated using 301 Chinook salmon from the challenge study that were not used for sequencing.

Our ultimate aim is to add a validated hypoxia-specific biomarker panel to similar panels for thermal, salinity, and general stress, as well as panels indicating different disease states (viral disease, inflammation/wounding response, humoral/cellular response, imminent mortality) applied simultaneously within a newly developed microfluidics-based salmon "Fit-Chip" tool (Miller et al. 2018). The Fit-Chips will then be applied to examine the synergistic interplay between stress and disease, to elucidate the combinations of stressors most impactful to salmon survival, and to identify habitats where fish are the most compromised. This information can then be used to develop plans for environmental mitigation.

Materials and Methods

Experimental set-up

The experiment was approved by the Fisheries, Oceans and the Canadian Coastguard (DFO) Pacific Region Animal Care Committee (2017-002) following the Canadian Council of Animal Care Standards. Sub-yearling Chinook salmon were provided by Big Qualicum Hatchery, Qualicum, British Columbia (BC), Canada, and were reared in communal tanks at the Pacific Biological Station, Nanaimo, BC. Juveniles were exposed to FW (< 14°C), light at the natural cycle, and fed a ration of commercial pellets (2% body mass, Bio-Oregon) every 1–2 days until used in the experiment. Four trials were conducted over the smoltification period, covering pre-smolt (March), smolt (May), and de-smolt (two trials, June and August) stages based on expected differences in seawater (SW) mortality, gill Na^+/K^+-ATPase activity, and body variables (see Houde et al. 2019a).

During the experiment, as detailed in Houde et al. (2019a) and in brief here, juveniles were exposed to 18 possible water treatments: three salinities (FW at 0 PSU, brackish (BW) at 20 PSU, and SW at 28 or 29 PSU), three temperatures (10, 14, and 18°C), and two dissolved oxygen (DO) concentrations (hypoxia 4□5 mg L^{-1} and normoxia > 8 mg L^{-1}) in all combinations. Each treatment was represented by two (replicate) 30 L pot tanks with tight fitting lids that limited gas exchange. Water at the desired salinity and temperature was divided into two PVC columns: one with media for typical aeration for normoxia and the other with a ceramic air stone delivering very small nitrogen bubbles (5–10 μm) for hypoxia. Nitrogen was supplied to the system using portable liquid units or compressed gas bottles (Praxair). Hypoxia conditions were measured to ensure target levels using nine DO probes, where a single probe was present in one of the two replicate tanks. Each probe was connected to a Point Four RIU3 monitor-controller (Pentair) for turning the nitrogen regulator on or off as required to keep DO in range. Collectively, the controllers were connected to a Point Four LC3 central water system (Pentair).

Juvenile handling

Prior to treatment exposure, 12□16 juveniles were acclimated to each tank (n = 18 tanks) for six days under the control conditions (i.e., FW, 13□14°C, normoxia), and fed a ration every day. Water conditions were then changed over 1□2 days. On day 1, salinated water was introduced over 2□3 h in morning, then temperature was changed by 2°C in the early afternoon, and dissolved oxygen was set to 6.5□8 mg L^{-1} in the mid-afternoon using the LC3 system. On the morning of day 2, temperature was changed by the remaining 2°C and DO was set to 4□5 mg L^{-1}. Juveniles were then exposed to full treatment conditions for six days and were fed rations with 48 h starvation before final sampling. On day six, juveniles were euthanized in an overdose of

TMS (250 mg L^{-1}, buffered for FW treatments) using normoxic water of the same salinity and temperature. Gill tissue from the left side was dissected, placed in RNAlater (Invitrogen) for 24 h in a 4°C fridge, and then stored in a -80°C freezer until used for RNA extraction.

As previously described by Houde et al. (2019a), the two SW-18°C-hypoxia tanks in the June, de-smolt trial (i.e., trial 3) had lower DO than intended in the SW treatment due to a programming failure (3.3–4.1 mg L^{-1} instead of 4–5 mg L^{-1}). After almost two days of exposure, 11 of 24 juveniles were dead or moribund, and the remaining 13 juveniles were ethically euthanized. Due to a poor separation between normoxia and hypoxia using the general hypoxia candidate genes previously applied (see Houde et al. 2019a), these fish were used as an 'extreme hypoxia' condition. Greater details on juvenile handling and sampling are described in Houde et al. (2019a)

RNA extraction and sample selection

Gill tissues from all of the samples described above were homogenized in TRIzol (Ambion) and 1–bromo–3–chloropropane (BCP) reagent with stainless steel beads using a mixer mill (Retch Inc., MM301) in 30 Hz for 3 min. The aqueous supernatant of the homogenate was removed and then used for extraction of RNA using the 'No-Spin Procedure' of MagMAX-96 Total RNA Isolation kits (Ambion) as per manufacturer's instructions and a Biomek FXP automation workstation (Beckman-Coulter). The resulting total RNA was stored at -80°C.

A subset of 24 individuals was selected for RNA-seq from the two de-smolt trials (Trials 3 and 4). The FW individuals were selected as there is an immediate need for application of the developed panel in freshwater. The more extreme temperatures were selected to ensure robustness of markers across different temperatures. Two individuals from each tank replicate were selected from Trial 3, and one individual from each tank replicate from Trial 4. Conditions

included were two temperatures (10°C and 18°C) and two DO conditions (normoxia and hypoxia).

Library preparation and sequencing

RNA-seq library preparation and sequencing was performed by D-Mark at the University of California, Davis, California, USA. Total RNA integrity of extracted gill tissue samples was measured using a BioAnalyzer 2100 (Agilent). Samples with RNA integrity numbers (RIN) ≥ 6 were selected for libraries and sequencing; one hypoxia-18°C individual from the June trial was substituted with a similar individual from the August trial to meet this standard.

Libraries were constructed using NEBNext Ultra RNA Library Prep for Illumina kits (New England Biolabs kits E7490 and E7530), as per manufacturer's instructions. Briefly, mRNA was enriched using oligo (dT) magnetic beads, fragmented, and reverse transcribed to cDNA. Samples were ligated with NEBNext multiplex oligo adaptor kits (New England Biolabs) for barcoding individual samples. Ligated DNA was then amplified by PCR. Libraries were quantified using a Qubit 2.0 fluorimeter and quality checked for insert size using a BioAnalyzer, then precisely quantified using KAPA Biosystems universal Illumina library quantification kit (Roche). Barcoded, prepared samples were pooled into two pools (with 10 and 14 individuals each) for sequencing on a HiSeq 4000 (Illumina) using paired-end (PE) 150 mode without PhiX with the aim to obtain approximately 10 million PE reads per sample. Samples from both DO conditions were present in approximate equal ratio in both lanes.

Sequence read processing

All bioinformatics analyses are reported in detail including all scripts on GitHub (see *Data Accessibility*). Raw reads were quality checked with FastQC 0.11.5 (Andrews, 2016), with

individual sample files compiled using MultiQC 1.0 (Ewels et al. 2016). Adaptors, poor quality sequences (Phred = 2, MacManes, 2014), and reads with a length less than 80 bp were removed using Trimmomatic v0.36 (Bolger et al. 2014) using flags *illuminaclip* (2:30:10), *slidingwindow* (20:2), *leading* (2) and *trailing* (2), and *minlen* (80). Trimmed reads were quality checked again with FastQC and MultiQC.

The Tuxedo protocol was followed for read alignments and assembling transcripts for quantification (Pertea et al. 2016). Trimmed PE reads were aligned to an indexed reference genome of Chinook salmon (Otsh_v1.0, RefSeq ID 6017098, Christensen et al. 2018) using the spliced aligner HISAT v2.1.0 (Kim et al. 2015) allowing up to 40 multi-mapping alignments (k = 40). The annotated reference genome contained 87,036 transcripts, and 82,000 intron chains. After alignment, the SAM file per individual was converted to a sorted BAM file with samtools v1.5 (Li et al. 2009). Alignments were then assembled into potential transcripts per individual with the reference genome as a guide using StringTie 1.3.4 (Pertea et al. 2015). Individual assemblies were merged to provide a final assembly, i.e. a combination of known transcripts from the reference genome annotation and novel transcripts not in the reference. The guided assembly was compared to the reference genome using *gffcompare* 0.10.4 from StringTie to identify novel features. This combined gff was then used to quantify read counts per transcript for each individual using StringTie. Counts per individual were merged into a single table and converted to transcript counts using the *prepDE.py* script provided by StringTie.

Statistical analysis for discovering candidate hypoxia genes

After converting to gene counts, RNA-seq analyses were conducted using R 3.4.4 (R Core Team, 2018). The transcript counts per individual were analyzed using the *edgeR* v.3.7 package

(Robinson et al. 2010) and general pipeline (Anders et al. 2013). Gene counts were filtered for low expressed genes using a 0.4 count-per-million (cpm) threshold, corresponding to a count of 5 reads in the sample with the fewest reads. A gene was removed if it was below this threshold in at least half the individuals (n = 12). Filtered counts were analyzed using multi-dimensional scaling (MDS) method to evaluate the effects of different factors (i.e., temperature, hypoxia, trial) on overall gene expression. Then, the filtered counts were normalized and dispersion estimated using a model design containing the four treatments with no intercept. A robust quasi-likelihood generalized linear model was then fit to the counts. Given the potential issues of separating thermal stress from hypoxic stress responses (Pörtner, 2010), quasi-likelihood F-tests used model paired contrasts within temperature, i.e. normoxia-10°C vs. hypoxia-10°C and normoxia-18°C vs. hypoxia-18°C. None of the genes were significant using false discovery rate ($q < 0.05$, Benjamini and Yekutieli 2001), so the unadjusted p-values were used. Significant genes ($p < 0.05$) in common for both contrasts were isolated using the *systemPipeR* package (Backman and Girke 2016) and retained if the $\log_2$ fold change direction was the same for both contrasts.

The subset of differentially expressed genes from above were subjected to a principal component analysis (PCA) using the scaled and normalized counts (cpm) of individuals. The PC axis associated with the separation of normoxia and hypoxia was identified. Candidate genes were ranked based on the significance of a Pearson correlation with this PC axis. The top 30 candidate genes based on correlation were examined for available mRNA sequences for several species of salmonids using a custom database (Akbarzadeh et al. 2018) and the public NCBI repository, as a goal of this work is to design primers that will work for multiple salmonid species.

Development of RT-qPCR assays for validating hypoxia biomarkers

Universal salmonid (across nine salmonid species) real-time qPCR assays were developed for the top 30 candidate genes discovered using the RNA-seq data. The assay primers and TaqMan probes for the sequences were produced using Primer Express 3.0.1 (Thermo Fisher Scientific, Waltham, MA) with primer melting temperature (Tm) between 58–60 ºC, and probe Tm between 68–70 ºC as default.

