Aquatic Toxicology 95 (2009) 258–272

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Hypoxia alters gene expression in the gonads of zebrafish (*Danio rerio*)☆,☆☆,◊

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ARTICLE INFO

Article history:
Received 27 May 2008
Received in revised form 26 August 2008
Accepted 30 August 2008

Keywords:
Reproduction
Zebrafish
Hypoxia
Gene expression
Microarray

ABSTRACT

The objectives of this study were to characterize gene expression responses to hypoxia in gonads of mature zebrafish (*Danio rerio*), and to start characterizing modes of action by which hypoxia could potentially alter reproduction. Adult male and female zebrafish were maintained under normoxia (7 mg O_2/L), moderate hypoxia (3 mg O_2/L), and severe hypoxia (1 mg O_2/L) for 4 and 14 days and changes in gene expression in gonadal tissues ($n = 5$ per sex per treatment) were evaluated using a commercial 21,000 gene zebrafish oligonucleotide microarray. Differentially expressed genes were determined using ANOVA ($p < 0.05$), and enriched gene ontology (GO) categories ($p < 0.01$) identified using GeneSpring GX software. Short-term (4 d) exposure to hypoxia affected expression of genes associated with the initial adaptive responses such as: metabolism of carbohydrates and proteins, nucleotide metabolism, haemoglobin synthesis, reactive oxygen species metabolism, and locomotion. Prolonged (14 d) hypoxia affected a suite of genes belonging to different GO categories: lipid metabolism, reproduction (e.g., steroid hormone synthesis), and immune responses. Results of the present study demonstrate that reproduction likely would be affected by hypoxia via multiple modes of action. These include previously hypothesized mechanisms such as modulation of expression of steroidogenic genes, and downregulation of serotonergic pathway. In addition, we propose that there are multiple other points of disruption of reproductive system function linked, for example, to reorganization of lipid transport and other mechanisms involved in responding to hypoxia (e.g., hydroxysteroid dehydrogenase alterations, downregulation of contractile elements, etc.).

1. Introduction

Certain aquatic habitats exhibit large, natural fluctuations in dissolved oxygen (e.g., large diurnal fluctuations in nutrient-rich tropical waters, seasonal hypoxia in ice-covered waters), and organisms inhabiting these environments have evolved a variety of physiological and behavioral strategies to cope with the lack of oxygen. Over the last few decades, anthropogenic activities have caused occurrence of hypoxia in habitats where it had been rare, thereby impacting organisms not accustomed to coping with prolonged hypoxia (Gray et al., 2002). The concern over the expansion of hypoxic environments has led to resurfacing of interest in understanding the adverse effects of, and adaptation to, hypoxia in aquatic organisms. Fish have been a subject of many hypoxia studies, as the majority of our understanding of the role of oxygen in physiology of aquatic organisms comes from work with fish (Wu, 2002).

Over the last few years new genomic tools, such as global gene expression profiling via microarrays, have been employed to characterize effects of hypoxia on development and physiology of fish (Ju et al., 2007b). Although few in number, the existing microarray-based studies have provided important insights into hypoxia responses. For example Ton et al. (2002) exposed zebrafish (*Danio rerio*) embryos to extreme hypoxia for 24 h and documented arrested development, together with a series of changes in gene expression consistent with a switch to anaerobic metabolism and downregulation of energy consumption (e.g., via downregulation of protein synthesis and channel arrest). They also demonstrated that hypoxia-induced changes in gene expression revert to normal upon a brief subsequent exposure to normoxia. van der Meer et al. (2005) focused on long-term adaptive responses to hypoxia

☆ Grant support: This work was supported by the Computational Toxicology Research Initiative sponsored within the Office of Research and Development of the U.S. Environmental Agency, and from the Office of Science Council and Policy within the Office of Pollution Prevention and Toxic Substances.
☆☆ This work has been subjected to review by the National Health and Environmental Effects Research Laboratory and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.
◊ The genomic data was deposited with NCBI's GEO (GEO accession number GSE10951).
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doi:10.1016/j.aquatox.2008.08.021

and selected the gills of the adult zebrafish as a target tissue. They demonstrated metabolic depression (as indicated by repression of genes in the tricarboxylic acid cycle and protein biosynthesis), and postulated that the induction of genes for lysosomal lipid (e.g., cholesterol) trafficking and degradation represent an adaptive response to hypoxia. Other studies have examined effects of hypoxia on global gene expression in multiple tissues (heart, muscle, liver, brain) in hypoxia-tolerant goby (*Gillisthys mirabilis*; Gracey et al., 2001) and medaka (*Oryzias latipes*; Ju et al., 2007a). In both studies the effects of hypoxia varied substantially among tissues and reflected the need for activity maintenance during hypoxia, as well as the extent of the hypoxic insult that each tissue was experiencing. For example, in liver, which is critical to an organism's survival, only a few genes were downregulated as compared to skeletal muscle (Gracey et al., 2001). To date, reproductive tissues have not been an object of microarray studies assessing effects of hypoxia in fish. This is not surprising, since other tissues (e.g., muscle, gills) are more obvious targets for study of adaptive and pathological repercussions of hypoxia. However, given the fact that fish commonly encounter hypoxic conditions in the inshore areas which serve as their spawning grounds, there is a need to better understand the modes of action of hypoxia and the extent of its effects on the reproduction, an important fitness trait that determines species survival.

