Paternal hypoxia exposure primes offspring for increased hypoxia resistance

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ABSTRACT

In a time of rapid environmental change, understanding how the challenges experienced by one generation can influence the fitness of future generations is critically needed. Using tolerance assays, transcriptomic and methylome approaches, we use zebrafish as a model to investigate transgenerational acclimation to hypoxia. We show that short-term paternal exposure to hypoxia endows offspring with greater tolerance to acute hypoxia. We detected two hemoglobin genes that are significantly upregulated by more than 7-fold in the offspring of hypoxia exposed males. Moreover, the offspring which maintained equilibrium the longest showed greatest upregulation in hemoglobin expression. We did not detect differential methylation at any of the differentially expressed genes, suggesting that another epigenetic mechanism is responsible for alterations in gene expression. Overall, our findings suggest that a 'memory' of past hypoxia exposure is maintained and that this environmentally induced information is transferred to subsequent generations, pre-acclimating progeny to cope with hypoxic conditions.

INTRODUCTION

Paradigm-shifting research has revealed that the life-history experiences of parents can influence the phenotype of their offspring through non-genetic mechanisms (Salinas et al., 2013; Bohacek and Mansuy, 2015; O'Dea et al., 2016; Dias and Ressler, 2013; Gapp et al., 2014; Radford et al., 2014; Burton and Metcalfe, 2014; Bonduriansky, 2012; Bonduriansky and Day, 2008). Non-genetically transmitted phenotypes can be generated by diverse environmental effects, affecting a wide array of offspring traits, both positively and negatively (O'Dea et al., 2016). Work on mice has demonstrated that learned fear responses (Dias and Ressler, 2013; Gapp et al., 2014) and metabolic alterations associated with undernourishment can be inherited via sperm (Radford et al., 2014). These studies suggest that parents can transmit information that may benefit offspring survival. Through this transgenerational plasticity (also known as transgenerational acclimation (Herman and Sultan, 2011; Marshall, 2008)), parents may provide offspring with increased tolerance to environmental perturbations, such as contaminants (Araujo et al., 2019; Kishimoto et al., 2017; Marshall, 2008), food shortages (Kishimoto et al., 2017; Weyrich et al., 2018), carbon dioxide (Allan et al., 2014; Shi et al., 2020; Lee et al., 2020), hypoxia (Ho and Burggren, 2012), but see (Truebano et al., 2018), salinity (Heckwolf et al., 2020, 2018), and temperature (Donelson et al., 2014, 2012; Veilleux et al., 2015; Salinas and Munch, 2012; Weyrich et al., 2018; Ryu et al., 2018). Studies have tended to focus on maternal transgenerational plasticity, or have exposed both parents to the environmental perturbation, making it impossible to disentangle the relative roles of mothers and fathers in altering offspring phenotype (Guillaume et al., 2016; Rutkowska et al., 2020). Thus, a better understanding of the specific role of paternal effects in transgenerational plasticity is needed, especially considering environmental specific information is likely transferred via sperm (see below). Further, the

underlying molecular processes have been identified in a just a few studies (Heckwolf et al., 2020; Kishimoto et al., 2017; Ryu et al., 2018; Shi et al., 2020; Strader et al., 2019; Veilleux et al., 2015). For example, metabolic genes are upregulated transgenerationally in the damselfish (*Acanthochromis polyacanthus*), suggesting shifts in energy production for maintaining performance at elevated temperatures (Veilleux et al., 2015). Potential epigenetic mechanisms for this transgenerational acclimation have been detected, via differential methylation of genes involved in energy homeostasis, mitochondrial activity, and oxygen consumption (Ryu et al., 2018). In a time of rapid environmental change, a better understanding of how environmental challenges experienced by organisms could increase the fitness of future generations to survive these same stressors is critically needed.

Hypoxia, defined as sufficiently low levels of oxygen to deprive tissues of oxygen, is a major physiological challenge (Diaz and Rosenberg, 2008; Long et al., 2015; Wang et al., 2016). The aerobic lifestyle of most animals requires a constant supply of sufficient oxygen, and low oxygen levels constitute a major environmental threat (Roesner et al., 2006). Hypoxic conditions precipitate conserved physiological effects in a wide array of vertebrates (Okumura et al., 2003; Saxena, 1995; Wu et al., 2003), but hypoxia is particularly well-studied in aquatic species. The total oxygen present in water at 30°C is only 0.5% that of air; hence, physiological stress of hypoxia challenges many freshwater and marine organisms, and hypoxia is one of the most widespread issues in aquatic habitats due to the rise of dead zones and climate change (Diaz and Rosenberg, 2008; Jenny et al., 2016). Thus, it is now more important than ever to understand how different aquatic species will response to environmental hypoxia.

The negative impacts of hypoxia on growth and reproduction are well-recognized (Townhill et al., 2017; Wu, 2002; Wu et al., 2003), with recent studies also discovering

transgenerational effects, whereby marine medaka fish (*Oryzias melastigma*) exposed to hypoxia show reproductive impairments in F1 and F2 generations, suggesting that hypoxia might pose a long-lasting threat to fish populations (Lai et al., 2019; Wang et al., 2016). But fishes, in particular, are also notable for their adaptive abilities to acclimate to hypoxic conditions (Alexander et al., 2017; Diaz and Rosenberg, 2008; Nikinmaa, 2002; Richards, 2011), including physiological, morphological, and phenotypic responses. Remarkably, goldfish (*Carassius auratus*) and crucian carp (*Carassius carassius*) exhibit gill remodeling in response to hypoxia exposure (Dhillon et al., 2013; Tzaneva et al., 2011). Zebrafish embryos and larvae modify cardiac activity and blood vessel formation in response to hypoxic exposures (Pelster, 2002). In addition to morphological alternations, gene expression can also be affected by hypoxic conditions. In zebrafish gill tissue, more than 300 genes are differentially expressed between hypoxia exposed individuals versus controls; these changes in gene expression are coupled with morphological changes in gill structure, such as increased surface area of gill tissue (van der Meer et al., 2005). Prolonged exposure to low oxygen has been shown to improve hypoxia tolerance in Murray cod (*Maccullochella peelii*; (Gilmore et al., 2020) and snapper (*Pagrus auratus*;(Cook et al., 2013)). Likewise, pre-acclimation of zebrafish larvae to mild hypoxia significantly improves their resistance to lethal hypoxia, and upregulation of some oxygen transport genes are associated with this acclimation (Long et al., 2015). Intriguingly, zebrafish offspring of males and females exposed to chronic hypoxia have a higher resistance to acute hypoxia than those of controls (Ho and Burggren, 2012), despite the offspring having never been exposed to hypoxia, suggesting that parents may pass on information that may pre-acclimate offspring to cope with hypoxic conditions.

Gene expression and transgenerational effects can be regulated by epigenetic modifications, including DNA methylation, histone modifications, and non-coding microRNAs (O'Dea et al., 2016). For example, in the marine medaka studies, transgenerational effects are associated with differential methylation in sperm and ovary, with altered gene expression in genes known to be associated with spermatogenesis and gene silencing (Wang et al., 2016) and cell cycle control and cell apoptosis (Lai et al., 2019). DNA methylation has also been investigated as a means underlying transgenerational effects of chemical exposures in zebrafish (Carvan et al., 2017) and the paternal methylome is believed to be stably transmitted to offspring in zebrafish (Jiang et al., 2013; Potok et al., 2013) without global reprogramming in primoridal germ cells (Ortega-Recalde et al., 2019; Skvortsova et al., 2019), potentially facilitating environmental specific information transfer.

Here we use zebrafish to further explore the phenomenon of transgenerational acclimation of hypoxia in fishes. We focus on paternal exposure as we predict that environmental specific information can be transferred via sperm, as observed in other studies (Dias and Ressler, 2013; Lamb et al., 2020; Wang et al., 2016). We test whether paternal exposure to hypoxia stimulates phenotypic responses in offspring, using behavioural phenotyping to identify resistance to acute hypoxia (time to loss of equilibrium). We then use RNA-Seq to identify candidate genes that are differentially expressed in control and hypoxic progeny, and whole genome bisulfite sequencing data to assess whether changes in DNA methylation underpin alterations in phenotype and gene expression (Figure 1).

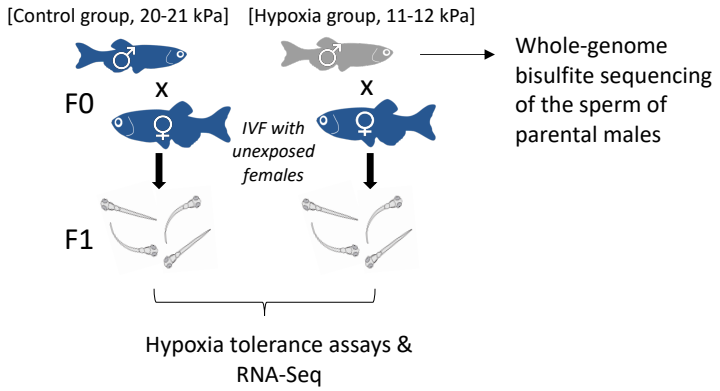

Figure 1. Experimental design. Groups of adult male zebrafish are maintained in normoxia (20-21 kPa, n = 20) or hypoxia (11-12 kPa, n = 20) for 14 days. Five males from each treatment were then used to create F1 progeny, crossing the males to unexposed females (n = 5 families/treatment), with half of the sperm used for whole genome bisulfite sequencing, to assess differential methylation (n = 3 per treatment). At 20-21 days post fertilization, offspring undergo acute hypoxia tolerance (6-8 offspring/family) assays and n = 3 offspring/treatment are used for differential gene expression analysis (RNA-Seq).

