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

3

4 Running title: Hypoxia biomarkers in salmonids

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23 **Abstract**

24 Identifying early gene expression responses to hypoxia (i.e., low dissolved oxygen) as a tool to
25 assess the degree of exposure to this stressor is crucial for salmonids, because they are
26 increasingly exposed to hypoxic stress due to anthropogenic habitat change, e.g., global
27 warming, excessive nutrient loading, and persistent algal blooms. Our goal was to discover and
28 validate gill gene expression biomarkers specific to the hypoxia response in salmonids across
29 multi-stressor conditions. Gill tissue was collected from 24 freshwater juvenile Chinook salmon
30 (*Oncorhynchus tshawytscha*), held in normoxia [dissolved oxygen (DO) > 8 mg L^{-1}] and hypoxia
31 (DO = 4□5 mg L^{-1}) in 10 and 18°C temperatures for up to six days. RNA-sequencing (RNA-seq)
32 was then used to discover 240 differentially expressed genes between hypoxic and normoxic
33 conditions, but not affected by temperature. The most significantly differentially expressed genes
34 had functional roles in the cell cycle and suppression of cell proliferation associated with
35 hypoxic conditions. The most significant genes (n = 30) were selected for real-time qPCR assay
36 development. These assays demonstrated a strong correlation ($r = 0.88$; $p < 0.001$) between the
37 expression values from RNA-seq and the fold changes from qPCR. Further, qPCR of the 30
38 candidate hypoxia biomarkers was applied to an additional 322 Chinook salmon exposed to
39 hypoxic and normoxic conditions to reveal the top biomarkers to define hypoxic stress.
40 Multivariate analyses revealed that smolt stage, water salinity, and morbidity status were relevant
41 factors to consider with the expression of these genes in relation to hypoxic stress. These hypoxia
42 candidate genes will be put into application screening Chinook salmon to determine the identity
43 of stressors impacting the fish.

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

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46

47 ## Introduction

48 Aquatic ecosystems are increasingly being affected by a complex mixture of anthropogenic
49 stressors (Hering *et al.* 2015). In recent years, the prevalence and intensity of aquatic hypoxic
50 episodes has increased worldwide, resulting in aquatic environments with large variations in
51 dissolved oxygen (Leveelahti *et al.* 2011; Robertson *et al.* 2014). Both natural and anthropogenic
52 drivers can be responsible for dissolved oxygen (DO) depletion in aquatic ecosystems. Natural
53 hydrodynamic conditions, such as the oxidation of organic matter and the release of gases (e.g.,
54 methane or carbon dioxide), may make certain aquatic ecosystems prone to oxygen depletion.
55 However, climate change can also reduce the solubility of oxygen because of increased thermal
56 stratification and enhanced microbial activity, all of which would cause DO depletion (Weinke
57 and Biddanda 2018). Moreover, agriculture, industry, and urbanization can lead to nutrient
58 loading (i.e., eutrophication) and consequent persistent algal blooms in aquatic ecosystems,
59 which also affect both the supply and uptake of DO (Friedrich *et al.* 2014). The combined effects
60 of warming and eutrophication in aquatic ecosystems may cause dramatic declines in DO, which
61 can lead to hypoxic conditions.

62 Hypoxia is defined as any level of DO that is low enough to negatively impact the behaviour
63 and/or physiology of an organism. However, the impacts of different hypoxia levels and their
64 effects on fish physiology or behavior is very species-specific (Pollock *et al.* 2007). For example,
65 cyprinid fish, such as the goldfish (*Carassius auratus*) and the crucian carp (*C. carassius*),
66 exhibit a striking capacity to survive and remain active for long periods under very low DO, even
67 tolerating anoxia (Gattuso *et al.* 2018). In contrast, DO below 5 mg/L is the threshold for
68 hypoxic stress in salmon (Lucas and Southgate. 2013; Olsvic *et al.* 2013; Del Rio *et al.* 2019).

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69

70 Hypoxia can adversely impact a wide variety of biochemical and physiological processes
71 in fish, such as cell cycle, fish growth, and aerobic metabolism (Zhang *et al.* 2009; Douxfils *et*
72 *al.* 2012). Hypoxia can also negatively impact normal biological functions, resulting in
73 suppressed development, reduced activity, feeding and growth, disturbed endocrine function, and
74 impaired reproductive performance (Callier *et al.* 2013; Wang *et al.* 2016; Abdel-Tawwab *et al.*
75 2019). Under extreme hypoxic conditions, mass mortality of wild populations can occur
76 (Douxfils et al. 2012; Long et al. 2015; Closs *et al.* 2016). Owing to the significance of hypoxic
77 stress in fish, the study of the effects of hypoxia on fishes has flourished, and received much
78 attention by fish physiologists (Pollock *et al.* 2007; Richards *et al.* 2009).

79 Salmon can be negatively impacted by hypoxia through impacts on swim performance,
80 growth, and development, which may ultimately affect fitness and survival. Typically, fish
81 respond to hypoxia through a number of behavioural, morphological, physiological, and
82 molecular changes. Behaviourally, fish can actively avoid hypoxic areas or reduce energy
83 demands by decreasing swimming activity. Morphologically, fish can adapt by increasing the
84 respiratory surface area on the gills. Physiological and molecular adaptations include enhanced
85 production of respiratory proteins (e.g. hemoglobin, myoglobin, and neuroglobin) to increase
86 oxygen carrying capacity, upregulation of genes encoding enzymes within the glycolytic
87 pathway, and downregulation of genes involved in aerobic energy production and other energy-
88 consuming processes (Tiedke *et al.* 2015). Differential regulation of genes involved in general
89 metabolism, catabolism, and the ubiquitin-proteasome pathway have also been demonstrated in
90 fish as responses to low DO (Ju *et al.* 2007; Zhang *et al.* 2012; Olsvik *et al.* 2013).

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

103 Measuring DO concentrations in aquatic ecosystems is one aspect of monitoring hypoxic
104 stress. However, frequent DO measurements over large regions and long periods of time are
105 impractical, unless sampling moorings are continuously used. Furthermore, such environmental
106 measurements do not directly assess whether hypoxic stress was experienced by a fish. We know
107 that low DO conditions have serious consequences for farmed salmon (e.g. Burt *et al.* 2012;
108 Solstorm *et al.* 2018), but it is not clear whether migratory fish inhabiting or moving through
109 such conditions can behaviourally moderate their exposure or whether they are also negatively
110 affected. The application of biomarkers to detect the specific stress response to low DO, and to
111 other stressors for that matter, could thus be an important approach (Zhang *et al.* 2012;
112 Akbarzadeh *et al.* 2018), and be a more integrative technique (Froehlich *et al.* 2015; Houde *et al.*
113 2019a). Biomarkers can be defined as measurable biochemical, cellular, and/or physiological

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114 changes in an organism caused by perturbations in the environmental conditions, such as
115 hypoxia. The ideal biomarker should be specific to the stressor of interest, be easy to assay, and
116 be relatively unaffected by sampling procedures (Zhang *et al.* 2012).

117 A major advantage of the biomarker approach is the ability to detect sub-lethal impacts at
118 low levels of stressor intensity. Moreover, the inferred consequences of sub-lethal exposure can
119 be made more robust because the biomarkers can also provide detailed physiological information
120 of an organism (Froehlich *et al.* 2015). Molecular ecologists have become interested in studying
121 early genetic responses of various organisms to stressors such as hypoxia (Nikinmaa and Rees
122 2005; Boswell *et al.* 2009). Several approaches have been used to identify hypoxia responsive
123 genes in fishes and their altered expression levels, including microarrays, real-time qPCR, and
124 proteomics (Rashid *et al.* 2017). RNA-seq has been more recently used to identify the
125 transcriptional responses of fish to hypoxia at the whole transcriptome level without a reference
126 genome (Olsvic *et al.* 2013; Long *et al.* 2015; Zhang *et al.* 2016).