To test the efficiency of the 30 discovered hypoxia genes across salmonid species, cDNA from RNA extractions of pooled tissues from each of the nine salmonid species, i.e. Chinook salmon (*O. tshawytscha*), coho salmon (*O. kisutch*), chum salmon (*O. keta*), pink salmon *(O. gorbuscha*), sockeye salmon (*O. nerka*), Atlantic salmon (*Salmo salar*), Artic charr (*Salvelinus alpinus*), rainbow trout (*O. mykiss*) and bull trout (*Salvelinus confluentus*), were serially diluted from 1/5 to 1/625 in five dilutions. Specific target amplification (STA), was performed to enrich targeted sequences within the pools using 3.76 µL 1X TaqMan PreAmp master mix (Applied Biosystems), 0.2 µM of each of the primers, and 1.24 µL of cDNA , as previously described (Akbarzadeh et al. 2018; Houde et al. 2019a). Samples were run on a 14 cycle PCR program, with excess primers removed with EXO-SAP-IT (Affymetrix), and then amplified samples were diluted 1/5 in DNA suspension buffer. The diluted samples and assays were run in singletons following the platform instructions (Fluidigm). For sample reactions, 3.0 µL 2X TaqMan mastermix (Life Technologies), 0.3 µL 20X GE sample loading reagent, and 2.7 µL STA product were used. For assay reactions, 3.3 µL 2X assay loading reagent, 0.7 µL DNA suspension buffer, 1.08 µL forward and reverse primers (50 uM), and 1.2 µL probe (10 uM) were used. The PCR was 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and then 60°C for

1 min. Data were extracted using the Real-Time PCR Analysis Software (Fluidigm) using Ct thresholds set manually for each assay. PCR efficiencies for each assay were calculated using $(10^{1/slope} - 1) \times 100$, where the slope was estimated by plotting the Ct over the serial dilutions of cDNA.

Assay efficiencies ranged between 0.71 and 1.56 among species. Two assays were not amplified in all salmonid species, including cyclin dependent kinase inhibitor 1B (CDKN1B) and receptor activity-modifying protein 1-like (RAMP1) in rainbow trout, and condensin-2 complex subunit G2 (NCAPG2) in chum and sockeye salmon. Structural maintenance of chromosome protein 4 (SMC4) did not amplify in Chinook salmon and Arctic charr, and generally showed poor efficiencies across the remaining salmonid species. Therefore, SMC4 was excluded from further analysis in the present study.

In total, 346 individuals were examined for gill gene expression. This number includes the RNA-seq samples from discovery analysis (n = 24) that were used for validating the transfer between platforms (RNA-seq to qPCR), and the extreme hypoxia samples that were either moribund or recently dead (n = 22). The rest of the data were comprised of 212 live-sampled juveniles from the four trials that had been exposed to the full treatment for six days, and 88 distressed juveniles (mostly from trials 1, 3, and 4, i.e. pre-smolt and de-smolt stages). The subset of juveniles (*n* = 43) used for analyzing the extreme hypoxia response were all from SW-18°C in trial 3: 11 live-sampled individuals exposed to extreme hypoxia for approximately two days, 11 distressed individuals exposed to extreme hypoxia, 11 live-sampled fish kept in normal oxygen for six days, and 11 live-sampled individuals exposed to hypoxia for six days.

Gill tissue homogenization, RNA extraction and quantification, cDNA synthesis, and STA were conducted as described above and previously (Houde et al. 2019a). The 96.96 gene expression

dynamic array (Fluidigm Corporation, CA, USA) was applied and generally followed Miller et al. (2016). qRT-PCR data were analysed with Real-Time PCR Analysis 3 Software (Fluidigm Corporation, CA, USA). The expression of the 29 discovered hypoxia genes were normalized to the expression of the housekeeping gene, S100 calcium binding protein (78d16.1), which was found to be the most suitable housekeeping gene in NormFinder analysis, as previously described (Houde et al. 2019a). Sample gene expression was normalized with the ΔΔCt method (Livak and Schmittgen, 2001) using the inter-array calibrator sample. Gene expression was then log transformed: $\log_2 (2^{-\Delta\Delta Ct})$.

Validating RNA-seq platform with qRT-PCR

To validate the translation of the discovered markers from RNA-seq platform to qRT-PCR, a correlation analysis was performed between the normalized counts (cpm) RNA-seq data and the fold changes obtained from the qRT-PCR platform for the same 24 samples used for RNA-seq. Fold changes in expression values of RNA-seq data (on the X-axis) were plotted against fold change values obtained from RT-qPCR (on the Y-axis) on a scatter graph.

Statistical analysis to validate hypoxia biomarkers

Analyses were performed using R 3.4.4 (R Core Team, 2018) at a significance level of $\alpha = 0.05$. As the expression levels of hypoxia genes were also influenced by water salinity and smolt stage, further analyses were separately carried out for each group. The dataset for each group was divided into a two-thirds training set and a one-third testing set. The training set was subjected to a Shrunken Centroid method (Tibshirani et al. 2002) to identify a classifier for hypoxia vs. normoxia fish for each group. This method uses an internal cross-validation for threshold selection and returned a reduced list of the most contributed genes for robust classification. Then, the selected genes were subjected to PCA analysis using the data from the training set that

excluded the 24 RNA-seq samples. This PCA was then applied to the testing set for visualization of unsupervised group separation within each group using the *fviz_pca* function of the *factoextra* R package (https://cran.r-project.org/web/packages/factoextra/index.html). The classification ability of the groups was also examined by subjecting the resulting biomarkers to linear discriminant analysis (LDA) using the training set, followed by determining classification performance on the testing set. A similar approach was used for examining mortality (dead or moribund vs. live), and the extreme hypoxia group of SW-18°C in trial 3.

Results

Sequencing and gene count overview

Across the 24 individuals for RNA-seq, there was a sequencing depth range of 9.4 to 16.9 million trimmed PE reads (9.4□17.0 million raw reads). The overall alignment rate of the PE reads to the reference Chinook salmon genome ranged from 78.2 to 80.5% per sample. The final gene transcript annotation file (gff), comprised of merged reference guided individual transcript assemblies, contained 8.5% novel introns, 21.3% novel exons, and 56.7% novel loci. Using this final assembly, there were counts for 127,887 transcripts. Application of the low expression filter retained counts for 48,768 genes. The MDS plot using the overall filtered RNA-seq data showed that dimension 1 (dim 1) clearly separated samples kept in 10 vs. 18ºC (i.e., large influence of temperature), and dimension 2 (dim 2) suggested separation of normoxia vs. hypoxia fish kept at 18 ºC (Fig. 1). No trial effect was seen on the overall gene expression data.

Discovery of candidate hypoxia genes

The number of upregulated and downregulated genes ($p < 0.05$) between normoxia and hypoxia treatments was similar within each temperature: at 10 °C, there were 1,926 upregulated and 1,441 downregulated genes from hypoxia. At 18 °C, there were 1,742 upregulated and 1,347

downregulated genes from hypoxia. In total, 349 genes were found to be differentially regulated in both comparisons, and when requiring that these genes follow the same direction of regulation, this included 240 genes (i.e., 96 upregulated genes and 144 downregulated genes).

The PCA using a total of 240 candidate genes revealed that normoxia and hypoxia separation occurred along PC1, as expected. There was also a separation of the 10 and 18°C groups along PC2 (Fig. 2). To avoid selecting genes responding to temperature, genes were excluded when they were correlated with PC2 ($p < 0.1$; Table 1). Downregulated genes from hypoxia showed higher correlations with PC1 than did upregulated genes. The top 20 downregulated genes (r = -0.85 to -0.95) and the top 10 upregulated genes (r = 0.73 to 0.81) were investigated further for assay development. The top 20 down-regulated genes were mostly involved in DNA synthesis, cell division, and cell cycle, while the top 10 up-regulated genes were involved in the cell cycle arrest, oxidative stress response, ion transport, cellular calcium regulation, peptide amidation, and neuropeptide processing (Table 2).

Correlation between RNA-seq and qRT-PCR data

The validation of the design of the primers for qPCR compared to the results from RNA-seq was conducted using a correlation analysis between the cpm values from the RNA-seq data and the fold changes obtained from the qRT-PCR platform for the same 24 samples used for both platforms. A high value for the Pearson correlation coefficient ($r = 0.88$; $p < 0.001$) indicated a positive correlation between platforms (Fig. 3). From 29 genes tested, 15 genes showed significant correlation ($p < 0.05$) between two platforms (Table S1).

Moreover, there was a high separation between hypoxia and normoxia along PC1 using the gene expression data of 24 independent RNA-seq samples (Fig. 4). Using the top eight genes obtained from the Shrunken Centroid method on 15 significant correlated genes, the

classification ability for normoxia was 100.0% and hypoxia was 75.0% with the average classification of 87.5%.

Hypoxia biomarkers

The qRT-PCR gene expression results for the 29 discovered hypoxia genes including all 300 live-sampled and distressed fish kept at different combinations of temperature, salinity and oxygen for six days, as well as 22 live-sampled and distressed fish exposed to extreme hypoxia for two days are presented in Fig. S1. Moreover, the initial results of the PCA analysis on all live-sampled and distressed fish together using the selected genes from Shrunken Centroid method showed that PC1 was associated with smolt stage, water salinity, and mortality (*data not shown*). Given the influence of smolt stage, salinity, and mortality on the candidate hypoxia biomarkers, we separated the data first by the three smolt stages (live-sampled only) and distressed and then by the three salinities such that there were 11 groups. Distressed fish in BW data was not analyzed due to too few samples (n=3) of normoxia fish in this salinity environment.

The results of the PCA analyses and LDA scores for normoxia vs. hypoxia within each group using the most contributed hypoxia biomarkers are shown in Figures S2-S5 and Table 3. Effectiveness of biomarkers were evaluated based on the ability to separate samples within the PCA and high scoring LDA values for both normoxic and hypoxic conditions.

Pre-smolt fish transferred to SW showed a distinct separation of normoxia and hypoxia groups along PC2, having the highest average LDA classification (87.5%) (Fig. S2a; Table 3). The pre-smolts in SW had higher LDA classification power than the LDA results for fish in FW or BW.

Smolts in FW or SW had distinct separations of normoxia and hypoxia samples along PC2 (Fig. S3a, b). The SW smolts showed the highest LDA classification (83.3%), although this was biased towards identifying samples in the hypoxia condition (Table 3). The most balanced LDA classification between hypoxic and normoxic classifications in the smolts was 75% as observed in smolts in FW (Table 3).

De-smolt fish in FW had a distinct separation for normoxia and hypoxia groups along PC2 with an average LDA classification of 83.3% but bias towards hypoxia (Fig. S4; Table 3). Although the highest average LDA classification for de-smolts was 87.5% (in SW), there was no clear separation for the PCA using the first two axes (Table 3). Moreover, both trials of de-smolt fish were also separated along PC2 in all salinity environments (Fig. S4).

Distressed fish in both FW and SW showed the highest PCA separation and classification ability with an average classification score of 90.0 and 88.9%, respectively (Fig. S5; Table 3).