MATERIALS AND METHODS

Fish husbandry

Breeding and husbandry took place within the Otago Zebrafish Facility (OZF), a temperature-controlled facility maintained at 25-27°C, pH 7-7.8 and conductivity 300-500 μS. Fish were maintained in a Tecniplast re-circulating system (Tecniplast, Varese, Italy) under a 14:10 light:dark photoperiodic cycle, with 30 minutes of simulated dawn and dusk at the start and end of each day. Acute hypoxia assays took place in the Zoology Department, where room temperature was controlled at 25°C, with a 13.5 hr (0700-2030 hr) light cycle with 30 min of simulated dawn and dusk. Zebrafish were fed twice daily with dry food (ZM000-400, size-dependent) and once daily with live rotifer (*Brachionus* spp. days 5-10 post fertilisation) or artemia (*Artemia salina;* days 10+ post fertilization*)*. All animals were collected and maintained

145 according to the standards of the Animal Ethics Committee for the University of Otago, New
146 Zealand (protocol no. AEC 44/16).

147
148 **Hypoxia exposure**
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150 In November 2016, nine-month-old male zebrafish (AB wild-type; n=20/treatment) were
151 exposed to hypoxic conditions (10.91-12.33 kPa pO_2) or control conditions (20-21 kPa), for two
152 weeks. Two glass tanks (36x29x26.5 cm) were separated into three zones (12 x 29 cm), with 10
153 fish in each outer compartment and two fine bubble diffusers and a filter positioned in the middle
154 compartment. The oxygen concentration of the treatment tank was maintained by using an
155 OxyGuard Mini probe (OxyGuard International, Denmark) and oxygen controller (Model
156 PR5714, PR Electronics, Denmark) that were connected to nitrogen and air cylinders (BOC Gas
157 Supplies, Food Fresh grade). An on/off relay output from the controller actuated a solenoid-
158 controlled flow of compressed nitrogen or compressed air (BOC Gas Supplies, New Zealand), to
159 maintain the system at 53.1-60 % air saturation (= 10.91-12.33 kPa or 4.38-4.95 mg/L) level
160 (Cook et al., 2013). Normoxic conditions in the control tank (>95% saturation) were maintained
161 by continually passing air through the bubble diffusers, connected to an air pump. The oxygen
162 concentration in the tanks was confirmed with a YSI 85 probe (YSI, Inc., Ohio, USA). Both
163 tanks were siphoned for waste and received 10% water changes every three days. Calibration of
164 the oxygen probe was checked daily and recalibrated as necessary.

165
166 **Hypoxia tolerance assays and analysis**
167
168 Progeny from five families per treatment (n=5 hypoxia, n=5 control) were generated by *in vitro*
169 fertilisation (IVF) (Johnson Sheri L. et al., 2018; Lamb et al., 2020) seven days after parental
170 exposure finished. Briefly, eggs and sperm were collected using abdominal massage – half the

sperm was used for IVF and the other half was stored for subsequent DNA extraction. Offspring were reared to 20-21 dpf when n=8 offspring per family (except family C5, where n = 6; total n = 77) were challenged by acute hypoxia assays in small acrylic chambers (100 mm H x 50 mm x 50 mm). A 0-1 kPa oxygen level was achieved by continually passing compressed nitrogen through a bubble diffuser for at least 10 minutes before assays and continuously passing the nitrogen through a valve in the top of the chambers during the assay. The oxygen concentration fluctuated between 0 and 1%, monitored using a fibre-optic oxygen probe (Foxy OR-125) attached to an Ocean Optics® USB 2000 spectrophotometer with USB-LS-450 light source and the manufacturer's software (OOI Sensors). Each fish was filmed for 240 seconds and recorded with a GoPro Hero 3+ camera. A total of 77 videos (n=37 control, n=40 hypoxia) were tracked using EthoVision XT behavioral tracking software, version 11.5 (Noldus Information Technology, Netherlands). Fish were then immediately euthanized by submersion in ice and stored in RNAlater (Invitrogen). One offspring from family C1 stayed at the bottom the entire assay, so this individual was removed from further analyses.

Resistance to acute hypoxia, defined as the first time that progeny lost equilibrium for 3 seconds or more during hypoxia assays (Ho and Burggren, 2012), and the loss of equilibrium frequency were analysed using R v. 3.5.1 (R Core Team, 2018). Loss of equilibrium > 3 s was initially modelled using a gaussian generalized linear model, and loss of equilibrium frequency as a poisson generalized linear model, with treatment (control vs. hypoxia) as a fixed effect. To account for multiple fry per family being tested, we also ran the models incorporating Treatment ID as a random effect, using a gaussian linear model for time to loss of equilibrium > 3 s and a poisson linear mixed effects model for the loss of equilibrium frequency. Significant heterogeneity was obvious in the time to loss of equilibrium, so a linear mixed model

194 incorporating heteroscedasity was compared to the model assuming homogeneity, by
195 incorporating the variance in Treatment into the model (Cleasby and Nakagawa, 2011).

196
197 **RNA-Seq library preparation and analysis**
198
199 Total RNA from six whole 20-21 dpf offspring (3 control offspring, 3 treatment offspring) was
200 extracted using a Zymo Duet extraction kit (Zymo, NZ). The integrity of RNA samples was
201 determined using an Agilent RNA 6000 Nano chip on an Agilent 2100 Bioanalyzer to check that
202 the samples had an RNA Integrity Number (RIN) value of 8–9. Total RNA concentration was
203 measured by Qubit 2.0 Fluorometer (Qubit RNA HS Assay Kit, Life Technologies). Samples
204 were sent to the Otago Genomics and Bioinformatics Facility at the University of Otago, under
205 contract to New Zealand Genomics Limited, for library construction and RNA sequencing.
206 Messenger RNA sequencing libraries were prepared using the Illumina TruSeq Stranded mRNA
207 sample preparation kit (Illumina), as per the manufacturer's instructions. RNA sequencing was
208 performed on the Illumina HiSeq2500 (Illumina, USA) machine with single-ended 100-bp reads
209 generating 17.4-20.0 million reads per sample.
210 Low quality reads and remaining adapters were trimmed using Trim Galore v0.6.4
211 [https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/] in a two-step process. First,
212 sequencing adaptors were removed, 10 bp were hard-trimmed from the 5' end to account for
213 sequence bias produced by PBAT library preparation, and last, low-quality ends from reads
214 (PHRED score <20) were removed (Bolger et al., 2014). The reads were then aligned to the
215 zebrafish genome (GCRz11) using HISAT2 v2.2.0 (Kim et al., 2015), informed with the
216 GCRz11.99 annotation. Expression was summarized sample by sample at the gene level using
217 featureCounts v2.0.0 (Liao et al., 2014). The analysis of differential expression was conducted in
218 R v.3.5.0 (R Core Team, 2018) using the DESeq2 package v1.24.0 (Love et al., 2014) without

the independent filtering option implemented in the *results* function (see https://github.com/OscarOrt/Paternal_hypoxia_Ragsdale_2020). Differentially expressed genes between offspring of fathers exposed to hypoxia and controls were extracted after correcting for multiple testing using False Discovery Rate cut-off of q=0.05 (Benjamini and Hochberg, 1995a). In order to test for over/under-representation of biological pathways which differentially expressed genes are involved, enrichment of Gene Ontology (GO) analysis terms was performed using Gorilla online accessed on August 4 2020 (Eden et al., 2009).

Whole genome bisulfite sequencing and analysis

DNA from six parental sperm samples was purified using a modified magnetic bead method (Peat et al., 2017). WGBS was undertaken using an adapted modified post-bisulfite adaptor tagging (PBAT) method (Miura et al., 2012; Peat et al., 2014). Briefly, bisulfite treatment was performed according to the EZ Methylation Direct Mag Prep kit (Zymo, D5044) instruction manual. Bisulfite treatment was performed before adaptor tagging, enabling simultaneous conversion of unmethylated cytosines, DNA fragmentation, and improving library preparation efficiency. Sequencing primers were added using random heptamer primers, and finally, sample-specific indexes and sequences required for Illumina flow-cells binding were added by PCR. Library integrity was assessed by agarose gel electrophoresis and a fragment analyser (Agilent) and sequenced on eight lanes (one flow cell) of an Illumina HiSeq using HiSeq2500 V4 sequencing of 100 bp single ended reads (in combination with 13 other samples, which were part of another study).