127 As most genes and proteins can be involved in several physiological pathways, it may not
128 be realistic to obtain such a specific predictor with a single biomarker. Miller *et al.* (2017)
129 demonstrated that the detection of a viral disease state in salmon could be accomplished by the
130 co-regulation of as few as seven biomarkers. This approach has led to the discovery of several
131 novel viruses in salmon (Mordecai *et al.* 2020) and the elucidation of the developmental pathway
132 of a natural disease outbreak, from a viral carrier state to disease causing death (Di Cicco *et al.*
133 2018). Recently, salmonid biomarker panels have been developed and validated for the
134 detection of thermal and salinity stress (Akbarzadeh *et al.* 2018; Houde *et al.* 2019a), but the
135 identification of a panel specific to hypoxic stress activated in gill tissue has proven to be more
136 difficult. This may be because of a lack of available transcriptional studies to use to identify

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137 candidate hypoxia biomarkers in salmon gill, whereas such studies were available for thermal
138 and salinity stress. For hypoxia, only two genes (i.e., *hypoxia inducible factor 1 alpha* [*hif1a*]
139 and *cytochrome C oxidase subunit 6B1* [*cox6b1*]) of the 24 candidate hypoxia genes applied to
140 the gill tissue of juvenile ocean-type Chinook salmon (*O. tshawytscha*) exposed to combinations
141 of salinity, temperature, and DO manipulations were primarily influenced by DO. However,
142 these two genes alone were not sufficient to separate salmon exposed to normoxia from those
143 exposed to hypoxia across differing salinities and temperatures.

144 Our objective here was to discover and validate a biomarker panel of at least eight genes
145 specific to the hypoxia response across several conditions. To this end, we applied RNA-seq
146 (Wang *et al.* 2009; Ozsolak and Milos 2011) with best practices for analysis (Conesa et al. 2016)
147 to discover additional candidate genes for the response to hypoxia in salmonids. It is well known
148 that the gill is a multifunctional organ (Evans et al. 2005). Oxygenation in the gill is only
149 dependent on the ambient oxygen level from direct contact with the external environment,
150 whereas oxygenation in all other tissues also depends on circulatory oxygen perfusion.
151 Therefore, temperature and hypoxia effects can only be separated using gill tissue. In this regard,
152 gill tissue is ideal for identifying specific hypoxia biomarkers in salmon. In total, gill tissue from
153 24 freshwater (FW) Chinook salmon juveniles were characterized by RNA-sequencing after
154 exposure to normoxia or hypoxia within 10 and 18°C temperatures (n = 6 fish per condition).
155 These fish were sourced from a previous exposure experiment (Houde *et al.* 2019a). TaqMan
156 assays were then designed to the top 30 candidate hypoxia genes (based on p-values) that were
157 consistent across temperatures. Biomarkers were validated using 301 Chinook salmon from the
158 challenge study that were not used for sequencing. Recent studies have shown that intermittent
159 diel cycles of hypoxia (12 h normoxia: 12 h hypoxia) might give different results as compared to

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160 constant (sustained) hypoxia (Borowiec et al. 2015). Therefore, in this study we exposed fish to
161 one hypoxia level and one hypoxia duration.
162
163 Our ultimate aim is to add a validated hypoxia-specific biomarker panel to similar panels
164 for thermal, salinity, and general stress, as well as panels indicating different disease states (viral
165 disease, inflammation/wounding response, humoral/cellular response, and imminent mortality)
166 applied simultaneously within a newly developed microfluidics-based salmon "Fit-Chip" tool
167 (Miller et al. 2018). The Fit-Chips will then be applied to examine the synergistic interplay
168 between stress and disease, to elucidate the combinations of stressors most impactful to salmon
169 survival, and to identify habitats where fish are the most compromised. This information can
170 then be used to develop plans for environmental mitigation.

171 **Materials and Methods**

172 *Experimental set-up*

173 The experiment was approved by the Fisheries and Oceans Canada (DFO) Pacific Region
174 Animal Care Committee (2017-002) following the Canadian Council of Animal Care Standards.
175 Sub-yearling Chinook salmon were provided by Big Qualicum Hatchery, Qualicum, British
176 Columbia (BC), Canada, and were reared in communal tanks at the Pacific Biological Station,
177 Nanaimo, BC. Juveniles were exposed to FW (< 14°C), light at the natural cycle, and fed a ration
178 of commercial pellets (2% body mass, Bio-Oregon) every 1–2 days until used in the experiment.
179 Four trials were conducted over the smoltification period, covering pre-smolt (March), smolt
180 (May), and de-smolt (two trials, June and August) stages based on expected differences in
181 seawater (SW) mortality, gill Na^{+}/K^{+}-ATPase activity, and body variables (see Houde *et al.*
182 2019a).

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183 During the experiment, as detailed in Houde et al. (2019a) and in brief here, juveniles
184 were exposed to 18 possible water treatments: three salinities (FW at 0 PSU, brackish (BW) at
185 20 PSU, and SW at 28 or 29 PSU), three temperatures (10, 14, and 18°C), and two dissolved
186 oxygen (DO) concentrations (hypoxia 4□5 mg L^{-1} and normoxia > 8 mg L^{-1}) in all combinations.
187 Each treatment was represented by two (replicate) 30 L pot tanks with tight fitting lids that
188 limited gas exchange. Water at the desired salinity and temperature was divided into two PVC
189 columns: one with media for typical aeration for normoxia and the other with a ceramic air stone
190 delivering very small nitrogen bubbles (5–10 μm) for hypoxia. Nitrogen was supplied to the
191 system using portable liquid units or compressed gas bottles (Praxair). Hypoxia conditions were
192 measured to ensure target levels using nine DO probes, where a single probe was present in one
193 of the two replicate tanks. Each probe was connected to a Point Four RIU3 monitor-controller
194 (Pentair) for turning the nitrogen regulator on or off as required to keep DO in range.
195 Collectively, the controllers were connected to a Point Four LC3 central water system (Pentair).
196
197 *Juvenile handling*
198 Prior to treatment exposure, 12□16 juveniles were acclimated to each tank (n = 18 tanks) for six
199 days under the control conditions (i.e., FW, 13□14°C, normoxia), and fed a ration every day.
200 Water conditions were then changed over 1□2 days. On day 1, higher salinity water was
201 introduced over 2□3 h in morning, then temperature was changed by 2°C in the early afternoon,
202 and dissolved oxygen was set to 6.5□8 mg L^{-1} in the mid-afternoon using the LC3 system. On
203 the morning of day 2, temperature was changed by the remaining 2°C and DO was set to 4□5
204 mg L^{-1}. Juveniles were then exposed to full treatment conditions for six days and were fed
205 rations with 48 h starvation before final sampling. On day six, juveniles were euthanized in an

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

210 As previously described by Houde et al. (2019a), the two SW/18°C-hypoxia tanks in the
211 June, de-smolt trial (i.e., trial 3) had lower DO than intended in the SW treatment due to a
212 programming failure (3.3–4.1 mg L^{-1} instead of 4–5 mg L^{-1}). After almost two days of exposure,
213 11 of 24 juveniles were dead or moribund, and the remaining 13 juveniles were ethically
214 euthanized. Because of a poor separation between normoxia and hypoxia using the general
215 hypoxia candidate genes previously applied (see Houde *et al.* 2019a), these fish were used as an
216 'extreme hypoxia' condition. Greater details on juvenile handling and sampling are described in
217 Houde *et al.* (2019a)

218 *RNA extraction and sample selection*

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

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

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229 were selected from Trial 3, and one individual from each tank replicate from Trial 4. Conditions
230 included were two temperatures (10°C and 18°C) and two DO conditions (normoxia and
231 hypoxia).

232 *Library preparation and sequencing*

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

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

249

250 *Sequence read processing*

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251 All bioinformatics analyses are reported in detail including all scripts on GitHub (see *Data*
252 *Accessibility*). Raw reads were quality checked with FastQC 0.11.5 (Andrews 2016), with
253 individual sample files compiled using MultiQC 1.0 (Ewels *et al.* 2016). Adaptors, poor quality
254 sequences (Phred = 2, MacManes 2014), and reads with a length less than 80 bp were removed
255 using Trimmomatic v0.36 (Bolger *et al.* 2014) using flags *illuminaclip* (2:30:10), *slidingwindow*
256 (20:2), *leading* (2) and *trailing* (2), and *minlen* (80). Trimmed reads were quality checked again
257 with FastQC and MultiQC.