In the present study we focus on characterizing hypoxia-induced gene expression changes in zebrafish gonads, in part because of the recent reports that hypoxia disrupts reproductive endocrine function in this species (Shang et al., 2006). While there has been plentiful evidence that hypoxic conditions suppress reproduction in fish (see review by Wu (2002)), only recently have investigators started defining mechanisms via which reproductive function in fish is altered. Some studies have described hypoxia-induced reductions in circulating sex steroids (testosterone, 11-ketotestosterone, and estradiol) and vitellogenin (VTG; egg yolk protein precursor) coupled with suppressed reproduction (Landry et al., 2007; Thomas et al., 2006; Wu, 2002), but the understanding of mechanisms via which hypoxia exerts these effects is limited. For example, it is unclear whether direct alterations of enzymes that regulate sex steroid biosynthesis represent a direct or indirect mechanism through which the reproductive suppression is mediated. There is, however, some evidence that other components of the hypothalamo–pituitary–gonadal (HPG) axis may be directly affected. For example, Thomas et al. (2007) demonstrated that hypoxia downregulates serotonin production, and hypothesized that the stimulatory serotonergic neuroendocrine pathway is a major site of hypoxia-induced reproductive inhibition in fish. Hypoxia has also been associated with downregulation of various steroidogenic genes, especially those in the cytochrome P450 group (e.g., *cyp11a*, *cyp19a*; Fradette and Du Souich (2004), Shang et al. (2006)). For example, the reduced abundance and activity of *cyp19a* and *cyp19b* (which convert testosterone to estradiol) during sexual differentiation in zebrafish exposed to hypoxia has been suggested as a mechanism by which hypoxia skews sex ratio in favor of males (Shang et al., 2006). Changes in expression of genes coding for steroidogenic enzymes have been observed upon exposure to variety of endocrine disrupting chemicals. Thus, it is important to better elucidate hypoxia-induced gene expression changes in fish gonads in order to discriminate biomarkers specifically indicative of exposure to endocrine disrupting chemicals from those associated with the effect of any type of stressor likely to adversely affect reproductive function.

The present study utilized commercially available oligonucleotide microarrays (with approximately 21,000 genes represented) to characterize in vivo responses of the zebrafish reproductive system to hypoxia, and to start characterizing mechanisms by which hypoxia might alter reproductive function in fish. The severity of hypoxia was varied by combining a range of oxygen concentrations (ranging from normoxia [7 mg dissolved O_2/L] to severe hypoxia [1 mg dissolved O_2/L]) with different durations of exposure (ranging from 4 to 14 d). Zebrafish were selected for this study because not only have they been the subject of many classic physiological studies of responses to reduced oxygen availability, but are relatively amenable to gene expression analyses because their genome has been sequenced.

2. Methods

2.1. Exposure

Reproductively mature zebrafish (*Danio rerio*; ab wild-type strain; 7 months old) were obtained from an on-site culture unit. Mixed groups of four males and four females were randomly loaded into 10 L tanks, receiving a continuous flow of Lake Superior water (1 μm filtered; 45 mL/min flow rate; 6.5 mg/L dissolved O_2/L). The hypoxic water treatments were prepared with the aid of an oxygen stripping column. Lake Superior water and nitrogen were continuously flowing in a counter-current manner, through a stripping column made of PVC pipe filled with PVC diffusion spheres to help disperse nitrogen into water, thereby depleting oxygen. The oxygen-depleted water from the stripping column was introduced at the top of a tilted glass re-aerator, which was partitioned so the water cascaded from top to bottom. As the water flowed down the re-aerator it successively regained oxygen. A peristaltic pump fitted with Teflon® tubing was used to continuously collect water from the re-aerator partitions containing the desired oxygen concentration and deliver it to the exposure tanks. The oxygen concentrations in exposure tanks containing fish were gradually lowered over a period of 4 h. The control (normoxic) tanks also received water from the re-aerator to ensure that the oxygen stripping process would not confound the results. Zebrafish were exposed to hypoxia for durations of 4 and 14 d. There were three oxygen treatments: normoxia (7 mg dissolved O_2/L), moderate hypoxia (3 mg dissolved O_2/L) and, severe hypoxia (1 mg dissolved O_2/L). Oxygen concentrations were measured daily using a YSI 55 oxygen meter (Yellow Springs, OH, USA). The measured concentrations of dissolved oxygen in the test system ranged from 75% to 118% of the target concentrations. The mean measured concentrations ± S.D. in the 4 d tanks were 5.76 ± 0.306 for the normoxia treatment, 2.232 ± 0.119 for the moderate hypoxia treatment and 1.18 ± 0.092 mg O_2/L water for the severe hypoxia treatment. The measured concentrations in the 14 d exposure tanks were: 6.17 ± 0.324 for the normoxia treatment, 2.711 ± 0.573 for the moderate hypoxia treatment and 1.16 ± 0.21 mg O_2/L water for the severe hypoxia treatment. Temperature was maintained at 25 °C, and fish were fed frozen brine shrimp daily. Each exposure tank contained four males and four females, and each time and oxygen content treatment combination was replicated twice (i.e., fish were held in two separate exposure tanks). The 4 and 14 d exposures were conducted concurrently. A subset of five biological replicates per sex from each of the three oxygen treatment groups was used for subsequent microarray analyses.