The PCA analysis on the extreme hypoxia group of SW-18°C in trial 3 showed a stronger separation of normoxia and live-extreme hypoxia exposed fish than normoxia and moderate hypoxia groups along PC1. Distressed extreme hypoxia exposed fish were also separated from other groups in PCA analysis (Fig. 5).

In sum, the conditions that showed the most conducive to separate fish kept in normoxic from hypoxic water were pre-smolt and smolt stages in SW, and de-smolt stage in FW. On the contrary, all conditions in BW, pre-smolt and smolt in FW, and de-smolt in SW were not able to highly separate fish kept in normoxic from hypoxic environment.

Discussion

Characterizing early genetic responses to hypoxia to facilitate the development of a tool to assess hypoxia exposure is crucial towards managing salmonid fish that are increasingly exposed to

hypoxic stress globally. This is particularly relevant considering the strong influence anthropogenic effects such as excessive nutrient loading can have on hypoxia levels and occurrence, for example on the west coast of North America. The present study aimed to discover and validate gill gene expression biomarkers specific to hypoxic stress in salmonids. Using high-throughput RNA-seq analysis, we discovered 30 candidate genes that were differentially expressed between hypoxia and normoxia conditions, but were not affected by temperature. The discovered genes were then designed to qRT-PCR primer design, and then used to query a larger set of sample by high-throughput microfluidics qPCR. These qPCR data were then used to characterize and identify the best markers for differentiating normoxic and hypoxic condition exposure.

Biological and molecular functions of candidate hypoxia genes

All of the top 20 downregulated genes in the hypoxia treatment discovered by the RNA-seq analysis were involved in functions related to DNA synthesis, cell division, and cell cycle. The concordant downregulation of these genes during hypoxia suggests that cell proliferation is suppressed under hypoxia in Chinook salmon gill tissue. Hypoxia has been shown to inhibit cell proliferation in numerous cell types (Hubbi and Semenza, 2015). A decreased number of cells may allow for decreased oxygen demand under hypoxia (Hubbi and Semenza, 2015). These hypoxia-suppressed genes may reflect a physiological alteration that halts cell proliferation and translational activities to allow the cells to preserve energy consumption under metabolic stress (Chi et al. 2006). The downregulation of genes involved in cell cycle progression occurs in response to acute and chronic hypoxia in zebrafish embryos (Ton et al. 2003) and in hepatic cells of hybrid striped bass (*Morone saxatilis* × *Morone chrysops*) (Beck et al. 2016). Therefore, the

downregulation of cell proliferation in the gill may be a physiological strategy in Chinook salmon to cope with hypoxic water.

Consistent with the suppression of genes involved in cell proliferation, the mRNA expression of genes involved in blocking the cell cycle progression i.e., transcription factor SOX-5 (SOX-5), cyclin dependent kinase inhibitor *1B* (CDKN1B), and malignant fibrous histiocytoma-amplified sequence 1 homolog (MFHAS1) were among the top 10 hypoxia-induced upregulated genes discovered in this study. Sox5 is well known for its function in controlling cell cycle and sequential generation of distinct corticofugal neuron subtypes (Hao et al. 2014). The upregulation of SOX5 occurs in response to hypoxia in human stem cells (Khan et al. 2007). Moreover, the upregulation of CDKN1B gene transcription, which blocks cell cycle progression from G1 to S phase, is also a well-known response to hypoxia (Wang et al. 2003; Peng et al. 2015). MFHAS1 (MASL1) is known as an oncogene that may play an important role in signal transduction and the control of cell growth in humans (Sakabe et al 1999). Collectively, this suggests that hypoxia may elevate expression of genes involved in inhibiting cell growth and proliferation, thus potentially reducing growth rates. Furthermore, this is in line with the lower growth performance of these juveniles kept in hypoxia compared to normoxia (see Houde et al. 2019a).

Top 10 hypoxia-induced upregulated genes included those involved in the oxidative stress response (e.g., *glutathione peroxidase 3*; GPx-3, and Acyl-coenzyme A thioesterase 9, mitochondrial; ACOT), ion transport (e.g., *receptor activity-modifying protein 1*; RAMP1), peptide amidation (e.g., *peptidylglycine alpha-amidating monooxygenase*; PAM), and neuropeptide processing (e.g., *endothelin-converting enzyme 2*; ECE-2). GPx-3 is a selenocysteine-containing protein with antioxidant properties. Hypoxia is known as a strong

transcriptional regulator of GPx-3 expression. RAMP1 is a member of the RAMP family of single-transmembrane-domain proteins, called receptor (calcitonin) activity modifying proteins (RAMPs). Chronic hypoxia was reported to enhance mRNA levels for RAMP1 in Sasco Sprague-Dawley rats (Qing et al. 2001; Cueille et al. 2005). It is stated that in hypoxia, upregulation of RAMP1 primarily regulates the calcitonin gene-related peptide reception (Qing and Keith 2003). Moreover, PAM catalyzes the amidation of peptides, a process important for peptide stability and biological activity. PAM-dependent amidation has the potential to signal oxygen, so that PAM is strikingly sensitive to hypoxia in cells (Sharma et al. 2009; Simpson et al 2015). PAM has catalytic activity on a range of substrates, in different cell types, and is as sensitive to hypoxia as the transcription factor, hypoxia inducible factor (HIF) (Simpson et al 2015). ECE-2 has been observed to be upregulated in response to hypoxia in juvenile salmon in this study. ECE-2 belongs to a family of membrane-bound metalloproteases involved in neuropeptide processing (Mzhavia et al. 2003). The true physiological function of ECE-2 remains largely to be determined (Ouimet et al. 2010), but it may be a novel HIF-target gene in hypoxia, where HIF-1 binds to transcription regulatory regions of the ECE-1 gene during hypoxia (Khamaisi et al. 2015). However, no information is available regarding this mechanism for the ECE-2 gene. The upregulation of GPX3, Sox5, CDKN1B, and ECE-2 observed in this study was in line with the higher expression of the transcription factor HIF-1A (see Houde et al 2019a) in response to hypoxia. Notably, and in line with observations from the current study in Chinook salmon, HIF-1A is a key regulator of the cellular and systemic homeostatic response to hypoxia through the activation of transcription of many genes, including those involved in cell cycle arrest (i.e. CDKN1B), antioxidant activity (i.e. GPX3), and other genes whose protein

products increase oxygen delivery or facilitate metabolic adaptation to hypoxia (Lando et al. 2003; Wang et al. 2003; Zagórska and Dulak, 2004).

Differing effects dependent on smolt stage and environmental salinity

Juvenile Chinook salmon exposed to normoxia and hypoxia in different conditions were separated to different levels of success using the biomarkers developed here. Gene expression patterns and multivariate analyses using the discovered hypoxia genes showed that at the level of hypoxia induced in our study, the hypoxia response in juvenile Chinook salmon was highly affected by the developmental process of three factors: smoltification (i.e., smolt stage), water salinity, and mortality status. It is known that metabolic responses can be affected by both smolt stage and salinity in salmon (Morgan and Iwama 1991; Hvas et al. 2018).

Across all smolt stages, the results showed lower classification ability of fish in BW than in FW or SW. It is known that salmon in BW are near their isosmotic environment (i.e., the gradient between blood and environmental salinity is lower), such that the oxygen consumption and metabolic rate or energy expenditure may be lower than that in FW or SW to maintain ion- and osmo-regulation (Morgan and Iwama 1991, 1998). These juveniles may have better aerobic performance and may experience less hypoxic stress in this environment (Hvas et al. 2018). This was in line with our previous observation of lower Chinook salmon juvenile mortality in BW than in either FW or SW (Houde et al. 2019a). Altogether, these results suggest that juvenile Chinook salmon are more resilient to low oxygen levels in brackish conditions than in either full salt water or fresh water.

Pre-smolt Chinook salmon were responsive to hypoxia in SW but not in FW. Juvenile salmonids that are physiologically unprepared for the transition from FW to SW (i.e., pre-smolts)

experience higher mortality in SW (Houde et al. 2019a,b). Pre-smolts in SW, as opposed to FW, may display higher oxygen consumption as a stress response, and higher energy expenditure to maintain ion- and osmo-regulation (Morgan and Iwana 1991, 1998). Furthermore, oxygen consumption typically is the lowest in the environment that is natural for a given life stage (Ern et al. 2014). It is likely that pre-smolt juvenile Chinook salmon in FW experienced lower physiological disturbance than those in SW, such that they were more prepared to cope with hypoxic conditions in FW than in SW.

Smolt and de-smolt juvenile Chinook salmon were responsive to hypoxia in both FW and SW. During smoltification, physiological adaptations to SW increase and adaptations to FW decrease. For example, the gill gene expression of SW and FW isoforms of Na^+/K^+-ATPase follows this pattern (Nilsen et al. 2007). De-smoltification occurs when smolts do not migrate to SW within their smolt window, and remain in FW too long, such that they revert to a more FW-adapted physiology. Although certain physiological features in de-smolts do revert back to FW forms, including ion- and osmo-regulation, other features remain in the SW form, e.g., higher metabolic rate (McCormick et al. 1998). Considering the lower oxygen requirements in the most natural environment for a particular life stage (Ern et al. 2014), it is possible that smolts and de-smolts both may be less able to cope with hypoxic conditions in FW and SW because they are not fully adapted to either environment.

The post-smolt stage was not examined in the present study but may reveal further insights on the response to hypoxia by salinity and smolt stage. De-smolts may occur in the hatchery setting, whereas smolts in the natural setting typically transition to SW and become post-smolts (McCormick et al. 1998). Therefore, the hypothesis of lower oxygen consumption or energetic cost in the natural salinity environment for a given life stage (Morgan and Iwana 1991,

1998; Ern et al. 2014), may be further examined using post-smolts, and this would be a valuable direction for future work. It is predicted that the post-smolt pattern may be similar to pre-smolts but in the opposite direction, i.e., post-smolts are more prepared to cope with hypoxic conditions in SW than in FW. Indeed, aerobic scope, but not standard or maximum metabolic rate, is lowest for post-smolt Atlantic salmon in SW than FW and BW (Hvas et al. 2018). Further studies examining post-smolts using the three salinities and the candidate hypoxia genes are warranted.

Across smolt stages and salinities, our results showed that three genes involved in cell cycle and DNA synthesis (i.e. anillin, CDKN1B, and Kif4) were among the most universal genes differentially expressed in response to hypoxia. However, some genes involved in ion transportation (i.e. RAMP1) were differentially expressed in saline water (i.e. brackish water and seawater). In pre-smolt fish kept in FW and BW, the hypoxia responsive genes were mostly involved in cell cycle and DNA synthesis, while in SW, the genes involved in ion transportation and oxidative stress also contributed to the hypoxia response. The smolt and de-smolt fish also showed differences in ion transportation genes related to hypoxia when in SW conditions. Previous studies demonstrated that the hypoxia exposure of gill in fish is associated with an ionoregulatory disturbance. Indeed, adjustments of gill morphology, which includes the morphological changes in the gills, along with possible changes in gill ventilation and perfusion to secure oxygen uptake, have a negative effect on the ionoregulatory status of the fish (Matey et al. 2008). Therefore, our results suggest that hypoxia may also affect the expression of osmoregulation and ionoregulation genes in gill tissue, which is important given that this tissue is the primary organ involved in both osmoregulation and ionoregulation (Houde et al 2019b).