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

272

273 *Statistical analysis for discovering candidate hypoxia genes*

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274 After converting to gene counts, RNA-seq analyses were conducted using R 3.4.4 (R Core Team,
275 2018). The transcript counts per individual were analyzed using the *edgeR* v.3.7 package
276 (Robinson *et al.* 2010) and general pipeline (Anders *et al.* 2013). Gene counts were filtered for
277 low expressed genes using a 0.4 count-per-million (cpm) threshold, corresponding to a count of 5
278 reads in the sample with the fewest reads. A gene was removed if it was below this threshold in
279 at least half the individuals (n = 12). Filtered counts were analyzed using multi-dimensional
280 scaling (MDS) method to evaluate the effects of different factors (i.e., temperature, hypoxia, and
281 trial) on overall gene expression. Then, the filtered counts were normalized and dispersions
282 estimated using a model design containing the four treatments with no intercept. A robust quasi-
283 likelihood generalized linear model was then fit to the counts. Given the potential issues of
284 separating thermal stress from hypoxic stress responses (Pörtner 2010), quasi-likelihood F-tests
285 used models with paired contrasts within temperatures, i.e., normoxia-10°C vs. hypoxia-10°C
286 and normoxia-18°C vs. hypoxia-18°C. No differential expression was observed when the false
287 discovery rate was applied ($q < 0.05$, Benjamini and Yekutieli 2001), and so for this discovery
288 work, the unadjusted p-values were used. Differentially expressed genes ($p < 0.05$) in common
289 for both contrasts were isolated using the *systemPipeR* package (Backman and Girke 2016) and
290 retained if the $\log_2$ fold change direction was the same for both contrasts.

291 The subset of differentially expressed genes from above were subjected to a principal
292 component analysis (PCA) using the scaled and normalized counts (cpm) of individuals. The PC
293 axis associated with the separation of normoxia and hypoxia was identified. Candidate genes
294 were ranked based on the significance of a Pearson correlation with this PC axis. The top 30
295 candidate genes based on correlation were examined for available mRNA sequences for several
296 species of salmonids using a custom database (Akbarzadeh *et al.* 2018) and the public NCBI

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297 repository, as a goal of this work is to design primers that will work for multiple salmonid
298 species.
299
300 *Development of RT-qPCR assays for validating hypoxia biomarkers*
301 Universal salmonid (across nine salmonid species) real-time qPCR assays (Table S1) were
302 developed for the top 30 candidate genes discovered using the RNA-seq data. The assay primers
303 and TaqMan probes for the sequences were produced using Primer Express 3.0.1 (Thermo Fisher
304 Scientific, Waltham, MA) with primer melting temperature (Tm) between 58–60 ºC, and probe
305 Tm between 68–70 ºC as default.
306 To test the efficiency of the 30 discovered hypoxia genes across salmonid species, cDNA
307 from RNA extractions of pooled tissues from each of the nine salmonid species, i.e. Chinook
308 salmon (*O. tshawytscha*), coho salmon (*O. kisutch*), chum salmon (*O. keta*), pink salmon *(O.*
309 *gorbuscha*), sockeye salmon (*O. nerka*), Atlantic salmon (*Salmo salar*), Artic charr (*Salvelinus*
310 *alpinus*), rainbow trout (*O. mykiss*) and bull trout (*Salvelinus confluentus*), were serially diluted
311 from 1/5 to 1/625 in five dilutions. Specific target amplification (STA), was performed to enrich
312 targeted sequences within the pools using 3.76 µL 1X TaqMan PreAmp master mix (Applied
313 Biosystems), 0.2 µM of each of the primers, and 1.24 µL of cDNA, as previously described
314 (Akbarzadeh et al. 2018; Houde et al. 2019a). Samples were run on a 14 cycle PCR program,
315 with excess primers removed with EXO-SAP-IT (Affymetrix), and then amplified samples were
316 diluted 1/5 in DNA suspension buffer. The diluted samples and assays were run in singletons
317 following the platform instructions (Fluidigm). For sample reactions, 3.0 µL 2X TaqMan
318 mastermix (Life Technologies), 0.3 µL 20X GE sample loading reagent, and 2.7 µL STA product
319 were used. For assay reactions, 3.3 µL 2X assay loading reagent, 0.7 µL DNA suspension buffer,

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320 1.08 µL forward and reverse primers (50 uM), and 1.2 µL probe (10 uM) were used. The PCR
321 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
322 1 min. Data were extracted using the Real-Time PCR Analysis Software (Fluidigm) using Ct
323 thresholds set manually for each assay. PCR efficiencies for each assay were calculated using
324 $(10^{1/slope} - 1) \times 100$, where the slope was estimated by plotting the Ct over the serial dilutions of
325 cDNA.

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

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

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342 normal oxygen for six days, and 11 live-sampled individuals exposed to hypoxia for six days.
343 The technical roadmap of the experimental method is illustrated in Fig. 1.
344 Gill tissue homogenization, RNA extraction and quantification, cDNA synthesis, and STA were
345 conducted as described above and previously (Houde *et al.* 2019a). The 96.96 gene expression
346 dynamic array (Fluidigm Corporation, CA, USA) was applied and generally followed Miller *et*
347 *al.* (2016). qRT-PCR data were analysed with Real-Time PCR Analysis 3 Software (Fluidigm
348 Corporation, CA, USA). The expression of the 29 discovered hypoxia genes were normalized to
349 the expression of the housekeeping gene, S100 calcium binding protein (78d16.1), which was
350 found to be the most suitable housekeeping gene in NormFinder analysis, as previously
351 described (Houde *et al.* 2019a). Sample gene expression was normalized with the ΔΔCt method
352 (Livak and Schmittgen 2001) using the inter-array calibrator sample. Gene expression was then
353 log transformed: $\log_2 (2^{-\Delta\Delta Ct})$.
354 *Comparing RNA-seq results to the designed qRT-PCR targets*
355 To validate the transfer of the discovered markers from RNA-seq platform to qRT-PCR, a
356 correlation analysis was performed between the normalized counts (cpm) of RNA-seq data and
357 the fold changes obtained from the qRT-PCR platform for the same 24 samples used for RNA-
358 seq. Fold changes in expression values of RNA-seq data (on the X-axis) were plotted against fold
359 change values obtained from RT-qPCR (on the Y-axis) on a scatter graph.
360 *Statistical analysis to validate hypoxia biomarkers*
361 Analyses were performed using R 3.4.4 (R Core Team, 2018) at a significance level of $\alpha = 0.05$.
362 As the expression levels of hypoxia genes were also influenced by water salinity and smolt stage,
363 further analyses were separately carried out for each group. The dataset for each group was
364 divided into a two-thirds training set and a one-third testing set. The training set was subjected to

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365 a Shrunken Centroid method (Tibshirani et al. 2002) to identify a classifier for hypoxia vs.
366 normoxia fish for each group. This method uses an internal cross-validation for threshold
367 selection and returned a reduced list of the most influential genes for robust classification. Then,
368 the selected genes were subjected to PCA analysis using the data from the training set that
369 excluded the 24 RNA-seq samples. This PCA was then applied to the testing set for visualization
370 of unsupervised group separation within each group using the *fviz_pca* function of the *factoextra*
371 R package (https://cran.r-project.org/web/packages/factoextra/index.html). The classification
372 ability was also examined by subjecting the resulting biomarkers to linear discriminant analysis
373 (LDA) using the training set, followed by determining classification performance on the testing
374 set. A similar approach was used for examining mortality (dead or moribund vs. live), and the
375 extreme hypoxia group of SW-18°C in trial 3.

376 **Data availability**

377 The analysis pipeline and scripts for the RNA-seq analysis is described in more detail on
378 GitHub: https://github.com/bensutherland/Simple_reads_to_counts. The RNA-seq reads have
379 been uploaded to the NCBI Sequence Read Archive (SRA) under BioProject PRJNA635140,
380 with accession numbers SAMN15020676-SAMN15020699.

381 https://www.ncbi.nlm.nih.gov/bioproject/PRJNA635140.