2.2. Tissue collection

Once the exposures were completed the zebrafish were euthanized in a buffered solution of tricaine methanesulfonate (MS 222; Finquel, Argent, Redmond, WA, USA). For females, the presence or absence of ovulated eggs was noted and the differences in the numbers of ovulating females between treatments was examined using Fisher's exact test (at $p < 0.05$; $n = 5–8$). Gonads

were removed using dissection tools washed with RNAseZap® (Ambion, Austin, TX, USA) between each sample, and immediately transferred to a pre-weighed microcentrifuge tube and snap-frozen in liquid nitrogen. Snap-frozen tissue samples were stored at −80 °C until analyzed. All laboratory procedures involving animals were reviewed and approved by the U.S. EPA Animal Care and Use Committee in accordance with Animal Welfare Act regulations and Interagency Research Animal Committee guidelines.

2.3. RNA isolation and microarray analyses

Gonads were thawed, homogenized, and extracted with TRI Reagent® (Sigma–Aldrich, St. Louis, MO, USA) following the manufacturer's protocol. Total RNA was quantified using a nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Integrity and purity of RNA samples was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and an RNA Pico Chip Kit (Agilent). A subset of gonad RNA was kept on site for real-time polymerase chain reaction (PCR) analyses focused on specific genes. Other aliquots of gonadal RNA from male and female fish exposed to hypoxia for 4 and 14 d (five biological replicates per sex from each of the three oxygen treatment groups) were submitted to an Agilent certified facility, MOgene, LC (St. Louis, MO, USA), for gene expression profiling.

We used a reference design for the microarray analyses, where RNA from each treatment was labeled with a Cy5 dye and hybridized onto arrays, together with Cy3 labeled RNA taken from a reference pool of RNA. The reference RNA was extracted from sexually mature male and female gonads and brains, and female livers. For the microarray analyses, total RNA was amplified using an Agilent low input linear amplification kit according to the process outlined by the manufacturer (Agilent Technologies). Amplified target cRNA (1–5 μg) was labeled with either cyanine-5 or cyanine-3 using ULS aRNA Flurorescent Labeling Kits from Kreatech according to the manufacturers protocol (Kreatech Biotechnology, Amsterdam, Netherlands). Concentrations of labeled cRNA and the label incorporation were determined using ND-1000 spectrophotometer. All of the labeling and post labeling procedures were conducted in ozone-free enclosure to ensure the integrity of the label. Labeled materials were set up for the fragmentation reaction according to Agilent protocol described in their processing manual, and hybridized overnight in the rotating oven at 65 °C in an ozone-free room. The Agilent Oligo Microarray Zebrafish slide, with approximately 21,000 genes represented, was used for the gene expression analyses (4 × 44 K format, product 015064). Labeled samples (825 ng) were co-hybridized using sureHyb chambers from Agilent. Wash conditions used were as outlined in the Agilent processing manual (protocol version 4.0) and the arrays were scanned using Agilent scanner (G2505B). Agilent feature extraction software was used for extracting array data; the initial data quality analyses were done using Rosetta Luminator software (Rosetta Biosoftware, Seattle, WA, USA).

2.4. Differentially expressed gene lists and data analyses

Output from Agilent's feature extraction software was imported using GeneSpring GX 7.3.1 software (Agilent Technologies). The enhanced Agilent FE Import function was used to flag low quality spots based on feature saturation, uniformity, pixel population consistency, and signal-to-noise ratio using procedures described in the Agilent Technologies Training Manual (version 4.5, May 2006). In order to group together samples that had similar expression profiles and determine whether replicate samples within a condition were more similar to each other than samples from a different condition, we generated a condition tree of all genes for all treatment combinations (similarity measure: Pearson correlation; Clustering algorithm: average linkage).