Mortality

The results of this study showed that the top candidate hypoxia genes were highly responsive to hypoxia in moribund and recently dead fish in both FW and SW environments, suggesting the active regulation of hypoxia genes in moribund and recently dead fish. These results confirm previous findings of post-mortem activity of hypoxia responsive genes in human (Ferreira et al. 2018), mouse and zebrafish (Pozhitkov et al. 2016). It has been previously suggested that the genes that function in DNA synthesis, deactivation of immune system, cell necrosis, stress response, and glycolysis of carbohydrates are also the major group of genes that show changes in transcription following death. Hypoxia is believed to play a major role in the initial pre- to post-mortem transition by activation of glycolysis (Ferreira et al. 2018). In our study, genes involved in cell cycle and DNA synthesis, oxidative stress, and neuropeptide processing were among the most significant contributed genes responsive to hypoxia in distressed fish in both FW and SW environments. The cellular hypoxic response to morbidity is likely due to reduced blood circulation and consequently a considerable reduction of available oxygen in cells. Our validated hypoxia biomarkers may be applicable to elucidating the causes of salmon mortality and the environmental habitats where salmon experience episodes of hypoxia. Although further confirmatory studies are required, these results demonstrate that this high-throughput gene expression approach may have a high potential for being a useful tool to discover hypoxic mortality in salmonids, and the synergistic relationships between hypoxic stress and disease, if applied on salmon Fit-Chips.

Extreme hypoxia

The most robust hypoxia response was observed in individuals exposed accidently to more severe hypoxic water in SW-18ºC than the fish exposed to the more moderate hypoxia regime designed in the original study as shown by the PCA analysis; this may suggest that the moderate

hypoxia regime was not strong enough to induce a consistent hypoxia response across all individuals. These results were accidently obtained on a limited number of fish. Conceivably, a stronger hypoxia across all treatments may have shown a more robust hypoxia response in salmon, but stronger hypoxia was not chosen due to the ethical considerations. Further experiments using stronger hypoxic water on different salinity, temperature and smolt stages are needed to confirm this hypothesis.

Conclusions

Previously, specific gill gene expression biomarkers have been identified for salinity and temperature stressors across multi-stressor conditions, but hypoxia biomarkers sufficient to differentiate fish exposed to moderate hypoxia relative to normoxia were not identifiable (Houde et al. 2019a). Here, candidate hypoxia genes were discovered through RNA-seq analysis, and then validated using microfluidics qPCR to reveal sets of biomarkers that were responsive to hypoxic stress in juvenile Chinook salmon. Moreover, the expression of these candidate hypoxia genes, and presumably of the hypoxia response, in juvenile Chinook salmon was affected by smolt stage, mortality, and water salinity. Our study is the first of its kind dealing with this range of factors from the gene expression perspective. From the suite of 30 candidate genes, different validated biomarker sets were identified that were optimal for each of the specific combinations of factors within the dataset (i.e., smolt stage, mortality, salinity). The genes involved in cell cycle and DNA synthesis were among the most universal biomarkers, but ion transportation genes were differentially expressed in saline water. Since the biomarkers were discovered on specific water salinity, smolt stage and water temperatures, no consistent gene expression model for all different salinity, smolt stage, and temperature groups was observed at the level of hypoxia exposure in our study. This study also suggests post-mortem regulation of hypoxia

genes in fish. The main challenge facing the development of a hypoxia gene expression biomarker panel for salmonids appears to be the relatively minor response compared to other stressors such as temperature, but the present study provides a set of biomarkers that along with factors smolt stage and salinity, can be used to determine whether an individual has recently experienced hypoxic stress. Moreover, our study confirms that fish might be highly resilient to mild hypoxia when they are in their natural salinity environment, but will still be stressed by extreme hypoxia conditions.

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Data availability

The analysis pipeline and scripts for RNA-seq analysis is described in more detail on GitHub: https://github.com/bensutherland/Simple_reads_to_counts

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Table 1. Correlations between 240 candidate gill hypoxia genes and their first two principal components. A principal component analysis used the scaled and normalized counts (cpm) of 240 genes. Displayed are Pearson correlations (*r*) and their significance (*p*) between the gene counts and the first two principal components (PC1 and PC2). Positive and negative correlations are upregulated and downregulated in hypoxia than normoxia, respectively. Results are sorted by *p*-value for PC1.

Gene name	Gene description	*r*1	*p*1	*r*2	*p*2
LOC112218460	anillin-like	-0.95189	8.81E-13	0.097139	0.651594
LOC112249990	ribonucleoside-diphosphate reductase subunit M2-like	-0.92312	1.33E-10	0.162189	0.448943

LOC112218878	aurora kinase B-like	-0.9088	8.17E-10	0.167254	0.434708
LOC112228646	ribonucleoside-diphosphate reductase large subunit-like	-0.89364	4.12E-09	0.259303	0.221116
LOC112226944	kinesin-like protein KIF2C	-0.89218	4.76E-09	0.23932	0.26004
top2a	DNA topoisomerase II alpha	-0.89049	5.60E-09	0.400934	0.052178
ncapd3	non-SMC condensin II complex subunit D3	-0.88624	8.34E-09	0.265898	0.209172
LOC112262552	histone H2A.V	-0.88545	8.96E-09	0.23774	0.263293
kif15	kinesin family member 15	-0.88468	9.61E-09	0.242711	0.253141
LOC112256655	rho GTPase-activating protein 11A-like	-0.8838	1.04E-08	0.113267	0.598212
ncapd2	non-SMC condensin I complex subunit D2	-0.88041	1.40E-08	0.392884	0.057543
LOC112259694	rab9 effector protein with kelch motifs-like	-0.88036	1.41E-08	0.008227	0.969567
ercc6l	ERCC excision repair 6 like, spindle assembly checkpoint helicase	-0.8803	1.42E-08	0.258103	0.223336
LOC112256480	tonsoku-like protein	-0.87516	2.20E-08	0.204184	0.338553
LOC112251434	lamin-B2-like	-0.87474	2.27E-08	0.368745	0.076207
LOC112215505	citron Rho-interacting kinase-like	-0.8736	2.50E-08	0.3016	0.152069
LOC112248619	proliferating cell nuclear antigen	-0.87069	3.16E-08	0.123867	0.564172
LOC112229914	claspin-like	-0.8681	3.89E-08	0.176494	0.409377
ndc80	NDC80, kinetochore complex component	-0.86564	4.71E-08	0.186882	0.381903
LOC112249909	mitotic checkpoint serine/threonine-protein kinase BUB1 beta-like	-0.86265	5.91E-08	0.364023	0.080341
lmnb1	lamin B1	-0.86212	6.15E-08	0.301504	0.152207
LOC112228013	condensin-2 complex subunit G2-like	-0.86067	6.85E-08	0.047134	0.82688
LOC112231350	chromosome-associated kinesin KIF4-like	-0.85421	1.09E-07	0.423934	0.038971
LOC112265693	structural maintenance of chromosomes protein 4-like	-0.8533	1.17E-07	0.448816	0.027813
actl6a	actin like 6A	-0.84991	1.47E-07	0.085557	0.691
LOC112253110	kinesin-like protein KIF11-B	-0.84744	1.74E-07	0.448441	0.02796
LOC112253851	lymphocyte-specific helicase-like	-0.84537	2.00E-07	0.071013	0.741599
rrm2	ribonucleotide reductase regulatory subunit M2	-0.84442	2.13E-07	0.189171	0.375994
ubr7	ubiquitin protein ligase E3 component n-	-0.84395	2.20E-07	0.123906	0.564047

	recognin 7 (putative)				
tyms	thymidylate synthetase	-0.84253	2.41E-07	-0.09206	0.668785
LOC112264801	transcription factor 19-like	-0.84228	2.45E-07	-0.05558	0.796434
gtse1	G2 and S-phase expressed 1	-0.84024	2.79E-07	0.415735	0.043335
espl1	extra spindle pole bodies like 1, separase	-0.83789	3.24E-07	0.477644	0.018251
LOC112224457	disks large-associated protein 5-like	-0.83749	3.32E-07	0.45812	0.024368
LOC112219045	inner centromere protein A-like	-0.83713	3.40E-07	0.467587	0.021223
LOC112230962	G2/mitotic-specific cyclin-B1-like	-0.83642	3.55E-07	0.360379	0.083645
LOC112249393	kinesin-like protein KIF23	-0.8302	5.19E-07	0.449583	0.027515
LOC112220852	DNA replication licensing factor mcm7-like	-0.8283	5.82E-07	-0.04832	0.822594
LOC112251353	nuclear autoantigenic sperm protein-like	-0.82811	5.88E-07	0.193195	0.365732
LOC112260003	histone H2A.Z	-0.82671	6.39E-07	0.379012	0.067777
LOC112228262	deoxycytidine kinase-like	-0.82584	6.72E-07	0.295568	0.160843
LOC112250026	mitotic checkpoint serine/threonine-protein kinase BUB1-like	-0.82543	6.88E-07	0.446334	0.028795
nuf2	NUF2, NDC80 kinetochore complex component	-0.8232	7.82E-07	0.206435	0.333139
LOC112262953	centromere-associated protein E-like	-0.82318	7.83E-07	0.454999	0.025483
LOC112239878	uncharacterized LOC112239878	-0.82244	8.17E-07	0.121091	0.573004
sass6	SAS-6 centriolar assembly protein	-0.82093	8.90E-07	-0.05939	0.782826
LOC112238559	uncharacterized LOC112238559	-0.81444	1.28E-06	0.488692	0.015386
neil3	nei like DNA glycosylase 3	-0.81113	1.52E-06	0.056919	0.791647
LOC112219506	malignant fibrous histiocytoma-amplified sequence 1 homolog	0.810857	1.55E-06	0.060474	0.778937
kifc1	kinesin family member C1	-0.80906	1.70E-06	0.483234	0.016752
LOC112245345	PCNA-associated factor-like	-0.8073	1.86E-06	0.336041	0.108392
LOC112229667	RNA polymerase II subunit A C-terminal domain phosphatase-like	-0.80719	1.88E-06	-0.09107	0.672133
ccnf	cyclin F	-0.80538	2.06E-06	0.515212	0.009981
LOC112256353	cyclin-A2-like	-0.80395	2.22E-06	0.267586	0.206185
iqgap3	IQ motif containing GTPase activating protein 3	-0.80357	2.26E-06	0.461015	0.023369