382 The qRT-PCR results and all the supplemental tables and figures are available at Figshare.

383 **Results**

384 *Sequencing and gene count overview*

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

396 *Discovery of candidate hypoxia genes*

397 The number of upregulated and downregulated genes ($p < 0.05$) between normoxia and hypoxia
398 treatments was similar within each temperature: at 10 °C, there were 1,926 upregulated and
399 1,441 downregulated genes from hypoxia. At 18 °C, there were 1,742 upregulated and 1,347
400 downregulated genes from hypoxia. In total, 349 genes were found to be differentially regulated
401 in both comparisons, and when requiring that these genes follow the same direction of
402 regulation, this included 240 genes (i.e., 96 upregulated genes and 144 downregulated genes).

403 The PCA using only the 240 candidate hypoxia-response genes revealed that normoxia
404 and hypoxia separation occurred along PC1, as expected given that these genes were selected
405 based on their hypoxia response. There was also a separation of the 10 and 18°C groups along
406 PC2 (Fig. 3). To avoid selecting genes responding to temperature in addition to hypoxia, genes
407 were excluded when they were correlated with PC2 ($p < 0.1$; Table S2). Downregulated genes

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408 from hypoxia showed higher correlations with PC1 than did upregulated genes. The top 20
409 downregulated genes (r = -0.85 to -0.95) and the top 10 upregulated genes (r = 0.73 to 0.81)
410 were investigated further for assay development. The top 20 down-regulated genes were mostly
411 involved in DNA synthesis, cell division, and cell cycle, while the top 10 up-regulated genes
412 were involved in the cell cycle arrest, oxidative stress response, ion transport, cellular calcium
413 regulation, peptide amidation, and neuropeptide processing (Table 1).

414 *Correlation between RNA-seq and qRT-PCR data*

415 The validation of the design of the primers for qPCR compared to the results from RNA-
416 seq was conducted using a correlation analysis between the $\log_2$ fold change from the RNA-seq
417 data and the $\log_2$ fold change of the qRT-PCR platform for the same 24 samples used for both
418 platforms. A high value for the Pearson correlation coefficient ($r = 0.88$; $p < 0.001$) indicated a
419 positive correlation between platforms (Fig. 4). From 29 genes tested, 15 genes showed
420 significant correlation ($p < 0.05$) between two platforms (Table S3).

421 Moreover, there was a high separation between hypoxia and normoxia along PC1 using
422 the gene expression data of 24 independent RNA-seq samples (Fig. 5). Using the top seven genes
423 obtained from the Shrunken Centroid method on 15 significant correlated genes, the
424 classification ability for normoxia was 100.0% and hypoxia was 75.0% with the average
425 classification of 87.5%.

426 *Hypoxia biomarkers*

427 The qRT-PCR gene expression results for the 29 discovered hypoxia genes including all 300
428 live-sampled and distressed fish kept at different combinations of temperature, salinity and
429 oxygen for six days, as well as 22 live-sampled and distressed fish exposed to extreme hypoxia
430 for two days are presented in Fig. S1. Moreover, the initial results of the PCA analysis on all

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431 live-sampled and distressed fish together using the selected genes from Shrunken Centroid
432 method showed that PC1 was associated with smolt stage, water salinity, and mortality (*data not*
433 *shown*). Given the influence of smolt stage, salinity, and mortality on the candidate hypoxia
434 biomarkers, we separated the data first by the three smolt stages (live-sampled only) and
435 distressed, and then by the three salinities such that there were 11 groups. Distressed fish in BW
436 data was not analyzed due to too few samples (n = 3) of normoxia fish in this salinity
437 environment.

438 The results of the PCA analyses and LDA scores for normoxia vs. hypoxia within each
439 group using the most contributing hypoxia biomarkers are shown in Figures S2-S5 and Table 2.
440 Effectiveness of biomarkers were evaluated based on the ability to separate samples within the
441 PCA and low LDA test error for both normoxic and hypoxic conditions.

442 Pre-smolt fish transferred to SW showed a distinct separation of normoxia and hypoxia
443 groups along PC2, having the highest average LDA classification accuracy (87.5%) (Fig. S2a;
444 Table 2). The pre-smolts in SW had higher LDA classification accuracy than the LDA results for
445 fish in FW or BW.

446 Smolts in FW or SW had distinct separations of normoxia and hypoxia samples along
447 PC2 (Fig. S3a, b). The SW smolts showed the highest LDA classification accuracy (83.3%),
448 although this was biased towards identifying samples in the hypoxia condition (Table 2). The
449 most balanced LDA classification between hypoxic and normoxic classifications in the smolts
450 was 75% as observed in smolts in FW (Table 2).

451 De-smolt fish in FW had a distinct separation for normoxia and hypoxia groups along
452 PC2 with an average LDA classification accuracy of 83.3% but biased towards hypoxia (Fig. S4;
453 Table 2). Although the highest average LDA classification accuracy for de-smolts was 87.5% (in

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454 SW), there was no clear separation for the PCA using the first two axes (Table 2). Moreover,
455 both trials of de-smolt fish were also separated along PC2 in all salinity environments (Fig. S4).

456 Distressed fish in both FW and SW showed the highest PCA separation and classification
457 ability with an average classification accuracy of 90.0 and 88.9%, respectively (Fig. S5; Table
458 2).

459 The PCA analysis on the extreme hypoxia group of SW/18°C in trial 3 showed a stronger
460 separation of normoxia and live-extreme hypoxia exposed fish than normoxia and moderate
461 hypoxia groups along PC1. Distressed extreme hypoxia exposed fish were also separated from
462 other groups in PCA analysis (Fig. 6).

463 In sum, the conditions that showed the most potential for separating fish kept in normoxic
464 from hypoxic conditions were pre-smolt and smolt stages in SW, and de-smolt stage in FW. On
465 the contrary, all conditions in BW, pre-smolt and smolt in FW, and de-smolt in SW were not able
466 to clearly separate fish kept in normoxic from hypoxic conditions.

467 Discussion

468 Characterizing early genetic responses to hypoxia to facilitate the development of a tool to assess
469 hypoxia exposure is crucial towards managing salmonid fish that are increasingly exposed to
470 hypoxic stress globally. This is particularly relevant considering the strong influence
471 anthropogenic effects, such as excessive nutrient loading, can have on hypoxia levels and
472 occurrence, for example on the west coast of North America. The present study aimed to
473 discover and validate gill gene expression biomarkers specific to hypoxic stress in salmonids.
474 Using high-throughput RNA-seq analysis, we discovered 30 candidate genes that were
475 differentially expressed between hypoxic and normoxic conditions, but were not affected by
476 temperature. The discovered genes were then designed to qRT-PCR assays, and then used to

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477 query a larger set of samples by high-throughput microfluidics qPCR. These qPCR data were
478 then used to characterize and identify the best biomarkers for differentiating exposure to
479 normoxic and hypoxic conditions.

480 *Biological and molecular functions of candidate hypoxia genes*

481 It should be noted that while transcript changes are expected to reflect protein and physiological
482 effects, a strong correlation between gene and protein expression is not always the case owing to
483 post-transcriptional and post-translational modifications (Maier *et al.* 2009; Schwänhausser *et al.*
484 2011; Kanerva *et al.* 2014). However, here we discuss transcripts in relation to their expected
485 protein products and functions. All of the top 20 downregulated genes in the hypoxia treatment
486 discovered by the RNA-seq analysis were involved in functions related to DNA synthesis, cell
487 division, and cell cycle. The concordant downregulation of these genes during hypoxia suggests
488 that cell proliferation may be suppressed in the Chinook salmon gill tissue. Hypoxia has been
489 shown to inhibit cell proliferation in numerous cell types (Hubbi and Semenza 2015). A
490 decreased number of cells may allow for decreased oxygen demand under hypoxia (Hubbi and
491 Semenza 2015). These hypoxia-suppressed genes may reflect a physiological alteration that halts
492 cell proliferation and translational activities to allow the cells to preserve energy consumption
493 under metabolic stress (Chi *et al.* 2006). The downregulation of genes involved in cell cycle
494 progression occurs in response to acute and chronic hypoxia in zebrafish embryos (Ton *et al.*
495 2003) and in hepatic cells of hybrid striped bass (*Morone saxatilis* × *Morone chrysops*) (Beck *et*
496 *al.* 2016). Therefore, the downregulation of cell proliferation in the gill may be a physiological
497 strategy in Chinook salmon to cope with hypoxic water.