Raw reads were trimmed in Trim Galore v0.6.4 as previously mentioned. Read mapping was performed using Bismark v0.22.3 (Krueger and Andrews, 2011) with the option --pbat

specified. Zebrafish genome version 11 (GRCz11) was used as reference. BAM files were deduplicated and reports containing methylation base calls were generated using the deduplicate_bismark and bismark_methylation_extractor scripts, respectively. The non-conversion rate during the bisulfite treatment was evaluated by calculating the proportion of non-CG methylation; by this measure, all libraries must have had a bisulfite conversion efficiency of at least 98.5% (Table S3).

CG methylation calls were analyzed in SeqMonk v1.47.0 [www.bioinformatics.babraham.ac.uk/projects/seqmonk]. To analyse methylation at gene level, probes with a minimum of 5 methylation calls were generated and the percentage methylation measured as number of methylated calls/total calls. CpG islands (CGIs) were identified using Gardiner-Garden & Frommer's criteria and previously published datasets. For the former, 200-bp windows moving at 1-bp intervals were considered CGIs if the Obs/Exp value was greater than 0.6 and a GC content greater than 50%. For the latter, data obtained by biotinylated CxxC affinity purification (Bio-CAP) and massive parallel sequencing was used to identify non-methylated CpG islands (Long et al., 2013).

Coupling between methylation and gene expression in differentially expressed genes (DEG) and at genome level was interrogated using methylation levels at transcription start sites (TSS). TSS were defined as 200 bp centered on the first nucleotide of an annotated mRNA, and a threshold of at least 20 methylation calls to be included in the analysis. DEG were divided into underexpressed and overexpressed, whereas coupling at genome level was assessed dividing gene expression levels into quartiles. Custom annotation tracks were generated using Gviz v1.28.3 (Hahne and Ivanek, 2016).

RESULTS

Hypoxia tolerance assays in unexposed offspring

Progeny of males exposed to moderate hypoxia for 14 days show a greater resistance to acute hypoxia than progeny of control males – time to loss of equilibrium was, on average, 32 seconds longer for progeny of hypoxia exposed males ($t = 2.52$, $p = 0.014$; confidence interval (CI) = 7.38, 58.83; Figure 2A) and the progeny of hypoxia exposed males lost equilibrium, on average, 9.18 times vs. 12.32 times in the control progeny ($t = -4.21$, $p < 0.0001$, CI = -0.433, -0.158; Figure 2B). However, when accounting for family (to avoid pseudo-replication due to multiple offspring per family being tested), the effect of time to loss of equilibrium becomes non-significant ($t = 1.67$, $p = 0.133$, CI = -12.66, 79.22; note this model accounts for observed heterogeneity in the data), though the effect remains for the number of times offspring lost equilibrium ($z = -2.46$, $p = 0.014$, CI = -0.567, -0.037). Hence, there are strong family effects (time to loss of equilibrium Treatment ID variance = 42.38; loss of equilibrium frequency Treatment ID variance = 0.025), with greater resistance to acute hypoxia being observed for just two families, H1 and H2 (Figure 2C and D).