LOC112229614	anillin-like	-0.80237	2.40E-06	0.484186	0.016507
LOC112250348	dnaJ homolog subfamily C member 5-like	-0.8023	2.41E-06	0.302088	0.151375
LOC112233066	targeting protein for Xklp2-like	-0.79965	2.75E-06	0.460572	0.023519
LOC112224264	kinesin-like protein KIF20B	-0.79701	3.14E-06	0.382112	0.065378
LOC112223799	receptor activity-modifying protein 1-like	0.79092	4.21E-06	-0.17556	0.411914
NA	E2F transcription factor 3	-0.78962	4.48E-06	-0.15758	0.462092
kif22	kinesin family member 22	-0.789	4.62E-06	0.402836	0.050969
LOC112215881	targeting protein for Xklp2-like	-0.78678	5.12E-06	0.441463	0.030803
LOC112264594	anillin-like	-0.78632	5.23E-06	0.48533	0.016216
cenpw	centromere protein W	-0.78442	5.71E-06	0.05837	0.786454
LOC112237254	denticleless protein homolog	-0.78403	5.82E-06	-0.02643	0.90244
pole2	DNA polymerase epsilon 2, accessory subunit	-0.78191	6.41E-06	-0.16531	0.440153
LOC112253106	rhotekin-2-like	-0.78139	6.56E-06	0.401285	0.051954
cep135	centrosomal protein 135	-0.78072	6.76E-06	0.353064	0.090585
LOC112228882	protein DEK-like	-0.78045	6.84E-06	0.266359	0.208353
LOC112250900	chromatin assembly factor 1 subunit A-like	-0.78001	6.98E-06	-0.21164	0.320825
NA	zinc finger protein 367-like	-0.77912	7.26E-06	0.512671	0.010419
pam	peptidylglycine alpha-amidating monooxygenase	0.778638	7.42E-06	0.049264	0.819183
LOC112221868	aurora kinase C-like	-0.77809	7.60E-06	0.492525	0.014483
NA	FAM214A-like	0.775327	8.59E-06	-0.02241	0.917231
LOC112228990	histone H2AX	-0.77484	8.78E-06	-0.00562	0.979211
racgap1	Rac GTPase activating protein 1	-0.77376	9.20E-06	0.542533	0.006161
LOC112230732	DNA polymerase theta-like	-0.77227	9.82E-06	-0.09497	0.658914
LOC112217492	mitotic checkpoint serine/threonine-protein kinase BUB1-like	-0.77218	9.85E-06	0.568198	0.003772
wdhd1	WD repeat and HMG-box DNA binding protein 1	-0.77202	9.92E-06	-0.18485	0.387201
LOC112218911	calphotin-like	0.767805	1.19E-05	-0.05576	0.795793
LOC112223116	cell division cycle-associated protein 3-like	-0.76487	1.34E-05	0.453099	0.026182
LOC112236066	glutathione peroxidase 3-like	0.764464	1.37E-05	-0.02272	0.916063

LOC112245091	dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3A	-0.76431	1.38E-05	0.118635	0.580867
LOC112232992	dual specificity protein kinase Ttk-like	-0.76341	1.43E-05	0.213132	0.31734
thumpd1	THUMP domain containing 1	-0.76011	1.63E-05	-0.10414	0.628187
LOC112231188	kinesin-like protein KIF20A	-0.75676	1.87E-05	0.554135	0.004959
rtel1	regulator of telomere elongation helicase 1	-0.75352	2.13E-05	-0.3122	0.137487
LOC112225439	chromatin assembly factor 1 subunit B-like	-0.75314	2.16E-05	-0.34447	0.099275
LOC112255192	cyclin-dependent kinase 1	-0.75261	2.21E-05	0.546964	0.005676
LOC112216690	endothelin-converting enzyme 2-like	0.750775	2.37E-05	0.253743	0.23153
eme1	essential meiotic structure-specific endonuclease 1	-0.74843	2.60E-05	0.328824	0.116672
LOC112226524	G-protein-signaling modulator 2-like	-0.74643	2.81E-05	0.26602	0.208956
LOC112214837	abnormal spindle-like microcephaly-associated protein homolog	-0.74507	2.96E-05	0.528526	0.007929
cdkn1b	cyclin dependent kinase inhibitor 1B	0.744469	3.02E-05	0.191772	0.369341
LOC112217828	serine/arginine-rich splicing factor 3	-0.74175	3.35E-05	-0.0636	0.767821
NA	UNKNOWN	-0.74136	3.40E-05	0.383668	0.064199
LOC112228552	ras-related and estrogen-regulated growth inhibitor-like protein	0.740487	3.51E-05	0.403917	0.050292
cenpo	centromere protein O	-0.73815	3.83E-05	-0.29485	0.161917
LOC112220417	suppressor APC domain-containing protein 2-like	-0.73592	4.16E-05	-0.22603	0.288222
stil	STIL, centriolar assembly protein	-0.73384	4.48E-05	0.256252	0.226791
LOC112221983	transcription factor E2F1-like	-0.73328	4.57E-05	0.040135	0.852291
LOC112215028	transcription factor SOX-5-like	0.73291	4.63E-05	0.216739	0.309024
LOC112241904	serine/threonine-protein kinase PLK1-like	-0.73168	4.84E-05	0.529272	0.007825
NA	UNKNOWN	0.729766	5.19E-05	0.059905	0.78097
LOC112264390	shugoshin 1-like	-0.72835	5.45E-05	0.582269	0.002833
gins3	GINS complex subunit 3	-0.72581	5.96E-05	-0.12713	0.55387
LOC112231339	peptidyl-prolyl cis-trans isomerase FKBP2-like	-0.72281	6.62E-05	-0.25111	0.236571
LOC112223647	angiotensin-converting enzyme-like	0.720183	7.24E-05	0.099537	0.643543

LOC112249961	ectonucleotide pyrophosphatase/phosphodiesterase family member 1-like	0.715024	8.62E-05	-0.16295	0.446778
LOC112262569	UPF0454 protein C12orf49 homolog	0.714297	8.83E-05	0.255886	0.227479
NA	UNKNOWN	-0.71355	9.05E-05	-0.17128	0.423564
dna2	DNA replication helicase/nuclease 2	-0.71169	9.63E-05	0.224184	0.292286
LOC112225466	centromere protein J-like	-0.7091	0.000105	0.035955	0.867533
LOC112228816	barrier-to-autointegration factor	-0.70864	0.000106	-0.15964	0.456202
LOC112253028	cGMP-dependent protein kinase 1-like	0.70798	0.000109	-0.1288	0.54864
LOC112227719	myb-related protein A-like	-0.70708	0.000112	-0.28867	0.171297
LOC112236577	long-chain-fatty-acid--CoA ligase 4-like	0.705743	0.000117	-0.15747	0.462419
LOC112248982	DNA damage-binding protein 1-like	-0.70312	0.000127	-0.25681	0.225749
LOC112235673	EEF1A lysine methyltransferase 4-like	-0.69907	0.000144	-0.24745	0.243706
LOC112232029	high mobility group protein B2-like	-0.69884	0.000145	-0.31407	0.135023
pold1	DNA polymerase delta 1, catalytic subunit	-0.69265	0.000176	-0.31135	0.13862
LOC112249379	nicotinamide riboside kinase 2-like	0.689692	0.000193	0.227891	0.284167
LOC112215182	semaphorin-3ab-like	0.689611	0.000193	0.419927	0.041059
LOC112242225	uncharacterized protein C17orf62 homolog	-0.6877	0.000204	-0.2815	0.182671
LOC112251159	regulator of G-protein signaling 4-like	0.684114	0.000227	0.446865	0.028583
dync1h1	dynein cytoplasmic 1 heavy chain 1	-0.67928	0.000262	-0.35053	0.093086
LOC112263008	apelin receptor A	0.679026	0.000264	0.376473	0.069793
LOC112263729	transcriptional repressor CTCF-like	-0.67812	0.000271	0.559862	0.004442
LOC112232096	synembryn-B-like	-0.67809	0.000271	0.248782	0.241094
NA	UNKNOWN	-0.67442	0.000301	-0.223	0.294917
LOC112236709	E3 ubiquitin-protein ligase TRIM71-like	-0.67409	0.000304	-0.0196	0.927555
LOC112249143	ADM-like	0.672756	0.000316	0.295191	0.161402
LOC112247189	hypoxia-inducible factor 1-alpha inhibitor	0.668775	0.000353	0.482602	0.016916
LOC112217507	midasin-like	-0.66769	0.000364	-0.16687	0.435784
mcm8	minichromosome maintenance 8 homologous recombination repair factor	-0.66736	0.000367	-0.26904	0.203645
NA	UNKNOWN	0.66693	0.000372	0.474682	0.019089

LOC112221551	semaphorin-3ab-like	0.660693	0.000441	0.184609	0.387823
NA	UNKNOWN	0.65839	0.000469	0.10629	0.621081
LOC112260474	Krueppel-like factor 4	0.65046	0.000579	0.222464	0.296101
LOC112262024	14-3-3 protein zeta-like	-0.64663	0.000639	-0.26286	0.214623
LOC112252887	nuclear receptor coactivator 4-like	0.645517	0.000658	-0.13894	0.517326
LOC112221822	lysine-specific demethylase 9-like	0.641114	0.000736	0.552184	0.005146
LOC112223699	histone H1.0-B-like	0.641012	0.000738	0.339695	0.104369
NA	DENN domain-containing protein 5B-like	0.638957	0.000777	0.121336	0.572222
LOC112227748	R-spondin-3-like	0.638326	0.000789	0.101093	0.63834
LOC112253174	hypoxia-inducible factor 1-alpha inhibitor	0.635982	0.000837	0.450921	0.027001
NA	plexin-A1-like	0.634565	0.000867	-0.1139	0.596167
LOC112217550	connective tissue growth factor-like	0.634026	0.000878	0.054465	0.800448
NA	UNKNOWN	0.633599	0.000888	-0.16494	0.441181
LOC112261313	15-hydroxyprostaglandin dehydrogenase [NAD(+)]-like	0.633544	0.000889	-0.31667	0.131641
NA	intersectin 1	-0.62847	0.001006	0.211586	0.320946
ankrd37	ankyrin repeat domain 37	0.628398	0.001008	0.363031	0.08123
NA	PI-PLC X domain-containing protein 1-lik	-0.62603	0.001067	-0.30754	0.143762
LOC112237955	E3 ubiquitin-protein ligase TRIM7-like	0.625981	0.001068	0.317822	0.130168
NA	meteorin-like protein	0.625568	0.001079	0.390335	0.059328
LOC112225560	multidrug resistance protein 1B-like	0.621334	0.001193	0.075296	0.726581
LOC112263666	ADM-like	0.619626	0.001242	-0.23664	0.265573
LOC112225033	rho GTPase-activating protein 20-like	0.610558	0.001531	0.474172	0.019236
LOC112245149	uncharacterized LOC112245149	0.608741	0.001596	0.009464	0.964991
LOC112252728	activating molecule in BECN1-regulated autophagy protein 1-like	-0.60802	0.001622	0.152281	0.477482
slc18a1	solute carrier family 18 member A1	0.607421	0.001644	0.137195	0.522646
plcd1	phospholipase C delta 1	0.605253	0.001726	-0.28573	0.175898
LOC112224548	interferon gamma receptor 1-like	0.604946	0.001738	0.470388	0.020359

Table 2. Top 20 downregulated and 10 upregulated candidate genes from the RNA-seq analysis, and the summary of qPCR TaqMan assay designs and efficiencies for candidate hypoxia stress genes. Genes are presented in order of the significance of the correlation with PC1, which separated normoxia and hypoxia (data are in Table S1). Presented are the forward (F), reverse (R), and probe sequences (P). Species abbreviations for efficieny: CK= Chinook salmon (*Oncorhynchus tshawytscha*), CO= Coho salmon (*O. kisutch*), CM= Chum salmon (*O. keta*), PK= Pink salmon (*O. gorbuscha*), SX= Sockeye salmon (*O. nerka*), AS= Atlantic salmon (*Salmo salar*), AC= Artic charr (*Salvelinus alpinus*), RT= Rainbow trout (*O. mykiss*), and BT= Bull trout (*Salvelinus confluentus*).