498 Consistent with the suppression of genes involved in cell proliferation, theexpression of
499 genes involved in blocking the cell cycle progression i.e., *transcription factor SOX-5* (*SOX-5*),

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500 *CDKN1B*, and *malignant fibrous histiocytoma-amplified sequence 1 homolog* (*MFHAS1*) were
501 among the top 10 hypoxia-induced upregulated genes discovered in this study. Sox-5 is well
502 known for its function in controlling cell cycle and sequential generation of distinct corticofugal
503 neuron subtypes (Hao *et al.* 2014). The upregulation of *SOX-5* occurs in response to hypoxia in
504 human stem cells (Khan *et al.* 2007). Moreover, the upregulation of *CDKN1B*, which blocks cell
505 cycle progression from G1 to S phase, is also a well-known response to hypoxia (Wang *et al.*
506 2003; Peng *et al.* 2015). MFHAS1 is known as an oncogene that may play an important role in
507 signal transduction and the control of cell growth in humans (Sakabe *et al.* 1999). Collectively,
508 this suggests that hypoxia may elevate expression of genes involved in inhibiting cell growth and
509 proliferation, thus potentially reducing growth rates. Furthermore, this is in line with the lower
510 growth performance of these juveniles kept in hypoxia compared to normoxia (see Houde *et al.*
511 2019a).

512 Top 10 hypoxia-induced upregulated genes included those involved in the oxidative
513 stress response (e.g., *glutathione peroxidase 3*; *GPx-3*, and *Acyl-coenzyme A thioesterase 9*,
514 *mitochondrial*; *ACOT*), ion transport (e.g., *RAMP1*), peptide amidation (e.g., *peptidylglycine*
515 *alpha-amidating monooxygenase*; *PAM*), and neuropeptide processing (e.g., *endothelin-*
516 *converting enzyme 2*; *ECE-2*). GPx-3 is a selenocysteine-containing protein with antioxidant
517 properties. Hypoxia is known as a strong transcriptional regulator of *GPx-3* expression.
518 Increased expression of oxidative stress genes under hypoxic conditions has been already
519 demonstrated in fish (Zhang *et al.* 2016). RAMP1 is a member of the RAMP family of single-
520 transmembrane-domain proteins, called receptor (calcitonin) activity modifying proteins
521 (RAMPs). Chronic hypoxia was reported to enhance mRNA levels for *RAMP1* in rats (Qing *et*
522 *al.* 2001; Cueille *et al.* 2005). It is stated that in hypoxia, upregulation of *RAMP1* primarily

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523 regulates the calcitonin gene-related peptide reception (Qing and Keith 2003). Moreover, PAM
524 catalyzes the amidation of peptides, a process important for peptide stability and biological
525 activity. PAM-dependent amidation has the potential to signal oxygen, so that PAM is strikingly
526 sensitive to hypoxia in cells (Sharma *et al.* 2009; Simpson *et al.* 2015). PAM has catalytic
527 activity on a range of substrates, in different cell types, and is as sensitive to hypoxia as the
528 transcription factor, hypoxia inducible factor (HIF) (Simpson *et al.* 2015). *ECE-2* has been
529 observed to be upregulated in response to hypoxia in juvenile salmon in this study. ECE-2
530 belongs to a family of membrane-bound metalloproteases involved in neuropeptide processing
531 (Mzhavia *et al.* 2003). The true physiological function of ECE-2 remains largely to be
532 determined (Ouimet *et al.* 2010), but it may be a novel HIF-target gene in hypoxia, where HIF-1
533 binds to transcription regulatory regions of the *ECE-1* gene during hypoxia (Khamaisi *et al.*
534 2015). However, no information is available regarding this mechanism for the *ECE-2* gene. The
535 upregulation of *GPX3*, *Sox-5*, *CDKN1B*, and *ECE-2* observed in this study was in line with the
536 higher expression of the transcription factor *HIF-1A* (see Houde *et al.* 2019a) in response to
537 hypoxia. Notably, and in line with observations from the current study in Chinook salmon, HIF-
538 1A is a key regulator of the cellular and systemic homeostatic response to hypoxia through the
539 activation of transcription of many genes, including those involved in cell cycle arrest (i.e.
540 *CDKN1B*), antioxidant activity (i.e. *GPX3*), and other genes whose protein products increase
541 oxygen delivery or facilitate metabolic adaptation to hypoxia (Lando *et al.* 2003; Wang *et al.*
542 2003; Zagórska and Dulak 2004). A recent transcriptome study in a catfish (*Pelteobagrus*
543 *vachelli*) identified 70 candidate genes in the HIF-1 signaling pathway in response to acute
544 hypoxia and reoxygenation (Zhang *et al.* 2017).

545 *Differing effects dependent on smolt stage and environmental salinity*

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

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

563 Pre-smolt Chinook salmon were responsive to hypoxia in SW but not in FW. Juvenile
564 salmonids that are physiologically unprepared for the transition from FW to SW (i.e., pre-smolts)
565 experience higher mortality in SW (Houde *et al.* 2019a,b). Pre-smolts in SW, as opposed to FW,
566 may display higher oxygen consumption as a stress response, and higher energy expenditure to
567 maintain ion- and osmo-regulation (Morgan and Iwana 1991,1998). Furthermore, oxygen
568 consumption typically is the lowest in the environment that is natural for a given life stage (Ern

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569 *et al.* 2014). It is likely that pre-smolt juvenile Chinook salmon in FW experienced lower
570 physiological disturbance than those in SW, such that they were more prepared to cope with
571 hypoxic conditions in FW than in SW.

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

583 The post-smolt stage was not examined in the present study but may reveal further
584 insights on the response to hypoxia by salinity and smolt stage. De-smolts may occur in the
585 hatchery setting, whereas smolts in the natural setting typically transition to SW and become
586 post-smolts (McCormick *et al.* 1998). Therefore, the hypothesis of lower oxygen consumption or
587 energetic cost in the natural salinity environment for a given life stage (Morgan and Iwana 1991,
588 1998; Ern *et al.* 2014), may be further examined using post-smolts, and this would be a valuable
589 direction for future work. It is predicted that the post-smolt pattern may be similar to pre-smolts
590 but in the opposite direction, i.e., post-smolts are more prepared to cope with hypoxic conditions
591 in SW than in FW. Indeed, aerobic scope, but not standard or maximum metabolic rate, is lowest

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592 for post-smolt Atlantic salmon in SW than FW and BW (Hvas *et al.* 2018). Further studies
593 examining post-smolts using the three salinities and the candidate hypoxia genes are warranted.

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

609 *Mortality*

610 The results of this study showed that the top candidate hypoxia genes were highly responsive to
611 hypoxia in moribund and recently dead fish in both FW and SW environments, suggesting the
612 active regulation of hypoxia genes in moribund and recently dead fish. These results confirm
613 previous findings of post-mortem activity of hypoxia responsive genes in human (Ferreira *et al.*

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614 2018), mouse and zebrafish (Pozhitkov *et al.* 2016). It has been previously suggested that the
615 genes that function in DNA synthesis, deactivation of immune system, cell necrosis, stress
616 response, and glycolysis of carbohydrates are also the major group of genes that show changes in
617 transcription following death. Hypoxia is believed to play a major role in the initial pre- to post-
618 mortem transition by activation of glycolysis (Ferreira *et al.* 2018). In our study, genes involved
619 in cell cycle and DNA synthesis, oxidative stress, and neuropeptide processing were among the
620 most significant contributed genes responsive to hypoxia in distressed fish in both FW and SW
621 environments. The cellular hypoxic response to morbidity is likely due to reduced blood
622 circulation and consequently a considerable reduction of available oxygen in cells. Our validated
623 hypoxia biomarkers may be applicable to elucidating the causes of salmon mortality and the
624 environmental habitats where salmon experience episodes of hypoxia. Although further
625 confirmatory studies are required, these results demonstrate that this high-throughput gene
626 expression approach may have a high potential for being a useful tool to discover hypoxic
627 mortality in salmonids, and the synergistic relationships between hypoxic stress and disease, if
628 applied on salmon Fit-Chips.