To generate lists of differentially expressed genes, each sex and duration of exposure combination was designated as a separate experiment in GeneSpring (resulting in the following "experiments": "4 d ovary", "4 d testis", "14 d ovary", "14 d testis"). The ratios of treatment and reference raw intensity data then were log transformed and normalized using locally weighted least squares regression (lowess) normalization, available in GeneSpring GX 7.3.1. To calculate more precise variance estimates we used the cross-gene error model available in GeneSpring. Differentially expressed genes for each "experiment" were determined using ANOVA ($p < 0.05$). No multiple comparison correction methods were used. The increased stringency of the multiple comparison correction methods in the presence of high noise and limited replicates has been shown to lead to committing Type II 'false negative' errors, thus overlooking many true differences in gene expression (Cole et al., 2003). Because the variability in gene expression in the gonad tissues of zebrafish is naturally very high due to the nature of the reproductive process (zebrafish are fractional, asynchronous spawners), and because the sample sizes used for this experiment were fairly small, the available multiple comparison correction methods tend to be too restrictive. For this reason, instead of applying multiple correction methods and thus constraining the analyses to a small number of genes that had very small variance and/or large changes in expression, we further refined the list of the differentially expressed genes by identifying biological processes (and genes within) that were altered by hypoxia. To accomplish this, representation of differentially expressed genes within defined gene ontology (GO) categories was compared to the overall representation of genes within that GO category on the microarray to determine whether hypoxia "enriched" their occurrence, relative to that which might be expected due to chance alone. The genes belonging to enriched GO categories were then used to generate hypotheses about potential modes of action by which hypoxia affects reproduction. Determination of statistically significant enrichment was calculated using GeneSpring GX 7.3.1 software ($p < 0.01$; Agilent Technologies). Enriched GO terms in biological process GO category were used to focus discussion of biological processes potentially impacted by hypoxia. Enriched GO categories associated with each time/oxygen concentration condition were examined primarily to investigate whether there were notable time- and concentration-related differences in the profiles of differentially expressed genes within each sex. To determine the extent of the effects of hypoxia on the reproductive endocrine system of zebrafish, the list of differentially expressed genes from the present study was compared to a list of 83 genes with known reproductive roles in the teleost hypothalamo–pituitary–gonadal (HPG) axis (Villeneuve et al., 2007). To generate a list of overlapping genes, the published annotation for the zebrafish oligonucleotide microarrays (G2518A, Agilent Technologies) was queried using gene and/or protein names, symbols, synonyms, and probe identifiers. For each of the four time/sex/oxygen concentration conditions examined, the number of differentially expressed genes overlapping with the literature-derived list was determined.

2.5. Real-time PCR

Six genes were selected as targets for follow-up investigation using quantitative real-time PCR: hypoxia-inducible factor 1a (*hif1a*), lactate dehydrogenase a4 (*ldha*), aldolase c (*aldoc*), transforming growth factor beta 2 (*tgfb2*), cytochrome P450, subfamily XIA, polypeptide 1 (*cyp11a1*) and hydroxy-delta-5-steroid dehydrogenase, 3 beta (*hsd3b*). These genes were selected based on either a

known role in response to hypoxia or in the regulation of the HPG-axis. The total RNA samples used for PCR analyses were aliquots of the samples used for microarray analyses. Subsamples (100 ng) of total RNA were converted to complementary DNA (cDNA) using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). The kit uses random primers and the reaction volume for all reverse transcription (RT) reactions was 50 μL. Thermocycler conditions for the RT reactions were as follows: 25 °C 10 min, 37 °C 120 min, 95 °C 5 min, 4 °C hold. Complementary DNA samples were stored at −80 °C until analyzed using real-time PCR.

Real-time PCR analyses for *ldha*, *aldoc* and *tgfb2* were conducted using Power SYBR® Green PCR Master Mix (Applied Biosystems). Template cDNA (2.5 μL; equivalent to 5 ng input RNA) was combined with 200 nM forward and reverse primers (see Supplementary Table S5) and 2X Master Mix in a 25 μL reaction. Amplification and quantification was conducted using a 7500 Real-Time PCR System (Applied Biosystems) using the following protocol: 95 °C 10 min, followed by 40 cycles of a) melt 95 °C 15 s, b) anneal and extend 60 °C 60 s. Following amplification, product specificity was verified by generating dissociation curves for all samples (dissociation; 95–60 °C; Applied Biosystems). Duplicate no template controls were analyzed along with each set of samples to confirm that the signals measured were not due to primer dimers.