Gene name	Function of gene	Assay name	Primers and TaqMan Probes	PCR Efficiency								
				CK	CO	CM	PK	SX	AS	AC	RT	BT
Down-regulated genes												
Anillin-like	Cell division	Anillin	F- TGGTGAATGGATTTGGAGCTT R- GGGTGGTTCCAATAGGACATGT P- CGCTTCTTTGTCCTGGAG	1.20	1.30	1.27	1.20	1.12	1.29	1.00	0.99	1.56
Ribonucleoside-diphosphate reductase subunit M2-like	DNA synthesis	RRM2	F- TGCTGCTAGTGATGGCATTGT R- TTTGGAAACCATAGAAGCATCTTG P- ATTTACACAGGAAGTCCAGG	1.28	1.38	1.27	1.07	1.15	1.34	1.10	1.00	1.47
Aurora kinase B-like	Cell division	AURKB	F- GAAATGTGGTCGCTTCGATGA R- CATCAGCCAACTCCTCCATGT P- CAGCGCACTGCTAC	1.26	1.42	1.29	1.14	1.21	1.37	1.00	1.07	1.50
Ribonucleoside-diphosphate reductase large subunit-like	DNA synthesis	RRM1	F- GCTGGAAGCAGGGTCTGAAG R- GTTGGCTGCAGGCTTGGT P- CGGGCATGTACTACCT	1.04	1.16	1.16	0.99	1.10	1.19	1.02	1.00	1.31
Kinesin-like protein KIF2C	Cell division	KIF2C	F- CGGCCAAACTGGAAGTGGTA R- TTCTGGCTCTTCCCTGAAAAGT P- AACTCACACAATGGGAG	1.11	1.20	1.18	1.09	1.05	1.24	0.98	1.02	1.27

Non-SMC condensin II complex subunit D3	Cell division	Ncapd3	F- TCACAGGTCCAGAAGAAAGTGTTC R- GCGAGCGCTTCTGTTCCA P- TCGAGAGCATGATTCC	1.09	1.19	1.11	1.08	1.01	1.17	0.99	1.00	1.33
Histone H2A.V	Cell division	H2AFV	F- CCCTCATCAAGGCCACCAT R- CGATCAGGGACTTGTGGATGT P- AGGCGGTGTGATCC	1.08	1.22	1.29	1.11	1.05	1.31	1.00	1.05	0.71
Kinesin family member 15-like	Cell division	Kif15	F- CAGGCAGGTCTTCTCCAAGCT R- AGTTTGGATGATAGCCTCCTTCTG P- CACAGGATCAGACTGC	1.17	1.22	1.15	1.01	1.03	1.21	0.99	1.02	1.31
Rho GTPase-activating protein 11A-like	Cell division	ARHGAP11A	F- TGATAATTACTCCCAACTCCAAGAGA R- CTTGCTGGAGCCATAACTTTGA P- AACTTCCTTTGGAATCTG	1.22	1.28	1.20	1.03	1.10	1.33	1.05	0.98	1.42
Rab9 effector protein with kelch motifs-like	Cell division	Rab9	F- CAGGCCCTCAATGACACCTT R- GGAGAGCTGTGGGTGAACCA P- CCTTCAACACAGACACC	1.26	1.16	1.26	1.03	1.05	1.08	1.02	1.03	1.03
ERCC excision repair 6 like, spindle assembly checkpoint helicase	Cell division	ERCC6L	F- TTGTATGGTCTCCACAGAGATGGT R- GTCTTCCCTAAGCCCATGTCAT P- TCAAGGAGGAATCCTAG	1.13	1.22	1.18	1.10	1.06	1.16	1.02	1.01	1.29
Tonsoku-like protein	DNA synthesis	Tonsl	F- CCAATGCTTGCACCAATCAG R- TCTGCCTCGCTATGTGGTACTG P- TGCGGGTCAGGGTT	1.15	1.39	1.26	1.03	1.13	1.30	1.02	0.86	1.52
Citron Rho-interacting kinase-like	Cell division	CIT	F- GATCTCTAGGTTTCAGCGCAAGA R- TGAGCTCCACATCCTTTTGGT P- ACCTGGAGTCAGTTCT	1.16	1.28	1.14	1.10	1.08	1.21	1.01	1.02	1.38
Proliferating cell nuclear antigen	DNA synthesis	PCNA	F- TTCTACTTGAACCAAACACAGATTCC R- TTTCTTCAGGATGGATCCCTGTA	1.11	1.20	1.12	1.11	1.26	1.17	1.04	0.99	1.26

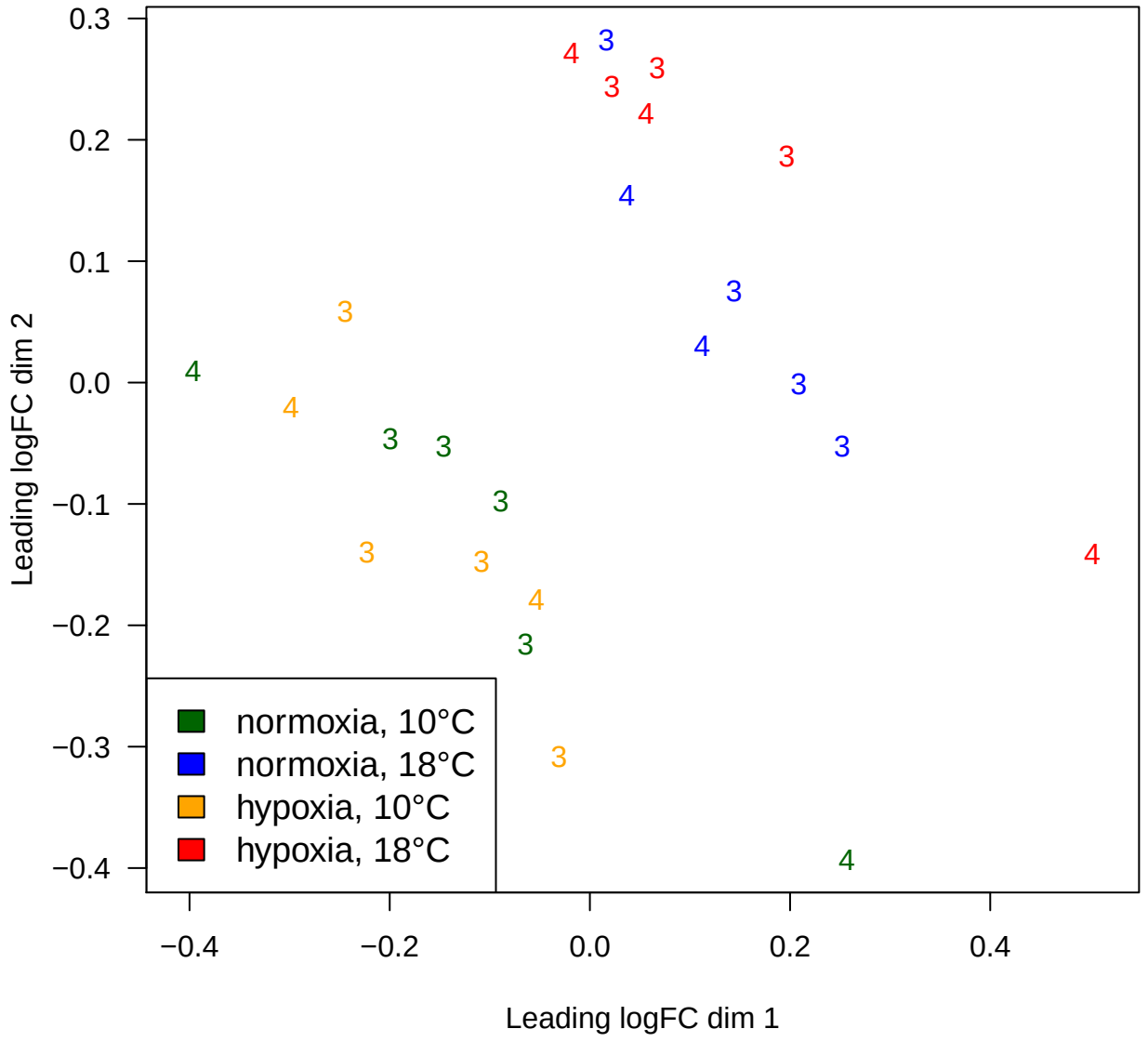
			P- TGTTTGAGGCTCGCTTG									
Claspin-like	DNA synthesis	Claspin	F- ATGCGGGCTGCCCTATC R- CTCTTGAAGAACTGGTCGATGCT P- CATGCCTGAGCCCAA	1.16	1.25	1.04	1.03	1.04	1.15	0.81	0.93	1.18
NDC80, kinetochore complex component	Cell division	Ndc80	F- CCGGCTCCAACACATCTTGT R- CTTCTCCCAGTTGATCCTCTCAA P- CACCCAGAAGTTCACTC	1.13	1.25	1.21	1.03	0.99	1.19	1.01	0.99	1.30
Lamin B1	Cell division	Lmnb1	F- CGAGCTCGCTGACGCTAGA R- CAACCTCGCGCGTTCTTT P- ATCTCTCGATGACACCGC	1.22	1.33	1.22	1.06	1.10	1.27	1.00	1.03	1.45
Condensin-2 complex subunit G2-like	Cell division	NCAPG2	F- CTGTGGCACTCGGCTGTTC R- GAGTCGAAGCCCGACCAA P- CGCTCTAGGAAACTT	1.21	1.17	-	1.31	-	1.70	1.20	0.99	1.36
Chromosome-associated kinesin KIF4-like	Cell division	Kif4	F- GAATCTGCAGCACGTCATCCT R- CAGCCAACGCCTCAATGG P- CAGCTCCAGGATGAGAGT	1.28	1.24	1.35	1.01	1.06	1.28	1.13	1.02	1.38
Structural maintenance of chromosomes protein 4-like	Cell division	SMC4	F- AGCGTGTGGCTGAGTTGGAT R- GATGTTGAAGCCAGCCATGA P- ATCACCACAGAGAGAGACA	-	1.83	1.99	1.66	1.48	1.55	-	1.07	1.85
Up-regulated genes												
Malignant fibrous histiocytoma-amplified sequence 1 homolog	Cell cycle arrest	MFHAS1	F- CCGAGGCCTGGGTGAAC R- TCAGCTGCTCCACAGAGAAGAA P- TCAGTGGCTGCTAGTC	1.13	1.23	1.29	0.99	1.14	1.17	0.95	1.02	1.36