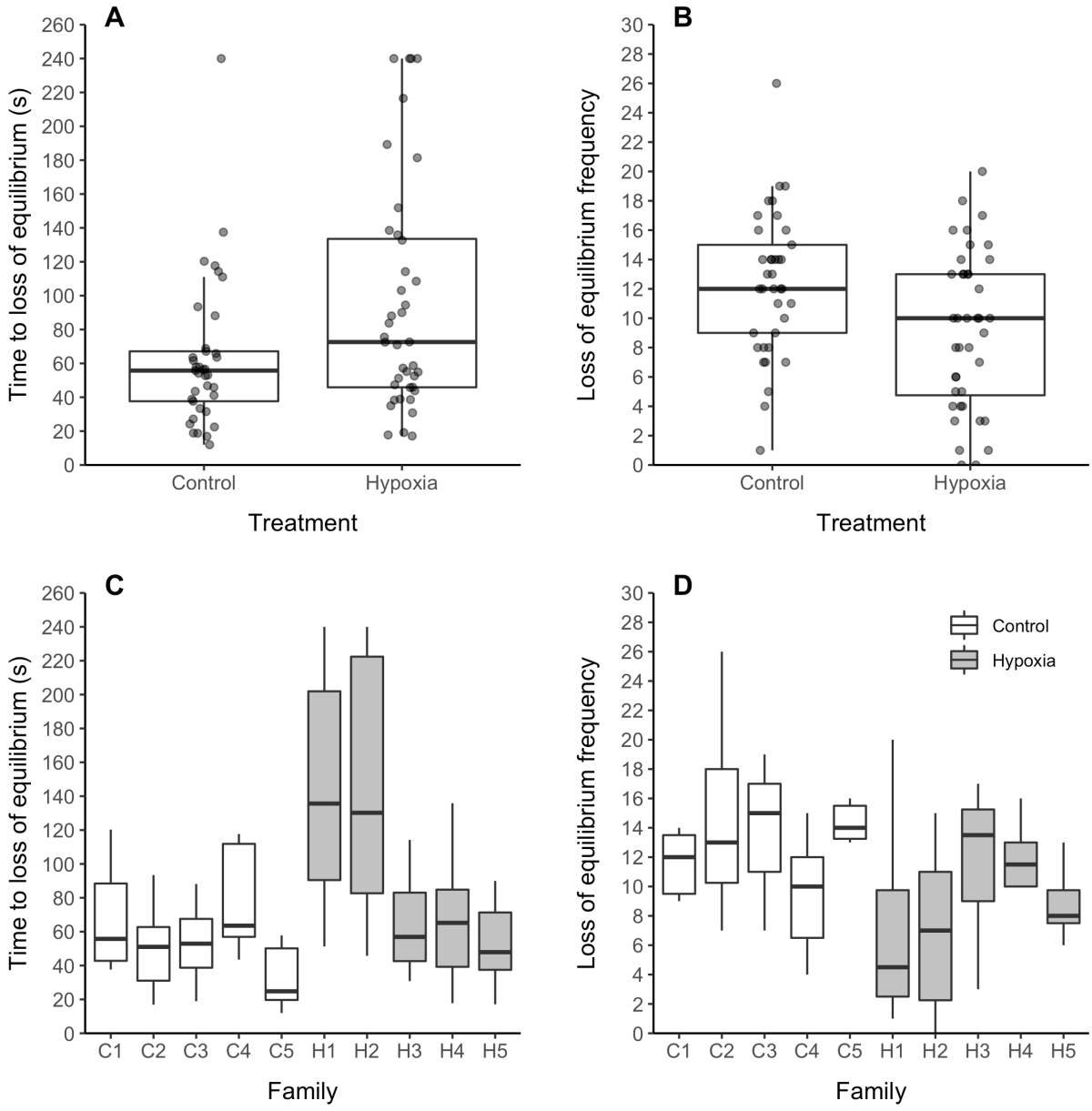


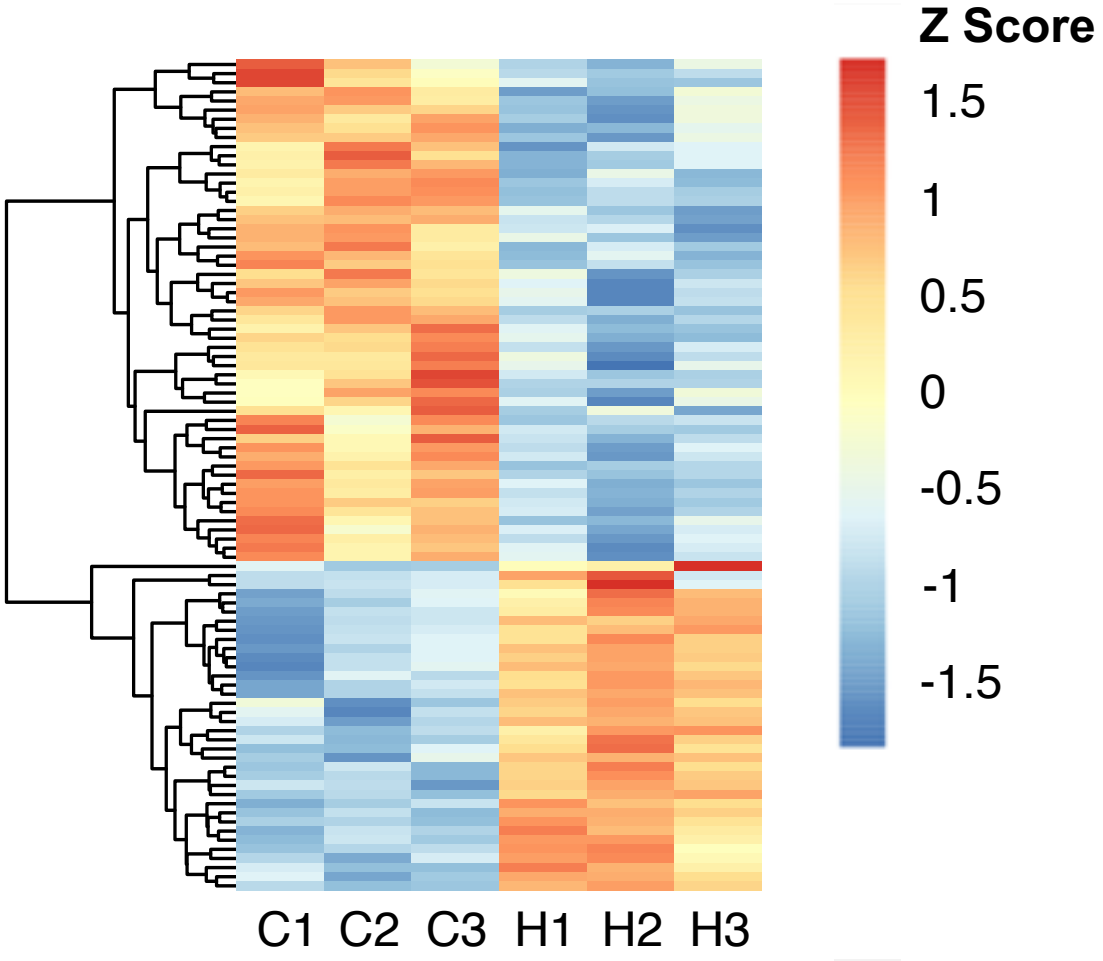
Figure 2. Hypoxia tolerance assays of unexposed offspring of control vs. hypoxia treated males. A and C) Time to first loss of equilibrium >3 sec, B and D) loss of equilibrium frequency – panels C and D are broken down by family. N = 6-8 progeny/family x 5 families/treatment. Tolerance assays done at 20-21 days post fertilization, following 14 day parental exposures (20-21 kPa normoxia control vs. 11-12 kPa hypoxia treatment). Boxes illustrate the interquartile range (medians, 25th and 75th percentiles), and whiskers illustrate 1.5 * the interquartile range, above and below the 75th and 25th percentiles.

Transcriptome wide gene expression patterns in unexposed offspring

We sequenced 17.4 – 20.0 million reads per sample. Mapping assigned 14.1 – 16.1 million reads to genes, representing 80.15 – 81.41% of reads uniquely assigned to a gene, with detected expression in 26,260 of the 32,057 genes in the GRCz11.99 annotation.

A total of 91 genes were significantly differentially expressed between the offspring of control and hypoxia exposed males (Figure 3; Table S1). Of the 91 differentially expressed genes, 36 genes were significantly upregulated, and 55 were significantly downregulated in the offspring of hypoxia exposed males (Figure 3). Eight genes exhibited greater than 4-fold change in differential expression (2 upregulated in hypoxia, 6 downregulated in hypoxia) (Figure 4). Most notably, two hemoglobin genes (*hbaa1* and *hbz* (*si:ch211-5k11.6*); Figure 4 and 5A) were upregulated by more than 12-fold in offspring of hypoxia exposed F0 males. Three additional genes were upregulated by more than 7-fold, but did not pass False Discovery Rate (FDR) correction – these included a third hemoglobin gene (*hbba1*; Figure 4 and 5A; Table S2) and a major histocompatibility gene (*mhc1ula*; Figure 4). Importantly, the observed upregulation in hemoglobin gene expression correlates with the differential family effects observed in our loss of equilibrium tolerance assays (compare Figure 2 with Figure 5A/Table S2). Sequenced offspring from the H1 and H2 families, the two families that show the greatest tolerance to acute hypoxia, also show the greatest upregulation in gene expression. Indeed, the sequenced H1 offspring never lost equilibrium within our 240s long assay and the sequenced H2 offspring first lost equilibrium at 216.5s, whereas the third hypoxia offspring, from family H3 (with a first loss of equilibrium at 181.5s), and the offspring from C1-C3 (first loss of equilibrium > 3 s at 38.8, 24.2 and 18.9s, respectively) show little to no expression at these hemoglobin genes.

315 Six genes were downregulated by more than 4-fold in offspring of hypoxia exposed
316 males (Figure 4), including *timm23b* (translocase of inner mitochondrial membrane 23 homolog
317 b (yeast)), *acot21* (acyl-CoA thioesterase 21), *znf1156* (zinc finger protein 1156), *cyt1l* (type I
318 cytokeratin), *irge1* (immunity-related GTP-ase), and *zgc:158417*.



319

320 **Figure 3. Differential gene expression in offspring of control vs. hypoxia treated males.** 91
321 genes are differentially expressed between the 20-21 day old offspring of hypoxia (n = 3) and
322 control (n = 3) males, with an FDR < 0.05. Red and blue colours represent higher and lower
323 expression, respectively.
324

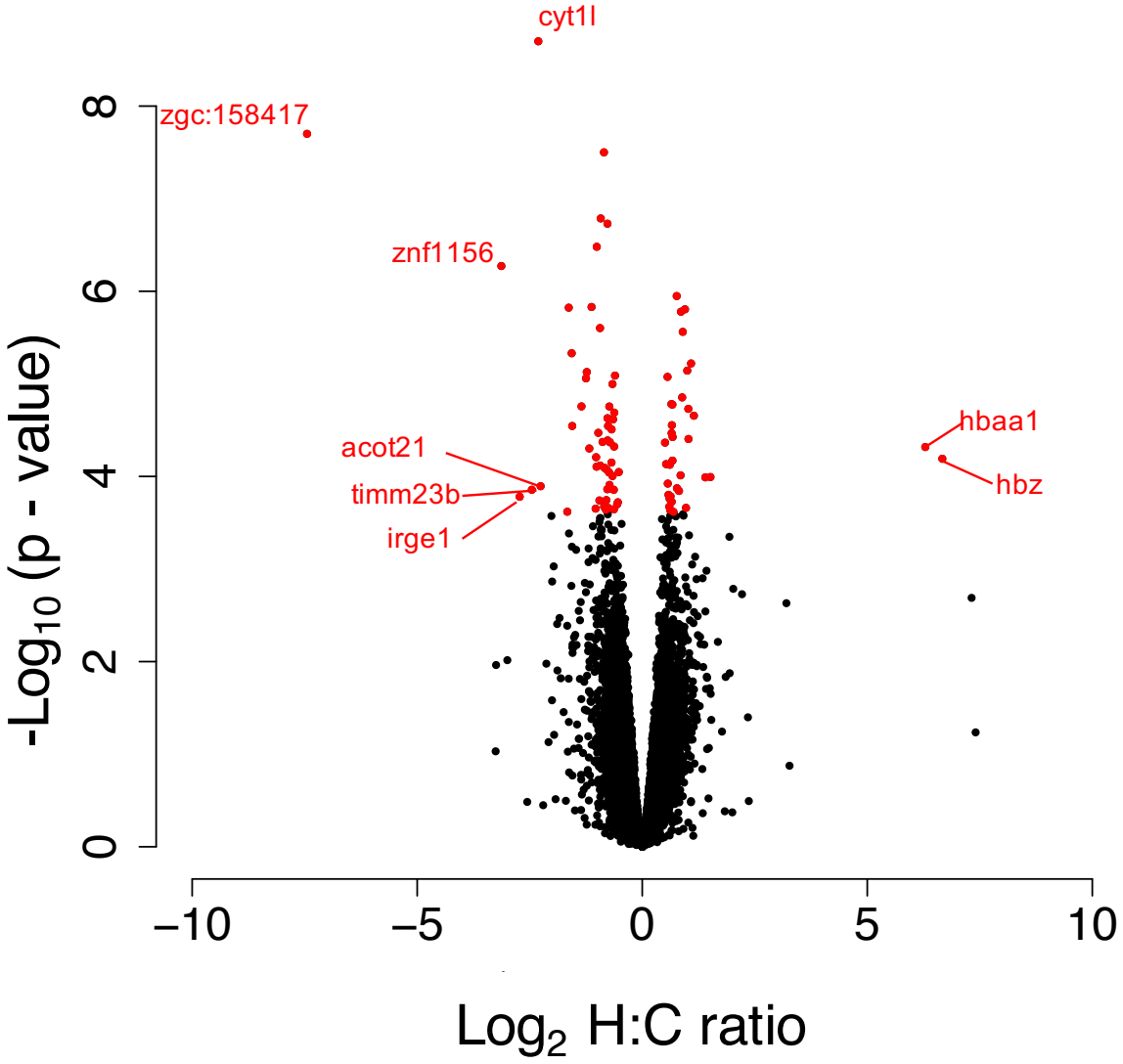


Figure 4. Volcano plot for differentially expressed genes in the offspring of control vs. hypoxia exposed males. 20-21 day old zebrafish F1 offspring from control (n = 3) and hypoxia (n = 3) treated males, showing the distribution of significance [-log10(p-value)] *vs*. fold change [log2(fold change)] for all genes. Each circle represents a gene, with significant genes (at 5% FDR) highlighted in red. Genes with greater than 4-fold change in expression between control and hypoxia treatments are labelled.