629 *Extreme hypoxia*

630 The most robust hypoxia response was observed in individuals exposed accidently to more
631 severe hypoxic conditions in SW/18ºC than the fish exposed to the more moderate hypoxia
632 conditions designed in the original study as shown by the PCA analysis; this may suggest that the
633 moderate hypoxia conditions were not strong enough to induce a consistent hypoxia response
634 across all individuals. These results were obtained on a limited number of fish due to an
635 instrument issue during laboratory handling. Conceivably, a stronger hypoxia across all
636 treatments may have shown a more robust hypoxia response in salmon, but stronger hypoxia was

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637 not chosen because of ethical considerations. In addition, the RNA-seq analysis across the
638 transcriptome revealed small fold-changes and no significant genes using false discovery rate (q-
639 values), supporting a weak hypoxia response using the experimental conditions, although this is
640 probably also impacted by sample size in the discovery component of this study. Further
641 experiments using stronger hypoxic conditions across different salinities, temperatures, and
642 smolt stages are needed to confirm this hypothesis.

643 **Conclusions**

644 Gill gene expression biomarkers have been previously identified for salinity and temperature
645 stressors across multi-stressor conditions, but hypoxia biomarkers sufficient to identify fish
646 exposed to moderate hypoxia (Houde et al. 2019a). Here, candidate hypoxia genes were
647 discovered through RNA-seq analysis, and then validated using microfluidics qPCR to reveal
648 sets of biomarkers that were responsive to hypoxic stress in juvenile Chinook salmon. Moreover,
649 the expression of these candidate hypoxia genes, and presumably of the hypoxia response, in
650 juvenile Chinook salmon was affected by smolt stage, induced mortality, and water salinity. Our
651 study is the first of its kind dealing with this range of factors from the gene expression
652 perspective. Following the biomarker discovery of 30 candidates by RNA-seq, different
653 validated biomarker sets were identified that were optimal for each of the specific combinations
654 of factors within the dataset (i.e., smolt stage, mortality, salinity). The genes involved in cell
655 cycle and DNA synthesis were among the most universal biomarkers, whereas ion transportation
656 genes were more specific signals within saline water. Since the biomarkers were discovered on
657 specific water salinity levels, smolt stages and water temperatures, no consistent gene expression
658 model for all conditions was observed at the level of hypoxia exposure conducted in our study.
659 Interestingly, this study also provides some evidence for post-mortem regulation of hypoxia

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660 genes in fish. The main challenge facing the development of a hypoxia gene expression
661 biomarker panel for salmonids appears to be the relatively minor response compared to other
662 stressors such as temperature, which made the removal of temperature response genes in this
663 study an important part of the design. The present study provides a set of biomarkers that, when
664 combined with factors smolt stage, salinity, and mortality, can be used to determine whether an
665 individual has recently experienced hypoxic stress. Moreover, our study confirms that fish might
666 be highly resilient to mild hypoxia when they are in their natural salinity environment, less so
667 when outside of the salinity environment expected for that development stage, and can be
668 stressed by extreme hypoxia conditions. The biomarkers identified here will be added to the
669 continued development of stressor-specific biomarkers being collected to understand the abiotic
670 and biotic stressors that salmonids face in their natural environment.

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936 **Table 1.** Top 20 downregulated and 10 upregulated candidate genes from the RNA-seq analysis, and efficiencies for candidate
937 hypoxia stress genes. Genes are presented in order of the significance of the correlation with PC1, which separated normoxia and
938 hypoxia (data are in Table S1). Species abbreviations for efficieny: CK= Chinook salmon (*Oncorhynchus tshawytscha*), CO= Coho
939 salmon (*O. kisutch*), CM= Chum salmon (*O. keta*), PK= Pink salmon *(O. gorbuscha*), SX= Sockeye salmon (*O. nerka*), AS= Atlantic
940 salmon (*Salmo salar*), AC= Artic charr (*Salvelinus alpinus*), RT= Rainbow trout (*O. mykiss*), and BT= Bull trout (*Salvelinus*
941 *confluentus*).
942

Gene name	Function of gene	Assay name	PCR Efficiency								
			CK	CO	CM	PK	SX	AS	AC	RT	BT
Down-regulated genes											
Anillin-like	Cell division	Anillin	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	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	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	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	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	1.09	1.19	1.11	1.08	1.01	1.17	0.99	1.00	1.33
Histone H2A.V	Cell division	H2AFV	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	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	1.22	1.28	1.20	1.03	1.10	1.33	1.05	0.98	1.42

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Rab9 effector protein with kelch motifs-like	Cell division	Rab9	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	1.13	1.22	1.18	1.10	1.06	1.16	1.02	1.01	1.29
Tonsoku-like protein	DNA synthesis	Tonsl	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	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	1.11	1.20	1.12	1.11	1.26	1.17	1.04	0.99	1.26
Claspin-like	DNA synthesis	Claspin	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	1.13	1.25	1.21	1.03	0.99	1.19	1.01	0.99	1.30
Lamin B1	Cell division	Lmnb1	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	1.21	1.17	-	1.31	-	1.70	1.20	0.99	1.36
Chromosome-associated kinesin KIF4-like	Cell division	Kif4	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	-	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	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	0.99	1.19	1.14	1.06	1.04	1.08	1.02	-	1.17
Peptidylglycine alpha-amidating monooxygenase_106571984	Peptide amidation	PAM	1.16	1.19	1.12	1.01	1.04	1.15	1.01	1.03	1.22

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protein family with sequence similarity 214, A	Cell division	FAM214A	1.21	1.34	1.19	0.98	1.05	1.18	1.01	0.98	1.12
Calphotin	Cellular calcium regulation	Calphotin	1.10	1.21	1.18	0.99	1.03	1.12	1.01	1.00	1.48
Gutathione peroxidase 3	Oxidative stress	GPX3	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	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	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	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	1.13	1.25	1.45	1.15	1.07	1.27	1.01	1.01	1.37

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Table 2. 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. Fish size (mean ± standard deviation) symbols are L for length and W for weight.

Treatment	Sample size		Fish size				LDA Classification ability (%)			Significant genes identified by Shrunken analysis	Function of genes
	Training	Testing	L (cm)		W (g)		N	H	Average		
			N	H	N	H					
RNA-seq samples	16	8	8.4±1.2	8.7±1.4	6.5±3.0	7.1±3.4	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	5.2±0.4	5.2±0.3	1.5±0.3	1.5±0.2	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	5.2±0.3	5.2±0.3	1.5±0.2	1.4±0.2	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	5.2±0.3	5.1±0.3	1.7±0.3	1.4±0.2	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	7.6±0.4	7.2±0.3	4.6±0.8	4.1±0.6	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	7.5±0.5	7.5±0.4	4.6±1.0	4.6±0.8	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	7.6±0.2	7.5±0.5	4.7±0.6	4.6±1.0	66.7	100.0	83.3	CDKN1B	Cell cycle arrest
										RAMP1	Ion transport
De-smolt											
FW	12	6	9.5±0.9	9.0±1.3	9.0±2.7	7.7±2.8	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	9.5±1.1	8.9±1.0	7.8±2.7	7.5±2.6	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	7.6±1.2	8.5±1.0	6.8±2.8	6.6±2.4	100.0	75.0	87.5	Calphotin	Cellular calcium regulation
										GPX3, ACOT9	Oxidative stress
										RAMP1	Ion transport
Distressed											
										KIF2C	Cell division and DNA synthesis
FW	17	9	8.3±0.9	8.0±1.0	6.1±2.1	5.5±2.2	80.0	100.0	90.0	MFHAS1	Cell cycle arrest
										GPX3	Oxidative stress
										ECE.2	Neuropeptide processing
SW	34	17	7.3±1.8	6.7±1.7	4.4±2.9	3.4±2.3	77.8	100.0	88.9	FAM214A, Kif4, ARHGAP11A, H2AFV, Claspin, Kif15	Cell division and DNA synthesis
										SOX.5, MFHAS1, CDKN1B	Cell cycle arrest

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	GPX3, ACOT9	Oxidative stress
	ECE.2	Neuropeptide processing
	RAMP1	Ion transport
	PAM	Peptide amidation

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Figure legends

Fig. 1. Technical road map of the experimental methods used for discovery (RNA-seq) and validation (q-PCR) of hypoxia biomarkers. Juvenile Chinook salmon were exposed to 18 possible water treatments in 30 L pot tanks (circles): 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 in two replicates. Four trials were conducted over the smoltification period, covering pre-smolt (March), smolt (May), and de-smolt 1 (June) and de-smolt 2 (August) stages. A subset of 24 FW individuals kept at two temperatures (10°C and 18°C) and two DO conditions (normoxia and hypoxia) was selected for RNA-seq from the two de-smolt trials (trials 3 and 4). A group of 212 live-sampled (no signs of morbidity), and 88 distressed juveniles (moribund and recently dead) from the four trials exposed to the full treatment were used for qRT-PCR. Twenty two individuals from two SW/18°C-hypoxia tanks in the trial 3 had lower DO than intended in the SW treatment (3.3–4.1 mg L^{-1} instead of 4–5 mg L^{-1}), and therefore were used as an 'extreme hypoxia' condition.