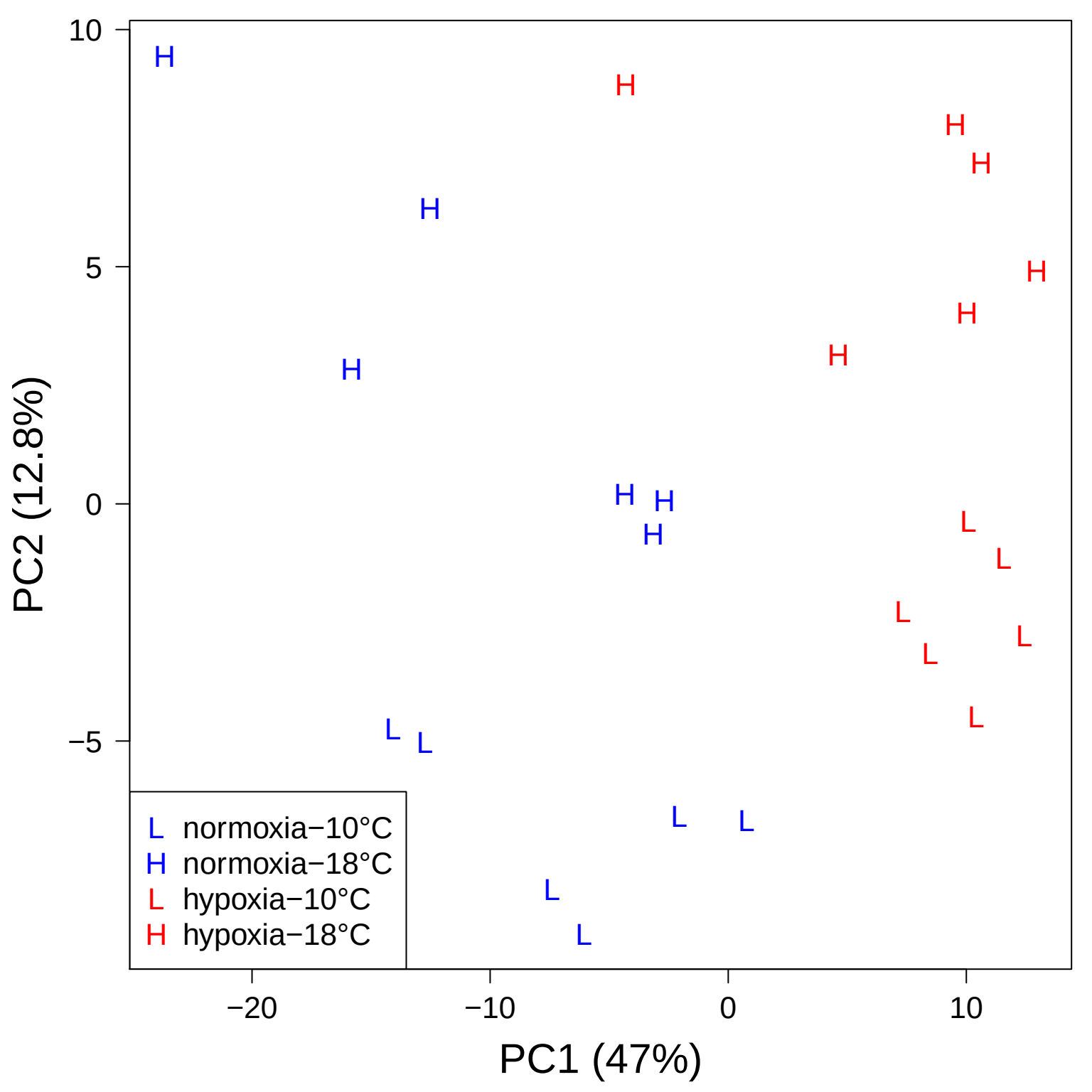
Receptor activity-modifying protein 1-like	Ion transport	RAMP1	F- CGAACCAAGTGGTGCAAGACT R- CCGGACATGCCTGGAAGA P- CTTCATCCAGATCCATTC	0.99	1.19	1.14	1.06	1.04	1.08	1.02	-	1.17
Peptidylglycine alpha-amidating monooxygenase_106 571984	Peptide amidation	PAM	F- TGTAGGAGATGCAGCCACAGA R- TCTTAACAGAGCGATGTTCAGCTT P- TGCTAAAGTTCTCCTCTGAC	1.16	1.19	1.12	1.01	1.04	1.15	1.01	1.03	1.22
protein family with sequence similarity 214, A	Cell division	FAM214A	F- GATGAGGAGTGTGTCTGTCAGTTCTC R- GAGCCTCAGGGCAACACTCT P- CCAGCGGAGCACCT	1.21	1.34	1.19	0.98	1.05	1.18	1.01	0.98	1.12
Calphotin	Cellular calcium regulation	Calphotin	F- CCTTCAAGCGTGTTGACATCA R- CAGTGTTCTCCCTGTAGCACTTCA P- TGTGTGGTGACCTGCA	1.10	1.21	1.18	0.99	1.03	1.12	1.01	1.00	1.48
Gutathione peroxidase 3	Oxidative stress	GPX3	F- AGGCCAGTCCTTCAGTGCAT R- GGCAGGACCAGGAGGTAACA P- TGGGCCTGGTAACC	1.25	1.16	1.11	1.08	1.05	1.20	0.85	0.92	1.21
Endothelin-converting enzyme 2	Neuropeptide processing	ECE-2	F- AGCAGACGCTGGGAGAGAAC R- CACGCCTGGTACGCCTTATAG P- CTGACAACGGAGGCC	1.16	1.25	1.20	1.10	1.09	1.20	1.04	1.02	1.38
Cyclin dependent kinase inhibitor 1B	Cell cycle arrest	CDKN1B	F- CGTCCTCAGCGAAATGGAA R- CCATTCGAATCTCCCGTTTAAT P- TCGATTTTTCAAGTCAAAC	1.23	1.31	1.20	1.07	1.07	1.21	1.04	-	1.38
Transcription factor SOX-5	Cell cycle arrest	SOX-5	F- GAGAGTACAAGGCCATTATGAGGAA R- GCCCGCCGAGGACAAG P- CGGCAGGAGATGAG	1.04	1.12	1.42	1.17	0.98	1.10	1.01	1.01	1.43
Acyl-coenzyme A thioesterase 9, mitochondrial	Oxidative stress	ACOT9	F- CAAGATGCACATGTCTCAGTACCA R- AGGGTCTCTGGCCACCATAA P- CCAGTTCTGGATGCTAC	1.13	1.25	1.45	1.15	1.07	1.27	1.01	1.01	1.37

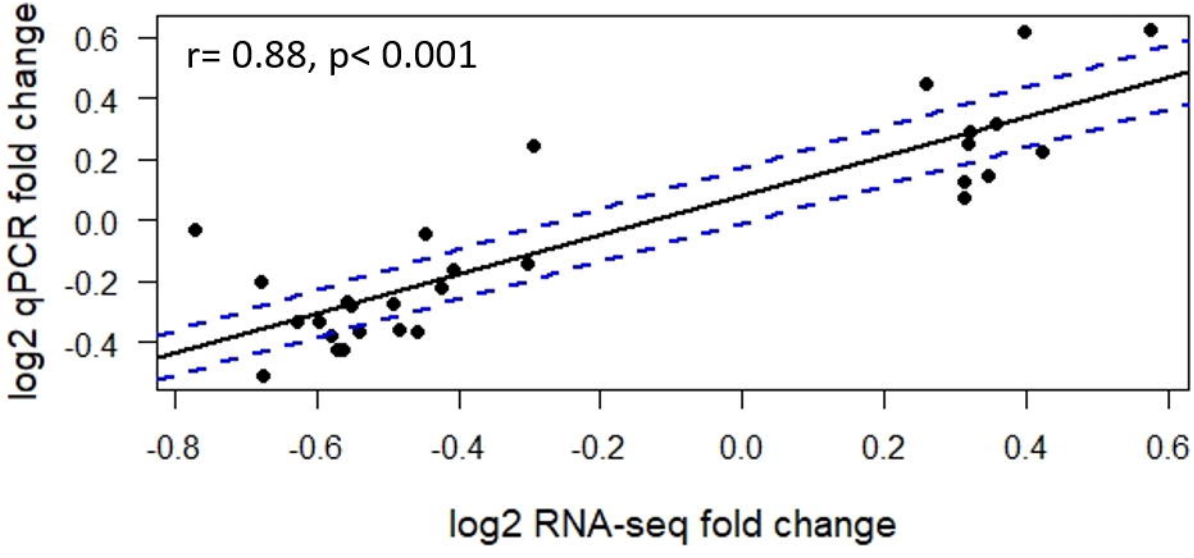
Table 3. Classification ability of the normoxia and hypoxia groups for 24 samples used in RNA-seq, and across the smoltification process of Chinook salmon, and the distressed (moribund/recently dead) fish using the hypoxia biomarkers. Biomarkers identified by Shrunken Centroid method for each group were placed into a linear discriminant analysis (LDA) for classifying groups within each treatment and mortality. LDA training set used two-thirds of the entire dataset. Presented are the classifications on the remaining one-third testing set. Columns are the LDA classification groups and rows are the real group. Salinity symbols are FW for freshwater, BW for brackish, and SW for seawater groups. Dissolved oxygen symbols are N for normoxia and H for hypoxia groups.