Of the genes we analysed, 13,698 were associated with a GO term (including 58 of the 91 differentially expressed genes). Nine GO terms were overrepresented in our differentially

335 expressed genes at a q-value cutoff of 0.1 (Table 1). Several significantly enriched GO terms
336 were associated with lytic or proteolytic activity (i.e. serine hydrolase activity, serine-type
337 endopeptidase activity, hydrolase activity, serine-type peptidase activity, glutamate
338 decarboxylase activity, endopeptidase activity, peptidase activity, peptidase activity, acting on L-
339 amino acid peptides). Proteolysis (GO:0006508 p-value = 7.43 x 10^{-04}; q-value = 0.7640) tended
340 to be over-represented. Genes associated with the process of aging (GO:0007568; p-value = 1.99
341 x 10^{-05}; q-value = 0.1640) as well as to the response to oxidative stress (GO:0006979; p-value =
342 9.20 x10^{-04}; q-value = 0.841) also tended to be over-represented.

343
344 Table 1: **Gene Ontology analysis terms associated with differentially expressed genes**. Only
345 terms with a False Discovery Rate q-value below 0.1 are displayed.
346

GO term	Sub-ontology	Description	Enrichment factor	p-value	FDR q-value
GO:0004252	function	serine-type endopeptidase activity	15.97	2.47 x 10^{-07}	7.55 x 10^{-04}
GO:0008236	function	serine-type peptidase activity	13.67	7.18 x 10^{-07}	1.10 x 10^{-03}
GO:0017171	function	serine hydrolase activity	13.67	7.18 x 10^{-07}	7.30 x 10^{-04}
GO:0016787	function	hydrolase activity	2.62	1.56 x 10^{-05}	1.19 x 10^{-02}
GO:0004351	function	glutamate decarboxylase activity	216.71	2.09 x 10^{-05}	1.28 x 10^{-02}
GO:0004175	function	endopeptidase activity	6.35	3.34 x 10^{-05}	1.70 x 10^{-02}
GO:0070011	function	peptidase activity, acting on L-amino acid peptides	4.84	8.66 x 10^{-05}	3.77 x 10^{-02}
GO:0008233	function	peptidase activity	4.64	1.19 x 10^{-04}	4.53 x 10^{-02}
GO:0009449	process	gamma-aminobutyric acid biosynthetic process	216.71	2.09 x 10^{-05}	1.28 x 10^{-02}

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348

Global methylation changes in the sperm of parental males and the relationship between methylation in parental sperm and gene expression in offspring

To explore potential mechanistic explanations underlying increased tolerance to acute hypoxia and differential gene expression in the zebrafish offspring, we conducted whole genome bisulfite sequencing (WGBS) of sperm from hypoxic *(n*= 3) and control males (*n*=3). We obtained an average of 67,891,334 reads per methylome (SEM ± 2,551,964) and a mapping efficiency of 45.02% (SEM ± 0.07%). Global cytosine-guanine (CG) dinucleotide methylation levels showed no differences between hypoxia and control samples (84.15% and 84.23%, respectively; $p >$ 0.05).

In jawed vertebrates, methylation of transcription start sites (TSS), and in particular regions enriched on CG dinucleotides (CGIs), is associated with transcriptional silencing (Peat et al., 2014). For two example cases, the upregulated hemoglobin cluster and the downregulated *acot21* gene, we characterized methylation at individual positions and CGI using statistical and experimental criteria (Gardiner-Garden and Frommer, 1987; Long et al., 2013). No differences in methylation levels were observed for these genes (Figure 5A). Next, we explored methylation levels for all differentially expressed genes. Using a threshold of 20 methylation calls we obtained 29 and 23 genes for the upregulated and downregulated groups. Whereas downregulated genes are hypermethylated and upregulated genes are hypomethylated for hypoxia and control samples, TSS methylation values remain stable when both groups of samples are compared (Figure 5B). Finally, we explored the global effect of DNA methylation on gene expression using expression quantiles. We found methylation levels in the parental sperm and gene expression in the F1 offspring are coupled in hypoxia and control samples (Figure 5C).

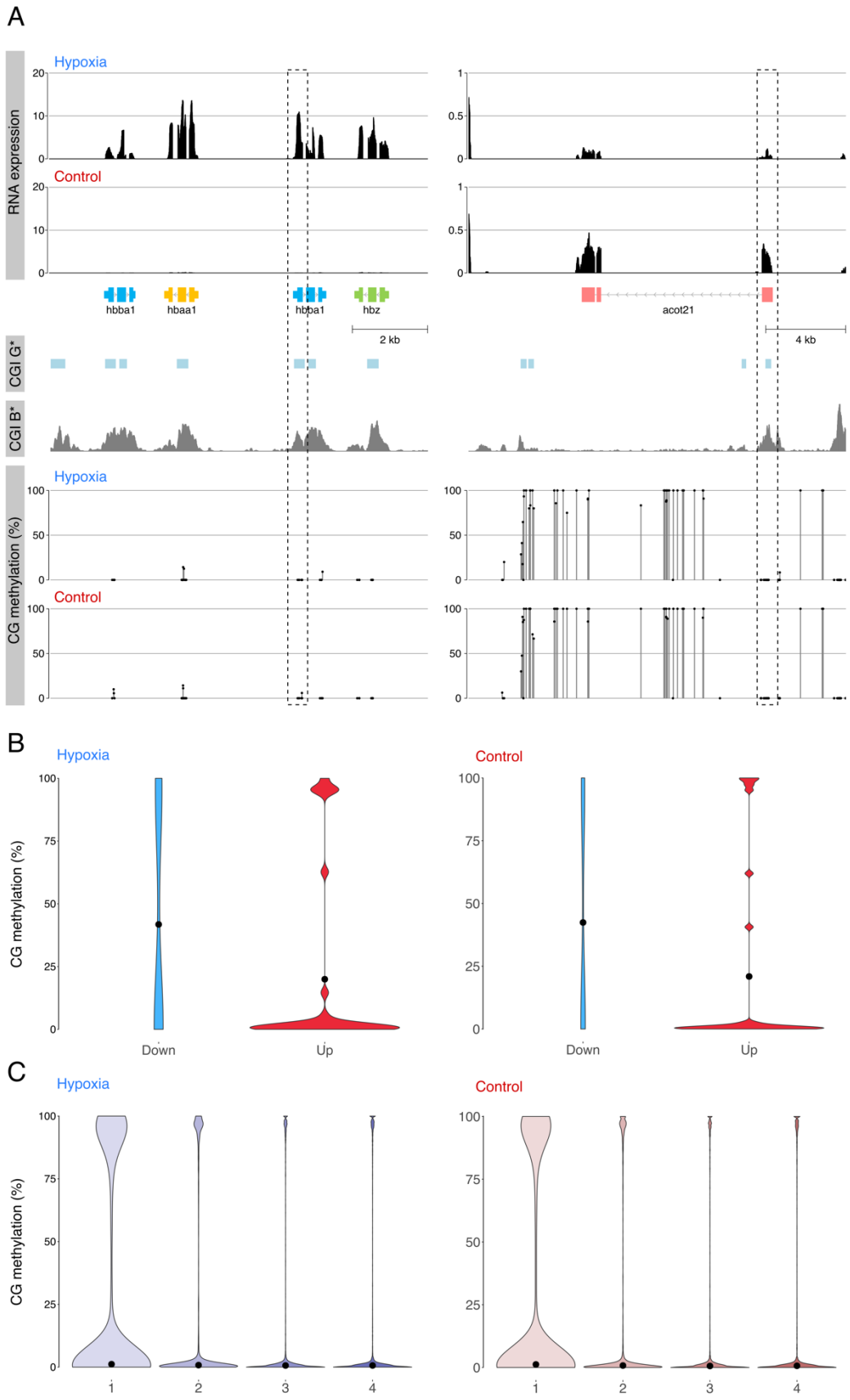

Figure 5. Global methylation changes and relationship between methylation in the sperm of hypoxia vs. control parental males and gene expression in the F1 progeny. A) Relationship between CpG methylation and RNA expression for Hb cluster and acot 21. CG methylation track shows methylation levels for dinucleotides with >5 calls; CGIs were predicted according to the Gardiner-Garden and Frommer criteria (CGI G*) and CxxC affinity purification (CGI B*). No changes in TSS methylation containing CGIs (dashed box) were observed for both genes (bottom). B) Violin plot showing distribution of methylation at transcription start sites (TSS) of differentially expressed genes in the F1 progeny. Downregulated genes are hypermethylated when compared to upregulated genes, however no clear differences were observed between both conditions. C) Violin plot showing distribution of methylation at TSS of genes classified into quartiles according to expression level (highest, 4). Each violin is scaled to the same maximum width (total area is not constant between violins) to demonstrate distributions for each quartile. Black dots denote the median.