Relative transcript abundance was estimated based on a standard curve generated by analyzing multiple dilutions of a gene-specific DNA amplicon, without correction for amplification efficiency. Amplicons used as standards were amplified from zebrafish cDNA (pooled ovary, testis, brain, and liver cDNA from control zebrafish) using the same gene-specific primers used for real-time PCR (Supplementary Table S5). Specificity of each amplicon (and the primer pair used to generate it) was verified using agarose gel electrophoresis. Dilution of the amplicon used as a standard was optimized to yield a standard curve over threshold cycle (Ct) numbers ranging from approximately 15 to 35, as most experimental samples fall within that range.

Abundance of *hif1a*, *hsd3b*, and *cyp11a1* mRNA was measured by quantitative real time polymerase chain reaction (PCR). Total RNA samples were diluted to 10 ng/μL for use in PCR assays. Zebrafish *hif1a*, *hsd3b*, *cyp11a1* cDNAs were amplified using a series of gene-specific primers (Supplementary Table S5), designed with the aid of PrimerExpress (Applied Biosystems) and synthesized by Integrated DNA Technologies (Coralville, IA, USA). Jumpstart Taq polymerase (Sigma D9307) was used for all PCR amplifications. The PCRs were conducted using the following protocol: initial melt was performed at 95 °C 6 min; then for 10 cycles, melt at 95 °C for 1 min, anneal at 55–45 °C for 1 min (anneal temperature decreased by 1 °C per cycle), extend at 72 °C 1 for min; and finally 30 cycles of melt at 95 °C for 1 min, anneal at 45 °C for 1 min, extend at 72 °C for 1 min. The PCR products were re-amplified using primers CYP11A T7-FW, CYP11A-W-RV, hif1aT7-FW, hif1a-W-RV and 3bhsdT7-FW, 3bhsd-W-RV respectively (Supplementary Table S5), resulting in products with a phage T7 RNA polymerase promoter. Gene-specific mRNA standards were prepared from T7 products using ultra-high yield in vitro transcription (MEGAscript®, Ambion, Austin, TX, USA). Size and purity of the mRNA standards were confirmed using an Agilent 2100 Bioanalyzer (with RNA 6000 NanoAssay Kit and RNA 6000 Nanochips). Real-time QPCR assays were performed in duplicate using a Taqman® EZ RT-PCR kit (Applied Biosystems), according to the manufacturer's recommended protocol. Each 25 μL reaction contained 50 ng total RNA, 150 nM of the appropriate probe, 200 nM forward primer, and 200 nM reverse primer. Samples were reverse-transcribed (50 °C for 2 min, 60 °C for 30 min, 95 °C for 5 min), followed immediately by 40 cycles of PCR amplification (melt at 94 °C for 20 s, anneal and extend at 58 °C for 60 s), using a 7500 Real Time PCR System (Applied Biosystems). A standard curve of known molar quantities of the appropriate gene-specific mRNA (10-fold dilution series, 50–5 × 10^7 copies) was used to calibrate the PCR data, and values interpolated from the curve were converted to copies of mRNA/ng total RNA for each sample.

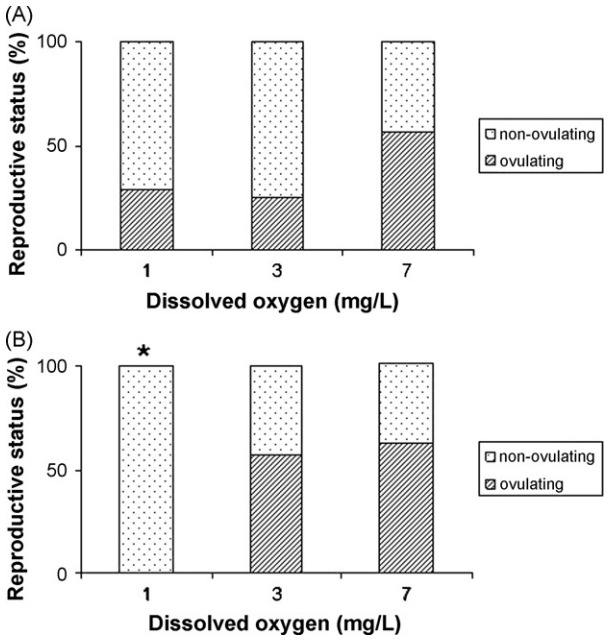

Fig. 1. Reproductive status of females after 4 days (A) and 14 days (B) of exposure to hypoxia. Prolonged exposure to severe hypoxia reduced percent of ovulating females (Fisher's exact test; control females compared with females from each hypoxia treatment; $n = 5–8$, $^{*}p < 0.05$).