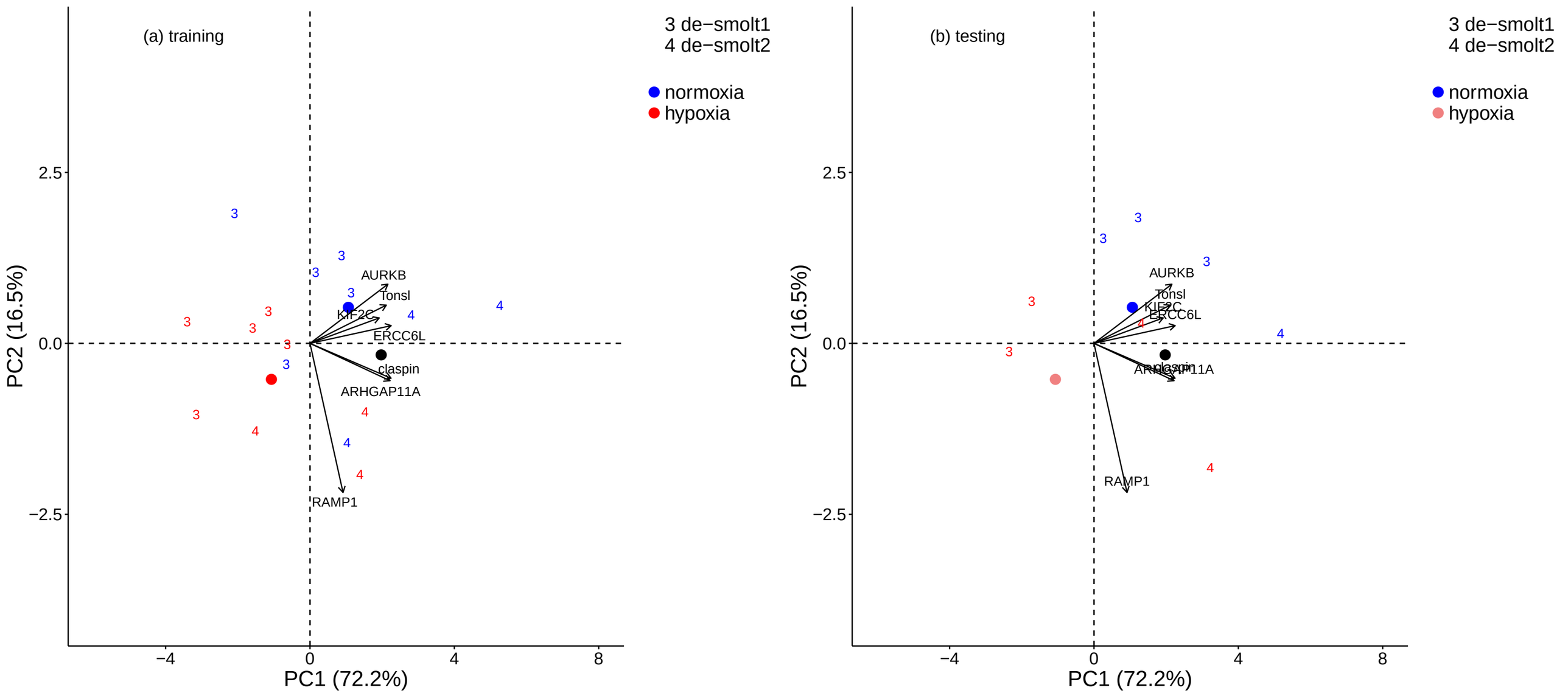
Treatment	Sample size		LDA Classification ability (%)			Significant genes identified by Sherunken analysis	Function of genes
	Training	Testing	N	H	Average		
RNA-seq samples	16	8	100.0	75.0	87.5	Claspin, Tonsl, ARHGAP11A, KIF2C, AURKB, ERCC6L	DNA synthesis, cell division, and cell cycle
						RAMP1	Ion transport
Pre-smolt							
FW	14	7	66.7	75.0	70.8	Ndc80, Kif4, Anillin, Ncapd3	DNA synthesis, cell division, and cell cycle
						Tonsl, NCAPG2, Anillin, KIF2C, CIT, Claspin, PCNA, Rab9, Lmnb1	DNA synthesis, cell division, and cell cycle
BW	12	6	66.7	66.7	66.7	SOX.5	Cell cycle arrest
						ACOT9	Oxidative stress
						Kif15, Anillin	DNA synthesis, cell division, and cell cycle
						CDKN1B	Cell cycle arrest
SW	13	6	75.0	100.0	87.5	RAMP1	Ion transport
						GPX3	Oxidative stress
						PAM	Peptide amidation
Smolt							
						PCNA, Rab9, Kif4, Claspin, Ncapd3, Ndc80	DNA synthesis, cell division, and cell cycle
FW	14	8	75.0	75.0	75.0	SOX.5	Cell cycle arrest
						GPX3	Oxidative s tress
						ECE.2	Neuropeptide processing
						Lmnb1, FAM214A	DNA synthesis, cell division, and cell cycle
BW	12	6	100.0	33.0	66.7	CDKN1B	Cell cycle arrest
						RAMP1	Ion transport
						Anillin, H2AFV, RRM1	DNA synthesis, cell division, and cell cycle
SW	12	6	66.7	100.0	83.3	CDKN1B	Cell cycle arrest
						RAMP1	Ion transport
De-smolt							
FW	12	6	66.7	100.0	83.3	Ndc80, Kif4, Anillin, ARHGAP11A, KIF2C, AURKB	DNA synthesis, cell division, and cell cycle
						FAM214A	DNA synthesis, cell division, and cell cycle
BW	20	10	40.0	60.0	50.0	ACOT9	Oxidative stress
						RAMP1	Ion transport
						Claspin, RRM1, Tonsl, FAM214A, Anillin, Rab9, CIT, Lmnb1	DNA synthesis, cell division, and cell cycle
						CDKN1B, SOX.5, MFHAS1	Cell cycle arrest
SW	31	16	100.0	75.0	87.5	Calphotin	Cellular calcium regulation
						GPX3, ACOT9	Oxidative stress
						RAMP1	Ion transport
Distressed							
FW	17	9	80.0	100.0	90.0	KIF2C	Cell division and DNA synthesis
						MFHAS1	Cell cycle arrest

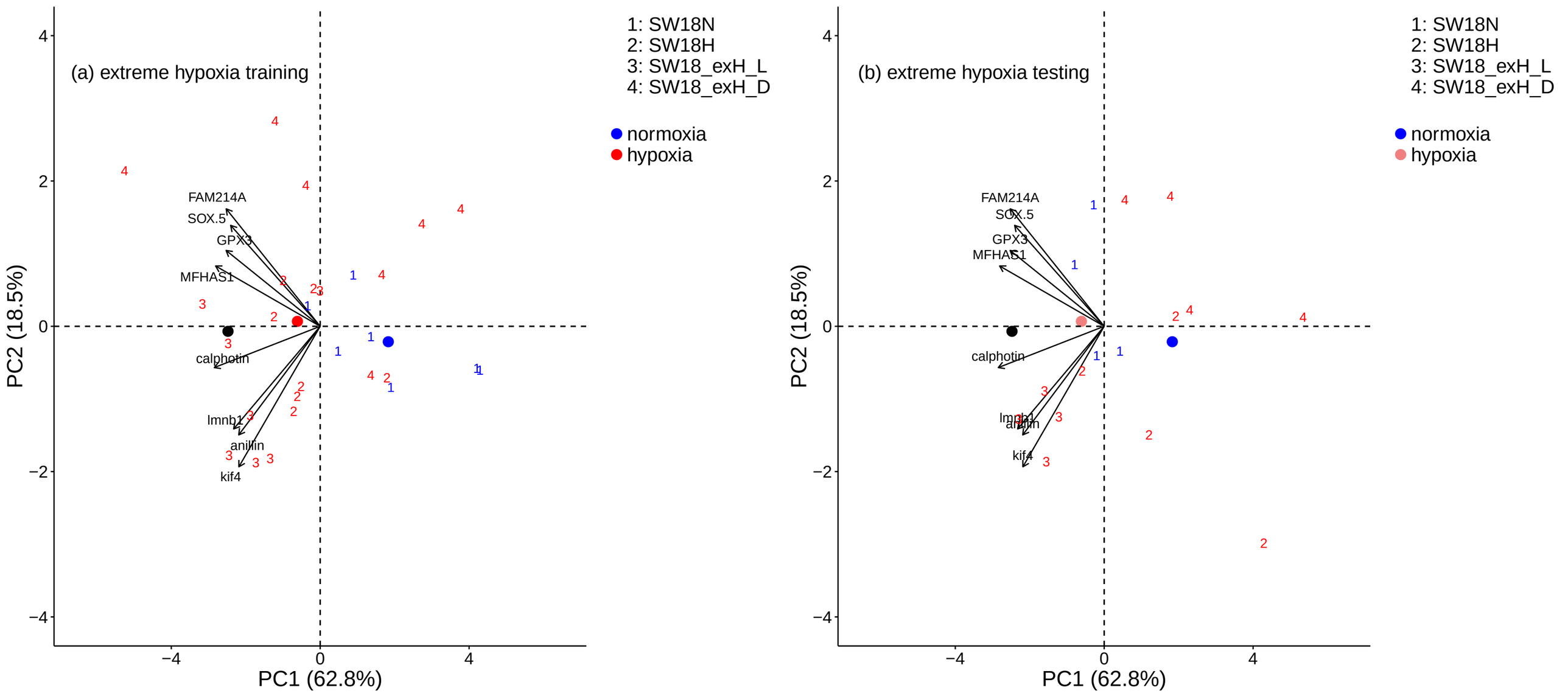
						GPX3	Oxidative stress
						ECE.2	Neuropeptide processing
						FAM214A, Kif4, ARHGAP11A, H2AFV, Claspin, Kif15	Cell division and DNA synthesis
						SOX.5, MFHAS1, CDKN1B	Cell cycle arrest
SW	34	17	77.8	100.0	88.9	GPX3, ACOT9	Oxidative stress
						ECE.2	Neuropeptide processing
						RAMP1	Ion transport
						PAM	Peptide amidation


0.3
0.2
0.1
0.0
−0.1
−0.2
−0.3
−0.4
Leading logFC dim 2
−0.4
−0.2
0.0
0.2
0.4
Leading logFC dim 1
normoxia, 10°C
normoxia, 18°C
hypoxia, 10°C
hypoxia, 18°C


10
5
0
−5
−20
−10
0
10
PC2 (12.8%)
PC1 (47%)
L normoxia−10°C
H normoxia−18°C
L hypoxia−10°C
H hypoxia−18°C


r= 0.88, p< 0.001
log2 qPCR fold change
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.8
-0.6
-0.4
-0.2
0.0
0.2
0.4
0.6
log2 RNA-seq fold change


(a) training
3 de−smolt1
4 de−smolt2
normoxia
hypoxia
AURKB
Tonsl
KIF2C
ERCC6L
claspin
ARHGAP11A
RAMP1
PC2 (16.5%)
PC1 (72.2%)
2.5
0.0
−2.5
−4
0
4
8
(b) testing
3 de−smolt1
4 de−smolt2
normoxia
hypoxia
AURKB
Tonsl
KIF2C
ERCC6L
ARHGAP11A
RAMP1
PC2 (16.5%)
PC1 (72.2%)
2.5
0.0
−2.5
−4
0
4
8


(a) extreme hypoxia training
1: SW18N
2: SW18H
3: SW18_exH_L
4: SW18_exH_D
normoxia
hypoxia
FAM214A
SOX.5
GPX3
MFHAS1
calphotin
lmnb1
anillin
kif4
PC2 (18.5%)
PC1 (62.8%)
(b) extreme hypoxia testing
1: SW18N
2: SW18H
3: SW18_exH_L
4: SW18_exH_D
normoxia
hypoxia
FAM214A
SOX.5
GPX3
MFHAS1
calphotin
lmnb1
anillin
kif4
PC2 (18.5%)
PC1 (62.8%)