DISCUSSION

We demonstrate that paternal exposure to hypoxia alters both the phenotypic response to hypoxia and gene expression in the offspring. Larvae of fathers that experienced moderate hypoxia-maintained equilibrium in acute hypoxia for longer than those of controls, indicating that paternal exposure stimulated a higher tolerance to hypoxic conditions. Using next-generation sequencing, we also detected significant changes in gene expression between control and hypoxia offspring, with two key hemoglobin genes upregulated in offspring of hypoxia exposed males –genes which may mediate the observed phenotypic differences, as they are involved in oxygen transport. This pattern of inheritance, through the paternal line, could have large evolutionary consequences as fathers are able to pass down valuable information to offspring that may enable better survival. However, the underlying mechanism for this transmission remains unknown, as we did not detect any differential methylation in the sperm of parental males at any of the differentially expressed genes.

Importantly, our study provides evidence of increased tolerance to acute hypoxia through paternal exposure, thus, adding to increasing evidence that environmental challenges experienced

405 by ancestors can provide progeny with environmental specific information that might allow
406 future generations to survive the same environmental challenge, i.e., transgenerational plasticity
407 (Donelson et al., 2014, 2012; Heckwolf et al., 2018; Herman and Sultan, 2011; Lee et al., 2020;
408 Marshall, 2008; Ryu et al., 2018; Veilleux et al., 2015). In a previous study, 20 dpf larvae
409 exposed to 4 kPa pO_2 revealed that offspring of parents exposed to hypoxia had longer time to
410 loss of equilibrium (hypoxia resistance; (Ho and Burggren, 2012)). In preliminary trials, we
411 found no behavioral differences using these oxygen parameters; thus, we increased the
412 magnitude of hypoxia, exposing larvae to ~1 kPa, which produced the expected phenotypic
413 differences. Importantly, the previous study (Ho and Burggren, 2012) did not differentiate
414 between maternal and paternal effects and did not provide a possible mechanism underlying this
415 phenotypic effect.

416 It is important to note that our study detects intergenerational acclimation, with potential
417 for transgenerational acclimation. A transgenerational study requires rearing fish through to
418 create an F2 generation. Indeed, few studies that claim transgenerational acclimation are truly
419 transgenerational, as the studies are conducted across a single generation (O'Dea et al., 2016).
420 Even if parents are exposed to environmental challenges prior to maturity, the primordial germ
421 cells are still exposed to the challenge as well. Regardless, our results suggest that parents are
422 passing on information that may benefit offspring survival, thus facilitating acclimation to
423 environmental conditions.

424 To try and understand the underlying mechanism priming progeny to better cope with
425 hypoxic conditions, we conducted transcriptomic analysis of the progeny. We detected 91 genes
426 that were differentially expressed in the offspring of paternal males that were exposed to
427 moderate hypoxia for two-weeks. Most notably, two hemoglobin genes (*hbaa1* and *hbz*)

exhibited over 7-fold differential expression, were upregulated in offspring of males exposed to hypoxia, with another hemoglobin gene, *hbba1*, also upregulated, though non-significantly. Remarkably, sequenced offspring from the H1 and H2 families, the two families that show the greatest tolerance to acute hypoxia also show the greatest upregulation in hemoglobin gene expression (Table S3). *hbaa1*, *hbz* and *hbba1* are all found on chromosome 3, and are part of the major hemoglobin locus responsible for heme binding, and are instrumental in oxygen transport (Ganis et al., 2012). Exposing fish to hypoxia is typically considered to improve hypoxia tolerance through alterations in hemoglobin, hemoglobin-O_2 binding affinities, or cardiac function to improve low O_2 performance (Cook et al., 2013). Thus, differential expression of hemoglobin genes (up-regulated in offspring from hypoxia exposed fathers) could indicate altered physiological mechanisms to combat low oxygen, which could be precipitating the increased tolerance to acute hypoxia in the H1 and H2 families.

The relationship between hypoxia and hemoglobin function has been studied in larval zebrafish, suggesting that zebrafish larvae might be able to upregulate hemoglobin concentration in response to chronic hypoxia (Schwerte et al., 2003). Under normoxic conditions, oxygen supply via diffusion seems to be sufficient to meet metabolic demands up to 12-14 dpf in zebrafish, but larvae appear to be able to use a circulatory system as a backup where necessary (Schwerte et al., 2003). Further, impaired hemoglobin function does not impair routine oxygen usage in normoxia or at moderate levels of hypoxia in 5-42 dpf larvae, but functional hemoglobin does allow larvae to sequester extra oxygen from water in extreme hypoxic conditions (Rombough and Drader, 2009). Larvae express embryonic/larval globins early in development, but somewhere between days 16 and 22 the embryonic/larval globins begin to decline (Ganis et al., 2012; Tiedke et al., 2011), and adult globin expression increases, with the

adult globin expression pattern nearly completely established by day 32dpf. *hbae5* (ENSDARG00000045142) is the only embryonic/larval hemoglobin to show any pattern of differential expression in our transcriptomic data. We found that this gene was highly expressed and upregulated twice as much in our hypoxia offspring than in our control, though not significantly differentiated after FDR correction ($p= 0.014$, $q = 0.391$). *hbae5* expression appears to peak around 22 dpf (Ganis et al., 2012), so upregulation of this gene should result in higher affinity for oxygen, which may help explain the increased tolerance to the acute hypoxia that we observed in our 20-21 dpf progeny. *hbae5* has also been shown to be up-regulated more than 5-fold by hypoxia in zebrafish larvae directly exposed to hypoxia; ((Long et al., 2015); note that hbae5 is called hbz in their study).

In addition to two genes upregulated over 7-fold in offspring of hypoxia exposed males, six genes were downregulated by more than 4-fold as well. These include *timm23b* (translocase of inner mitochondrial membrane 23 homolog b (yeast)), an integral component of membranes. Gene *acot21* (acyl-CoA thioesterase 12), is a key component of acyl-CoA metabolic processes with thiolester hydrolase activity and found in the cytoplasm. Gene *znf1156* (zinc finger protein 1156) has metal ion binding functions. Gene *zgc:158417* and *irge1* (immunity-related GTP-ase family) are predicted to be integral component of membranes and used in GTP binding. Gene *cyt1l* (type I cytokeratin) is predicted to have structural molecule activity. While there is little information on these downregulated genes, expression changes of such significant magnitude are likely to have large consequences.

An HRGFish database (Rashid et al., 2017) reports 50 key genes that are altered through hypoxia, but we did not find any of them to be differentially expressed in our transcriptomic data (Table S1, showing data for 46 of the 50 HRGFish genes referenced for the zebrafish),

474 suggesting that the genes that are altered by direct exposure to hypoxia may be quite different to
475 those that may be passed on to future generations of hypoxia exposed ancestors. Importantly,
476 transcriptomic studies of F1 and F2 adult tissues, like heart, gill, brain and liver, might reveal
477 differential expression of some of these key hypoxia genes.

478 Interestingly, our GO analysis highlighted an overrepresentation of genes associated with
479 aging and oxidative stress. Studies have previously hypothesized a link between hypoxia (and
480 effects on respiration), oxidative stress (and free radical production) and aging in humans
481 (Katschinski, 2006; Valli et al., 2015), with several studies demonstrating age-related changes in
482 the hypoxia inducible factor system.

483 DNA methylation is known to be altered by hypoxia exposure in fishes (Lai et al., 2019;
484 Wang et al., 2016) and other vertebrates (Childebayeva et al., 2019; Zhang et al., 2017). Thus,
485 we hypothesized that hypoxia might produce changes in the paternal methylome, which are
486 inherited to the offspring, explaining the observed tolerance to acute hypoxia and differential
487 gene expression. Interesting, we found methylation levels and gene expression were coupled in
488 our hypoxia and control samples, despite the different tissues of origin for the methylome and
489 RNA expression data. This coupling supports a common pattern between the paternal methylome
490 and expression levels of the embryo. Yet, we observed no differences in methylation levels in
491 any differentially expressed genes, including the hemoglobin cluster, suggesting that sperm
492 methylation patterns are not responsible for changes in the observed changes in gene expression
493 in our study, or perhaps through epigenetic control away from the focal genes. The medaka
494 studies (Lai et al., 2019; Wang et al., 2016) exposed fish to a similar level of hypoxia, but for a
495 much longer period, the entire 3-month life cycle, which may explain why they observed
496 differential methylation in the sperm and we did not. If alterations to DNA methylation are not

497 responsible for the changes in gene expression observed in our study, then perhaps histone
498 modifications or miRNAs are at play. miRNA in medaka have also been identified as a potential
499 underlying mechanism of transgenerational testis impairment induced by hypoxia by targeting
500 genes associated with stress responses, cell cycle, epigenetic modifications, sugar metabolism,
501 and cell motion (Tse et al., 2016).