3. Results

Some degree of hypoxia-related mortality was observed in the severe hypoxia treatment at all time-points tested (4 d – two males and one female, 14 d – one male and two female mortalities). Still, surviving animals in the severe hypoxia treatment did not seem stressed from a behavioral perspective (e.g., feeding and swimming appeared normal). The reproductive status of females (measured as percent of the ovulating females) exposed to hypoxia for 4 d was not affected significantly (Fig. 1), but there was a trend toward reduced ovulation rates (approximately 60% of control fish were ovulating vs. 25% of fish from hypoxic treatments). Prolonged (14 d) exposure to hypoxia reduced percent of ovulating females, underlined by the complete absence of ovulating females in the severe hypoxia treatment (1 mg O_2/L).

A condition tree (similarity measure: Pearson correlation; clustering algorithm: average linkage) of all genes for all treatment combinations shows that the males and females clustered sepa-

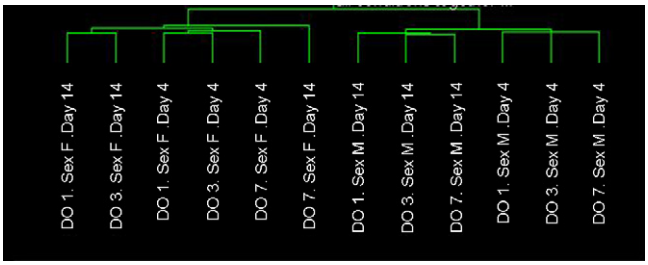

Fig. 2. Condition tree of all genes by time/tissue/sex combination (Similarity measure: Pearson correlation; Clustering algorithm: average linkage).

Table 1
Testis–gene ontology (GO) categories significantly enriched (Fisher's test, $p < 0.01$) upon exposure to hypoxia for 4 days.

Type of comparison	Gene ontology category description	Genes in category	Genes in list in category	p value
Set 1: overall effect of hypoxia (ANOVA) – based on 1520 differentially expressed probes				
	Response to hypoxia	22	8	0.000976
	Transition metal ion homeostasis	125	22	0.00735
	Iron ion homeostasis	125	22	0.00735
	Iron ion transport	123	22	0.00605
	Monosaccharide catabolism	79	17	0.00214
	Main pathways of carbohydrate metabolism	106	20	0.00469
	Macromolecule metabolism	2520	294	0.00251
	Hexose catabolism	79	17	0.00214
	Glucose catabolism	79	17	0.00214
	Glucose metabolism	91	17	0.00965
	Glycolysis	65	17	0.000196
	Carbohydrate catabolism	92	18	0.0048
	Anaerobic glycolysis	8	4	0.00535
	Tricarboxylic acid cycle intermediate metabolism	20	8	0.000463
	RNA metabolism	371	60	0.000177
	mRNA metabolism	232	36	0.0066
	mRNA processing	212	36	0.00142
	RNA processing	303	53	0.0000535
	Nuclear mRNA splicing, via spliceosome	176	32	0.000798
	RNA splicing	180	32	0.00118
	RNA splicing, via transesterification reactions	176	32	0.000798
	RNA splicing, via transesterif. with bulged adenosine as nucleophile	176	32	0.000798
	DNA metabolism	309	46	0.00522
	DNA replication	71	19	0.0000598
	Alcohol catabolism	84	17	0.00422
	Glutamine family amino acid metabolism	17	6	0.00507
	Positive chemotaxis	5	3	0.009
	Regulation of positive chemotaxis	5	3	0.009
	Positive regulation of positive chemotaxis	5	3	0.009
	Induction of positive chemotaxis	5	3	0.009
Set 2: normoxia vs. severe hypoxia (Tukey's test) – based on 856 differentially expressed probes				
	Cellular physiological process	6110	385	0.00975
	Regulation of erythrocyte differentiation	2	2	0.00345
	Cell homeostasis	193	22	0.00213
	DNA replication	71	12	0.000801
	Response to metal ion	21	5	0.0064
	Response to cadmium ion	17		0.00237
	Response to organic substance	15	4	0.0096
	Response to hypoxia	22	7	0.000184
	Homeostasis	201	22	0.00354
	Cell homeostasis	193	22	0.00213
	Cell ion homeostasis	143	17	0.00427
	Ion homeostasis	145	17	0.00492
	Cell ion homeostasis	143	17	0.00427
	Cation homeostasis	138	17	0.00295
	Di-, tri-valent inorganic cation homeostasis	134	17	0.00215
	Iron ion homeostasis	125	17	0.000994
	Metal ion homeostasis	135	17	0.00233
	Transition metal ion homeostasis	125	17	0.000994
	Iron ion homeostasis	125	17	0.000994
	Iron ion transport	123	17	0.000827
	Metabolism	4652	301	0.00908
	Cellular metabolism	4297	286	0.00197
	Catabolism	299	28	0.00973
	Carbohydrate catabolism	92	14	0.0009
	Alcohol catabolism	84	13	0.00116
	Monosaccharide catabolism	79	13	0.00064
	Energy derivation by oxidation of organic compounds	123	15	0.00557
	Main pathways of carbohydrate metabolism	106	15	0.00129
	Macromolecule metabolism	2520	183	0.00043
	Monosaccharide metabolism	109	14	0.00459
	Monosaccharide catabolism	79	13	0.00064
	Hexose catabolism	79	13	0.00064
	Hexose metabolism	108	14	0.00422
	Hexose catabolism	79	13	0.00064
	Glucose catabolism	79	13	0.00064
	Glucose metabolism	91	13	0.00244
	Glucose catabolism	79	13	0.00064
	Glycolysis	65	13	0.0000848
	Carbohydrate catabolism	92	14	0.0009
	Monosaccharide catabolism	79	13	0.00064
	main pathways of carbohydrate metabolism	106	15	0.00129