Fig. 2. Multi-dimensional scaling (MDS) plot to evaluate the effects of different factors (i.e. temperature, hypoxia, trial) on overall gene expression (3= trial 3, de-smolt; 4= trial 4, de-smolt).

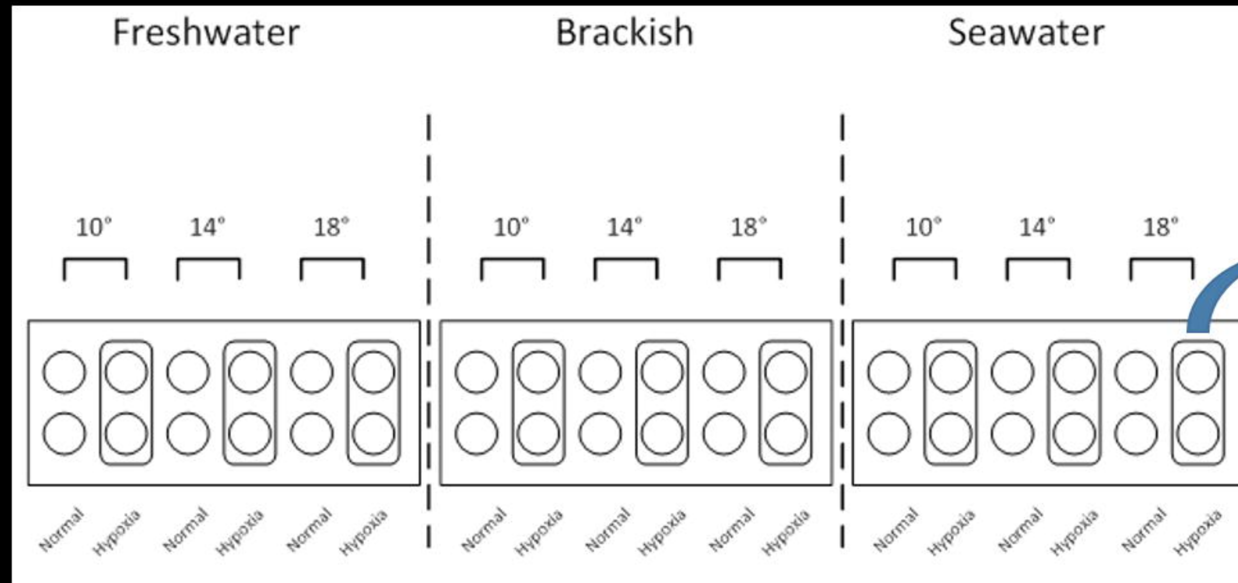
Fig. 3. Canonical plot of the first two principal components of 240 candidate gill hypoxia genes.

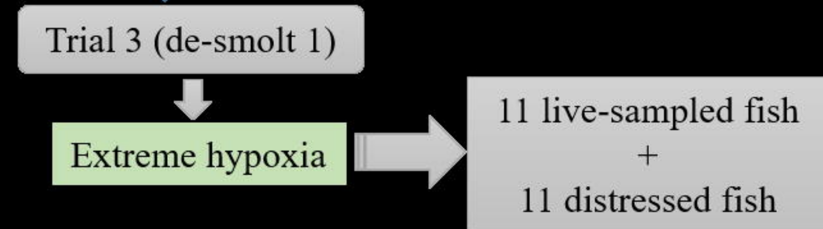
Fig. 4. Validating RNA-seq platform with qRT-PCR data using the same 24 samples used in RNA-seq. Fold changes of gene expression detected by RNA-seq were plotted against the data of qRT-PCR. The reference line indicates the linear relationship between the results of RNA-seq and qRT-PCR.

Fig. 5. Canonical plots of the first two principal components of the identified hypoxia biomarkers using the 7 hypoxia biomarkers returned by the Shrunken Centroid method on significant correlated genes between RNA-seq and qRT-PCR platforms for 24 samples used in RNA-seq analysis. Temperature treatment symbols are 10 and 18 °C groups. Principal component analysis was performed on the training set (left panel) and then applied to the testing set (right panel). Centroids are represented by the largest point of the same colour. Arrows represent loading vectors of the biomarkers using the training set.

Fig. 6. Canonical plots of the first two principal components using 8 hypoxia biomarkers returned by the Shrunken Centroid method for the extreme hypoxia group of SW/18°C in trial 3. 11 live individuals exposed to extreme hypoxia for approximately two days (SW18_exH_L), 11 dead or moribund individuals exposed to extreme hypoxia (SW18_exH_D), 11 live fish kept in normal oxygen for six days (SW18N), and 11 live individuals exposed to designed hypoxia for six days (SW18H). Principal component analysis was performed on the training set (left panel) and then applied to the testing set (right panel). Centroids are represented by the largest point of the same colour. Arrows represent loading vectors of the biomarkers using the training set.

Experimental design and tank layout





Discovery

RNA-seq

24 fish

Normoxia
Hypoxia

10°
18°

Freshwater

Trial 1 (pre-smolt)

Trial 2 (smolt)

Trial 3 (de-smolt 1)

Trial 4 (de-smolt 2)