502 In conclusion, our study demonstrates that paternal exposure to hypoxia is able to endow
503 offspring with a higher resistance to hypoxic conditions and that upregulation of genes at the
504 hemoglobin gene complex on chromosome 3 may explain these phenotypic effects. This study is
505 the first to demonstrate that paternal exposure alone can mediate these changes in phenotype and
506 gene expression. These findings suggest that paternal inheritance may be providing offspring
507 with valuable environmental specific information (a 'memory') that could increase their survival,
508 though it is unclear how this information is transmitted as we did not detect any differential
509 methylation in the sperm of control and hypoxia treated males. Regardless, the variability in
510 tolerance to acute hypoxia that we observed between hypoxia families suggests that progeny of
511 different genotypes respond to hypoxia in different ways, phenotypically (i.e., an epigenotype x
512 environment interaction). In other words, environmental specific information is transferred
513 through some genotypes, but not others, suggesting that pre-acclimation to hypoxic conditions
514 may be driven by an interaction between the genotype, the epigenotype and the environment.

515
516
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J.C., and T.K. collected the data. A.R., S.L.J., O.O. and L.D. performed the analyses. A.R. and S.L.J led the writing of the manuscript, with assistance from O.O. and L.D. for bioinformatics aspects, and all the authors contributed to revisions. **Competing interests.** The authors declare no competing financial interests. **Data and materials accessibility.** The raw phenotypic data can be found at https://osf.io/xpy8j/. The accession number for the RNA-Seq and WGBS datasets reported in this paper is GEO:GSE160662. The source code for the bioinformatic analysis is publicly available on GitHub https://github.com/OscarOrt/Paternal_hypoxia_Ragsdale_2020.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at...

Table S1. Results of differential gene expression analysis
Table S2. Read counts mapping to four hemoglobin genes
Table S3. Whole genome bisulfite sequencing summary data

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783

Table S1. Differentially expressed genes between the offspring of control vs. hypoxia exposed males.

Gene_ID	log2FoldChange	pvalue	padj	direction	gene_symbol	gene_description
ENSDARG00000101671	-7.447901496	1.99E-08	0.000186241	downinhypoxia	zgc:158417	zgc:158417
ENSDARG00000098800	-3.130931401	5.34E-07	0.001425146	downinhypoxia	znf1156	zinc finger protein 1156
ENSDARG00000014975	-2.72345402	0.00016574	0.041287001	downinhypoxia	irge1	interferon-inducible GTPase 5
ENSDARG00000100375	-2.453048439	0.00013931	0.036657692	downinhypoxia	timm23b	translocase of inner mitochondrial membrane 23 homolog b (yeast)
ENSDARG00000036832	-2.309127267	2.00E-09	0.000037	downinhypoxia	cyt1l	type I cytokeratin, enveloping layer, like
ENSDARG00000042513	-2.257793529	0.0001275	0.036091399	downinhypoxia	acot21	acyl-CoA thioesterase 21
ENSDARG00000104890	-1.664931261	0.00024063	0.049653703	downinhypoxia	LOC103910514	NACHT, LRR and PYD domains-containing protein 3-like
ENSDARG00000100504	-1.63260871	1.50E-06	0.002588816	downinhypoxia	ppp1r27b	protein phosphatase 1, regulatory subunit 27b
ENSDARG00000088717	-1.56758552	4.67E-06	0.005820696	downinhypoxia	ecrg4b	ECRG4 augurin precursor b
ENSDARG00000041645	-1.555587305	2.85E-05	0.015228636	downinhypoxia	a2ml	alpha-2-macroglobulin-like
ENSDARG00000078878	-1.349850562	1.75E-05	0.01216431	downinhypoxia	si:ch73-244f7.3	si:ch73-244f7.3
ENSDARG00000059651	-1.250202425	8.73E-06	0.007763018	downinhypoxia	cidec	cell death inducing DFFA like effector c
ENSDARG00000101479	-1.232335842	7.45E-06	0.007735341	downinhypoxia	LOC103910140	CD59 glycoprotein-like
ENSDARG00000088625	-1.176010143	4.99E-05	0.019840159	downinhypoxia	si:ch211-142d6.2	si:ch211-142d6.2
ENSDARG00000093193	-1.126255412	1.48E-06	0.002588816	downinhypoxia	chia.6	chitinase, acidic.6
ENSDARG00000075827	-1.035496215	0.00022261	0.048352334	downinhypoxia	f7i	coagulation factor VIIi
ENSDARG00000005179	-1.024794268	6.21E-05	0.024155494	downinhypoxia	dglucy	D-glutamate cyclase
ENSDARG00000090899	-1.015231326	7.85E-05	0.026664488	downinhypoxia	LOC108180718	syncollin-like
ENSDARG00000043729	-1.010559912	3.31E-07	0.001029956	downinhypoxia	plac8.1	placenta associated 8, tandem duplicate 1
ENSDARG00000074118	-0.976528595	3.38E-05	0.016774298	downinhypoxia	ftr15	finTRIM family, member 15
ENSDARG00000060350	-0.951169523	0.00018177	0.043538956	downinhypoxia	apoda.2	apolipoprotein Da, duplicate 2
ENSDARG00000079727	-0.938155384	2.50E-06	0.003590744	downinhypoxia	selenop2	selenoprotein P2
ENSDARG00000018478	-0.936033991	7.62E-05	0.02638081	downinhypoxia	agxtb	alanine--glyoxylate and serine--pyruvate aminotransferase b
ENSDARG00000045834	-0.921661494	1.63E-07	0.000696718	downinhypoxia	si:dkey-14d8.7	si:dkey-14d8.7
ENSDARG00000024503	-0.881635247	4.24E-05	0.018319171	downinhypoxia	c6ast3	six-cysteine containing astacin protease 3
ENSDARG00000056765	-0.850236732	3.16E-08	0.000196709	downinhypoxia	ela2l	elastase 2 like
ENSDARG00000033854	-0.847319022	0.00021264	0.047864722	downinhypoxia	abrab	actin binding Rho activating protein b
ENSDARG00000039393	-0.83487661	8.09E-05	0.026986919	downinhypoxia	si:ch211-240l19.5	si:ch211-240l19.5
ENSDARG00000041569	-0.805620387	0.0002295	0.048352334	downinhypoxia	ces2a	carboxylesterase 2a
ENSDARG00000029587	-0.798510485	0.00018023	0.043538956	downinhypoxia	msra	methionine sulfoxide reductase A
ENSDARG00000018263	-0.789477991	8.43E-05	0.027625412	downinhypoxia	pdia2	protein disulfide isomerase family A, member 2
ENSDARG00000086254	-0.775585332	0.00013736	0.036657692	downinhypoxia	hhla2b.2	HERV-H LTR-associating 2b, tandem duplicate 2
ENSDARG00000009153	-0.77272349	1.86E-07	0.000696718	downinhypoxia	pla2g1b	phospholipase A2, group IB (pancreas)
ENSDARG00000030616	-0.771999685	2.35E-05	0.014143122	downinhypoxia	nfe2l1a	nuclear factor, erythroid 2-like 1a
ENSDARG00000030915	-0.769078697	4.08E-05	0.018319171	downinhypoxia	cpa1	carboxypeptidase A1 (pancreatic)
ENSDARG00000042993	-0.759414196	2.85E-05	0.015228636	downinhypoxia	prss1	serine protease 1
ENSDARG00000077058	-0.748679878	0.00021958	0.048263741	downinhypoxia	LOC100334908	elastase-1-like
ENSDARG00000002396	-0.737186432	9.06E-05	0.028683515	downinhypoxia	cry-dash	cryptochrome DASH
ENSDARG00000007276	-0.731121935	1.76E-05	0.01216431	downinhypoxia	ela3l	elastase 3 like
ENSDARG00000015123	-0.727327583	0.00012357	0.035516831	downinhypoxia	dnase1l4.1	deoxyribonuclease 1 like 4, tandem duplicate 1
ENSDARG00000102442	-0.719815554	4.27E-05	0.018319171	downinhypoxia	folr	folate receptor
ENSDARG00000105411	-0.707858646	0.00013593	0.036657692	downinhypoxia	si:ch211-113d11.5	si:ch211-113d11.5
ENSDARG00000057437	-0.684000585	7.08E-05	0.025948145	downinhypoxia	apodb	apolipoprotein Db
ENSDARG00000090428	-0.68164618	3.10E-05	0.016098467	downinhypoxia	ctrb1	chymotrypsinogen B1
ENSDARG00000103687	-0.66146725	1.01E-05	0.008540483	downinhypoxia	sycn.2	syncollin, tandem duplicate 2
ENSDARG00000068680	-0.66001916	9.90E-05	0.030324771	downinhypoxia	ctrl	chymotrypsin-like
ENSDARG00000020123	-0.648795043	2.42E-05	0.014143122	downinhypoxia	coq8aa	coenzyme Q8A, genome duplicate a
ENSDARG00000039730	-0.63234854	0.0001393	0.036657692	downinhypoxia	zgc:112160	zgc:112160
ENSDARG00000076011	-0.630164232	0.00022674	0.048352334	downinhypoxia	si:ch211-149b19.3	si:ch211-149b19.3
ENSDARG00000033832	-0.625143351	4.76E-05	0.019532834	downinhypoxia	ampd1	adenosine monophosphate deaminase 1 (isoform M)
ENSDARG00000009443	-0.621954227	2.05E-05	0.013179559	downinhypoxia	zgc:92137	zgc:92137
ENSDARG00000040432	-0.604641591	8.15E-06	0.007763018	downinhypoxia	klf2b	Kruppel-like factor 2b
ENSDARG00000053097	-0.55692281	0.00019994	0.046118082	downinhypoxia	hsf2	heat shock transcription factor 2
ENSDARG00000088711	-0.54155565	0.0001904	0.044465458	downinhypoxia	lgals1l1	lectin, galactoside-binding, soluble, 1 (galectin 1)-like 1
ENSDARG00000101160	-0.521525439	8.97E-05	0.028683515	downinhypoxia	apoa4a	apolipoprotein A-IV a
ENSDARG00000053358	0.506539149	4.31E-05	0.018319171	upinhypoxia	basp1	brain abundant, membrane attached signal protein 1
ENSDARG00000030614	0.53052294	7.36E-05	0.02637128	upinhypoxia	syt1a	synaptotagmin Ia
ENSDARG00000099203	0.562147944	8.42E-06	0.007763018	upinhypoxia	atp1b2a	ATPase Na+/K+ transporting subunit beta 2a
ENSDARG00000055216	0.567916067	0.00011944	0.034868517	upinhypoxia	tuba1c	tubulin, alpha 1c
ENSDARG00000020795	0.583776186	0.00015794	0.040423002	upinhypoxia	rac3b	Rac family small GTPase 3b
ENSDARG00000007654	0.601581979	0.00021243	0.047864722	upinhypoxia	nsfa	N-ethylmaleimide-sensitive factor a
ENSDARG00000039647	0.607944755	0.00017374	0.042710585	upinhypoxia	slc6a1b	solute carrier family 6 member 1b
ENSDARG00000027419	0.612777357	7.48E-05	0.02637128	upinhypoxia	gad1b	glutamate decarboxylase 1b
ENSDARG00000025106	0.619971087	0.00023034	0.048352334	upinhypoxia	ppp3cb	protein phosphatase 3, catalytic subunit, beta isozyme
ENSDARG00000076364	0.628462284	0.00016385	0.041287001	upinhypoxia	si:dkey-178k16.1	si:dkey-178k16.1
ENSDARG00000103937	0.648166679	1.66E-05	0.01216431	upinhypoxia	ndrg4	NDRG family member 4
ENSDARG00000022995	0.652667549	3.41E-05	0.016774298	upinhypoxia	clic4	chloride intracellular channel 4
ENSDARG00000099257	0.654101819	0.00018766	0.044379337	upinhypoxia	si:ch73-119p20.1	si:ch73-119p20.1
ENSDARG00000102453	0.658745469	2.80E-05	0.015228636	upinhypoxia	slc1a2b	solute carrier family 1 member 2b
ENSDARG00000059775	0.670043489	1.67E-05	0.01216431	upinhypoxia	slc32a1	solute carrier family 32 member 1
ENSDARG00000017673	0.671384509	6.75E-05	0.025230895	upinhypoxia	nova2	NOVA alternative splicing regulator 2
ENSDARG00000086222	0.675190414	3.75E-05	0.017981649	upinhypoxia	nat16	N-acetyltransferase 16
ENSDARG00000100166	0.694355451	0.00024185	0.049653703	upinhypoxia	si:dkeyp-9d4.3	si:dkeyp-9d4.3
ENSDARG00000059945	0.766331798	1.13E-06	0.002588816	upinhypoxia	sv2a	synaptic vesicle glycoprotein 2A
ENSDARG00000068745	0.771799195	0.00013402	0.036657692	upinhypoxia	map4l	microtubule associated protein 4 like
ENSDARG00000051970	0.816336471	0.00014345	0.037223718	upinhypoxia	smu1b	SMU1 DNA replication regulator and spliceosomal factor b
ENSDARG00000114797	0.852062415	9.73E-05	0.030303635	upinhypoxia	rab3b	RAB3B, member RAS oncogene family
ENSDARG00000000503	0.860377519	1.66E-06	0.002588816	upinhypoxia	stx1b	syntaxin 1B
ENSDARG00000015537	0.886970984	1.40E-05	0.011381432	upinhypoxia	gad2	glutamate decarboxylase 2
ENSDARG00000097196	0.900381828	2.74E-06	0.003659608	upinhypoxia	CR847531.1	NA
ENSDARG00000105201	0.949578108	1.57E-06	0.002588816	upinhypoxia	adcyap1r1a	adenylate cyclase activating polypeptide 1a (pituitary) receptor type I
ENSDARG00000040123	0.97270037	0.00021835	0.048263741	upinhypoxia	zfpm2a	zinc finger protein, FOG family member 2a
ENSDARG00000098880	1.000025811	7.19E-06	0.007735341	upinhypoxia	pclob	piccolo presynaptic cytomatrix protein b
ENSDARG00000030376	1.024003877	1.86E-05	0.012425665	upinhypoxia	grin2bb	glutamate receptor, ionotropic, N-methyl D-aspartate 2B, genome duplicate b
ENSDARG00000057660	1.02770991	3.94E-05	0.018319171	upinhypoxia	dhx57	DEAH (Asp-Glu-Ala-Asp/His) box polypeptide 57
ENSDARG00000012610	1.085030009	6.02E-06	0.007033416	upinhypoxia	saga	S-antigen; retina and pineal gland (arrestin) a
ENSDARG00000053205	1.147549538	2.21E-05	0.01375797	upinhypoxia	ppfia4	PTPRF interacting protein alpha 4
ENSDARG00000079997	1.401524233	0.00010231	0.03034154	upinhypoxia	si:zfos-223e1.2	si:zfos-223e1.2
ENSDARG00000100339	1.515219022	0.00010157	0.03034154	upinhypoxia	arf4b	ADP-ribosylation factor 4b
ENSDARG00000079078	6.289376582	4.81E-05	0.019532834	upinhypoxia	si:ch211-5k11.8	hbz
ENSDARG00000097011	6.667965594	6.44E-05	0.024565914	upinhypoxia	hbaa1	hemoglobin, alpha adult 1
Extra genes 4-fold up but not padj						
ENSDARG00000097238	7.203654746	NA	NA			hbba1
ENSDARG00000089087	7.317595408	0.00204913	0.168364492			hbba1
ENSDARG00000059039	7.407254142	0.05813054	0.640297745			mhc1ula