Table 1
(*Continued*).

Type of comparison	Gene ontology category description	Genes in category	Genes in list in category	*p* value
	Glycolysis	65	13	0.0000848
	Isocitrate metabolism	2	2	0.00345
	Nitrogen metabolism	216	22	0.00825
	RNA metabolism	371	38	0.000552
	mRNA metabolism	232	25	0.0024
	mRNA processing	212	25	0.000668
	RNA processing	303	33	0.000432
	mRNA processing	212	25	0.000668
	Nuclear mRNA splicing, via spliceosome	176	22	0.000631
	RNA splicing	180	22	0.000856
	RNA splicing, via transesterification reactions	176	22	0.000631
	RNA splicing, via transesterit. with bulged adenosine as nucleophile	176	22	0.000631
	Nuclear mRNA splicing, via spliceosome	176	22	0.000631
	DNA replication	71	12	0.000801
	DNA-dependent DNA replication	29	6	0.00599
	pyrimidine deoxyribonucleotide metabolism	2	2	0.00345
	Pyrimidine deoxyribonucleotide metabolism	2	2	0.00345
	Monosaccharide metabolism	109	14	0.00459
	Alcohol catabolism	84	13	0.00116
	Organic acid metabolism	234	25	0.0027
	Carboxylic acid metabolism	234	25	0.0027
	Isocitrate metabolism	2	2	0.00345
	Amino acid metabolism	155	19	0.00182
	Amino acid biosynthesis	33	7	0.00262
	Glutamine family amino acid biosynthesis	12	4	0.00401
	Glutamine family amino acid metabolism	17	6	0.000285
	Glutamine family amino acid biosynthesis	12	4	0.00401
	Proline metabolism	3	2	0.00995
	Proline biosynthesis	2	2	0.00345
	Amine biosynthesis	44	8	0.00368
	Amino acid and derivative metabolism	182	20	0.00505
	Amino acid metabolism	155	19	0.00182
	Regulation of erythrocyte differentiation	2	2	0.00345
	Negative regulation of erythrocyte differentiation	2	2	0.00345
	Response to methylmercury	12	4	0.00401
	Nitrogen compound biosynthesis	44	8	0.00368
Set 3: normoxia vs. moderate hypoxia (Tukey's test) – based on 318 differentially expressed probes				
	Gametogenesis	99	6	0.0093
	Negative regulation of programmed cell death	62	5	0.0053
	Negative regulation of apoptosis	59	5	0.00428
	Anti-apoptosis	33		0.000297
Set 4: altered in both severe and moderate hypoxia – based on 99 differentially expressed probes				
	Post-embryonic hemopoiesis	1	1	0.00219
	Organic acid transport	24	2	0.00436
	Carboxylic acid transport	24	2	0.00981
	Macromolecule biosynthesis	512	9	0.00629
	Protein biosynthesis	462	9	0.00629
	dsRN A fragmentation	1	1	0.00629
	miRNA-mediated gene silencing, production of miRNAs	1	1	0.00629
	Pre-microRNA processing	1	1	0.00629
	miRNA-mediated gene silencing	1	1	0.00629

rately (Fig. 2). For males, the clustering occurred first according to the duration of exposure, and then according to the severity of hypoxia (with hypoxic treatments clustering together (Fig. 1)). In contrast, the females exposed to normoxia for 14 d clustered away from all other treatments. The females exposed to moderate and severe hypoxia for 14 d clustered together. All 4 d females clustered together, with hypoxic treatments clustering together within the 4 d group.