Validation

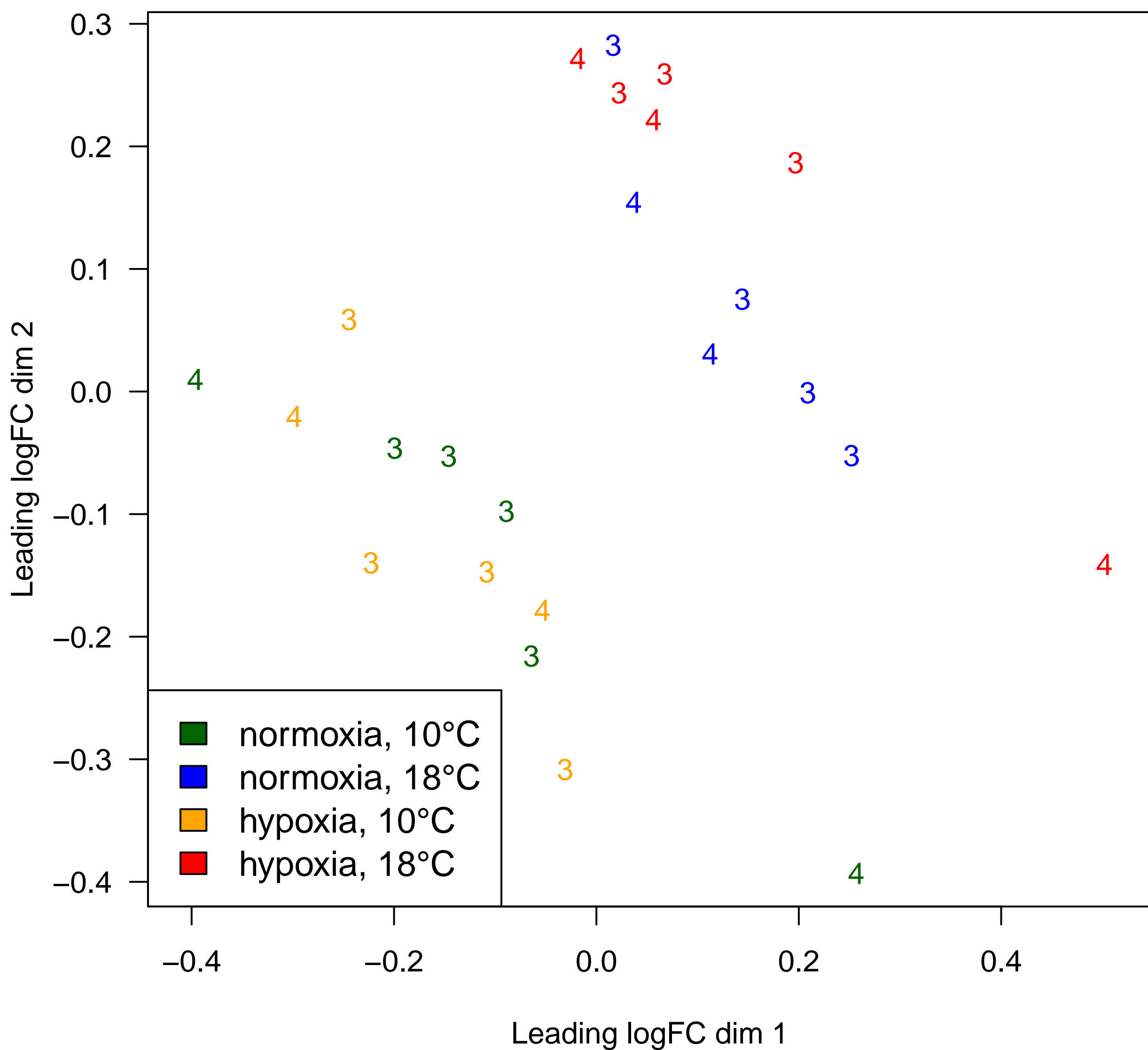
qRT-PCR

Freshwater
Brackish
Seawater

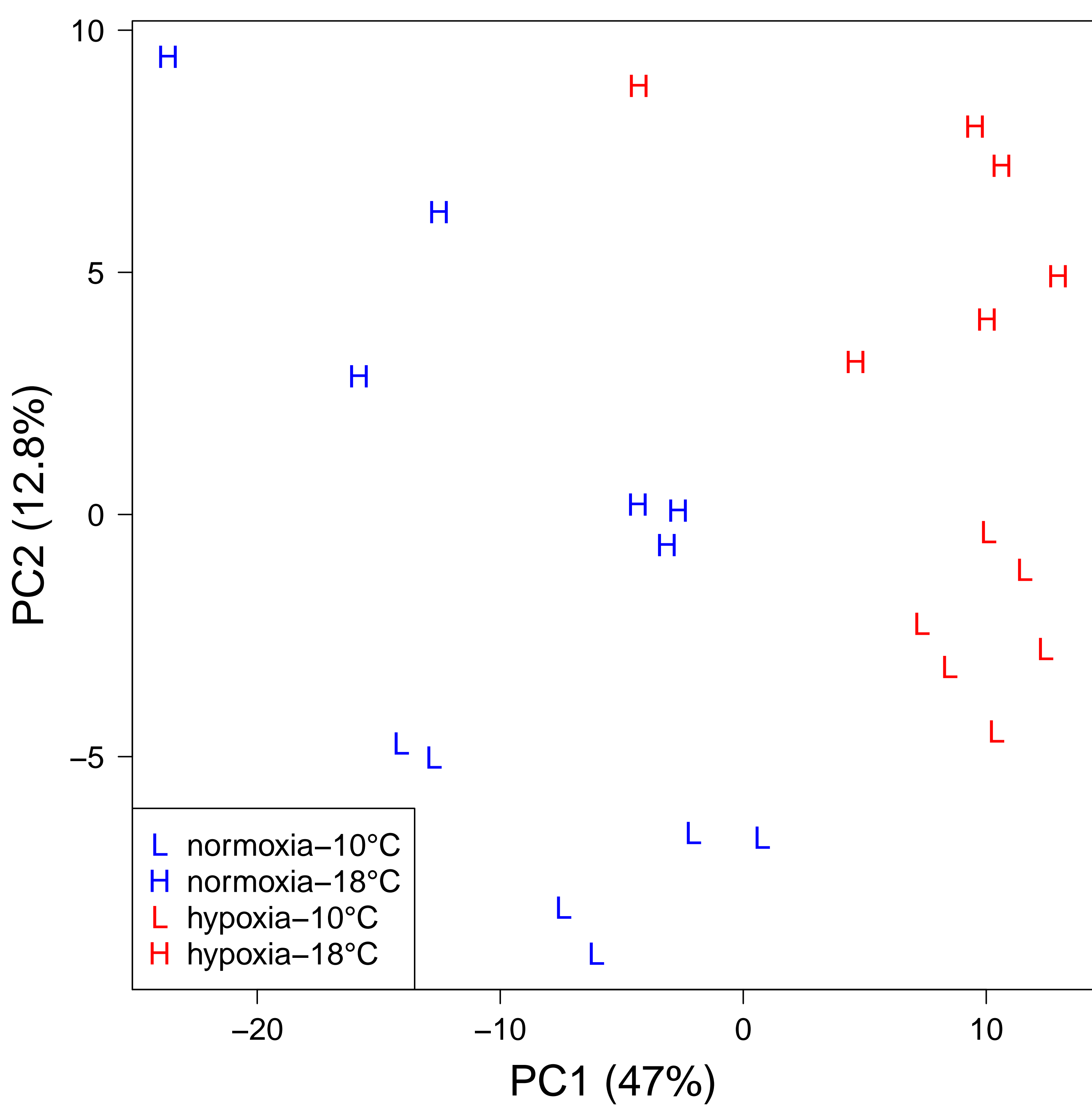
10°
14°
18°

Normoxia
Hypoxia

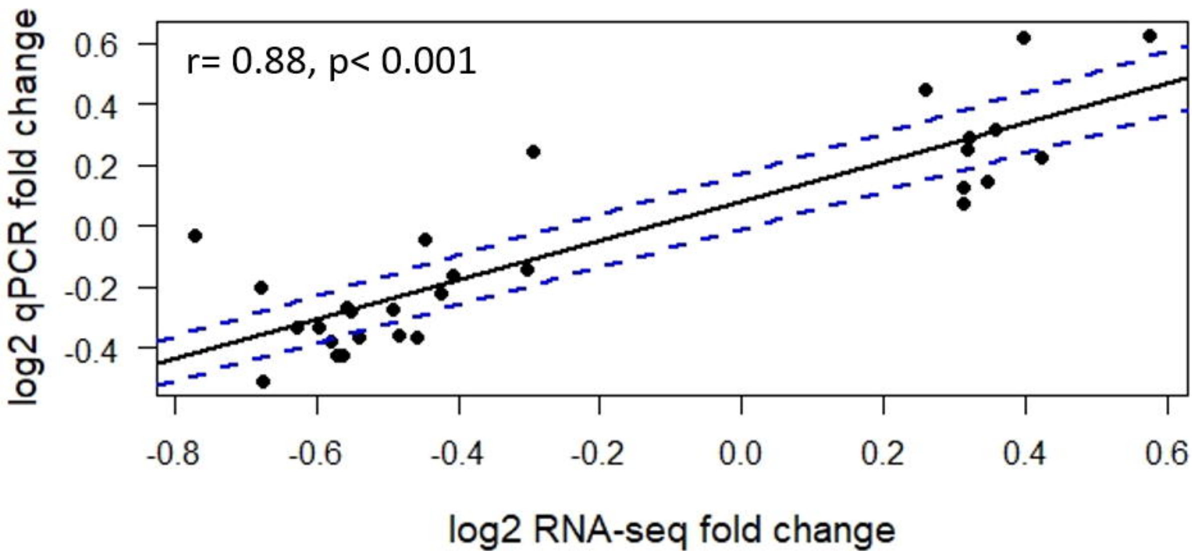
212 live-sampled fish
+
88 distressed fish



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



PC2 (12.8%)
PC1 (47%)
10
5
0
−5
−20
−10
0
10
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%)
2.5
0.0
−2.5

PC1 (72.2%)
−4
0
4
8

(b) testing

3 de−smolt1
4 de−smolt2

normoxia
hypoxia

AURKB
Tonsl
KIF2C
ERCC6L
claspin
ARHGAP11A
RAMP1

PC2 (16.5%)
2.5
0.0
−2.5

PC1 (72.2%)
−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

PC1 (62.8%)

PC2 (18.5%)

(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

PC1 (62.8%)

PC2 (18.5%)