Table S2: **Read counts mapping to four haemoglobin genes located on a small region of chromosome 3**. * genes are not significantly differentially expressed but show strong expression in samples H1 and H2 who were the most resilient to loss of equilibrium in hypoxic environment. Note that there are two versions of hbba1 due to genome duplication, and they are physically separated on chromosome 3.

Ensembl ID	Gene name	C1	C2	C3	H1	H2	H3
ENSDARG00000079078	*hbz*	0	0	2	9	303	1
ENSDARG00000097011	*hbaa1*	2	1	17	671	1427	14
ENSDARG00000097238	*hbba1**	0	0	8	341	974	8
ENSDARG00000089087	*hbba1**	1	3	13	227	1169	21

Table S3. Whole genome bisulfite sequencing of the sperm of hypoxic vs. control males

The table lists the general sequencing statistics as well as the number of cytosine calls at either CG dinucleotides ('CG') or in other sequence contexts ('non-CG'), for the samples used in the experiments, mapped against the Zebrafish genome version 11 (GRCz11) . Details of bioinformatic processing are provided in the Methods section. The frequency of non-CG methylation indicates the maximum rate of non-conversion during the bisulfite treatment step; by this measure, all libraries had a bisulfite conversion efficiency of at least 98.52%.

Sample	Reads number	Mapping effi	Total unique map	Methylated CG calls	Unmethylated CG calls	Total CG calls	% CG Methylation	Methylated non-CG calls	Unmethylated non-CG calls	Total non-CG calls	Non-CG Methylation
WGBS_ZF_hypoxia_01	71407777	44.80%	32002473	40595086	7701958	48297044	84.05%	2891863	204518174	207410037	1.39%
WGBS_ZF_hypoxia_02	63581861	44.90%	28517341	37211081	6924223	44135304	84.31%	2802223	186459744	189261967	1.48%
WGBS_ZF_hypoxia_03	74348252	44.90%	33365526	41945900	7945201	49891101	84.07%	2933887	212658962	215592849	1.36%
WGBS_ZF_control_01	60567819	45.10%	27287633	34647909	6480315	41128224	84.24%	2529456	176922946	179452402	1.41%
WGBS_ZF_control_02	62901530	45.20%	28453313	35787583	6746571	42534154	84.14%	2558652	183685718	186244370	1.37%
WGBS_ZF_control_03	74540770	45.20%	33692759	42528134	7911181	50439315	84.32%	3154853	216091870	219246723	1.44%