The 4 d hypoxia treatment (compared to normoxia) resulted in differential expression of 1520 gene in the testis (Supplemental Table S1) and 480 in ovary (Supplemental Table S3). After 14 d of treatment, 524 probes in the testis (Supplemental Table S2) and 1627 probes in the ovary (Supplemental Table S4) were differentially expressed in animals held under hypoxic conditions compared with those held in normoxia treatments. The post hoc comparisons with the normoxic treatment (within each time/sex combination) showed that the number of genes changed by exposure to severe hypoxia consistently exceeded the number of genes altered by the moderate hypoxia (Tables 1–4). For all treatments there was a considerable overlap between genes altered by both severe and moderate hypoxia. The number of common genes was smaller in 4 d exposed fish (11–31% probes overlapped) than in 14 d exposed fish (with 18–66% of altered probes overlapping between moderate and severe hypoxia). The changes in gene expression were most prominent in the 14 d ovary; this also was the tissue where the overlap between genes altered by severe and moderate hypoxia was the highest.

GO enrichment analyses were conducted to examine which biological processes were altered by severe vs. moderate hypoxia (compared to normoxia), and to determine alterations common to both severe and moderate hypoxia. The significantly enriched GO biological process categories for each sex/time combination are presented in Tables 1–4 (Table 1: 4 d testis; Table 2: 14 d testis; Table 3: 4 d ovary; Table 4: 14 d ovary). Each table contains the

Table 2
Testis–gene ontology (GO) categories significantly enriched (Fisher's test, $p < 0.01$) upon exposure to hypoxia for 14 days.

Type of comparison	Gene ontology category description	Genes in category	Genes in list in category	*p* value
Set 1: overall effect of hypoxia (ANOVA) – based on 524 differentially expressed probes				
	Exocrine system development	22	5	0.000535
	Pancreas development	19	6	0.000019
	Exocrine pancreas development	12	4	0.000408
	Hormone metabolism	28	6	0.000207
	Retinol metabolism	7	3	0.00102
	Cellular lipid metabolism	132	11	0.00322
	Steroid metabolism	27	5	0.00144
	Polyisoprenoid metabolism	6	3	0.000596
	Terpenoid metabolism	6	3	0.000596
	Terpene metabolism	6	3	0.000596
	Terpenoid metabolism	6	3	0.000596
	Apocarotenoid metabolism	5	3	0.000305
	Vitamin A metabolism	13	3	0.00722
	Retinol metabolism	7	3	0.00102
	Retinal metabolism	5	3	0.000305
	Biosynthesis	880	46	0.000497
	Cellular biosynthesis	814	43	0.000622
	Macromolecule biosynthesis	512	27	6.76E−03
	Carbohydrate biosynthesis	53	6	0.00648
	Translational termination	4	2	0.00582
	Protein myristoylation	4	2	0.00582
	Protein amino acid myristoylation	4	2	0.00582
	N-terminal protein myristoylation	4	2	0.00582
	Purine ribonucleoside monophosphate biosynthesis	13	3	7.22E−03
	Purine ribonucleoside monophosphate metabolism	13	3	0.00722
	Alkene metabolism	8	3	0.00159
	Digestive tract morphogenesis	10	3	0.00325
	Gut morphogenesis	10	3	0.00325
	Gut development	11	3	0.00325
		5	3	0.009
Set 2: normoxia vs. severe hypoxia (Tukey's test) – based on 322 differentially expressed probes				
	Steroid metabolism	27	4	0.0014
	Biosynthesis	880	29	0.0014
	Cellular biosynthesis	814	26	0.00387
	Lipid biosynthesis	63	5	0.00596
	Protein myristoylation	4	2	0.00198
	Protein amino acid myristoylation	4	2	0.00198
	N-terminal protein myristoylation	4	2	0.00198
	Cellular lipid metabolism	132	7	0.011
	Larval or pupal development (sensu Insecta)	23	3	0.00831
	Secretion	105	7	0.0032
	Secretory pathway	98	7	0.00217
	Vesicle-mediated transport	162	8	0.0101
	ER to Golgi transport	26	3	0.0117
	Cobalt ion homeostasis	43	5	0.0011
	mRNA splice site selection	7	2	0.00667
Set 3: normoxia vs. moderate hypoxia (Tukey's test) – based on 154 differentially expressed probes				
	Exocrine system development	22	4	0.0000926
	Pancreas development	19	6	4.09E−08
	Exocrine pancreas development	12	4	6.83E−06
	Alkene metabolism	8	3	0.0000731
	Cellular biosynthesis	814	17	0.00857
	Cellular lipid metabolism	132	6	0.00357
	Hormone metabolism	28	5	0.0000128
	Retinol metabolism	7	3	0.0000461
	Steroid metabolism	27	3	0.00327
	Isoprenoid metabolism	15	3	0.000561
	Polyisoprenoid metabolism	6	3	0.0000266
	Terpenoid metabolism	6	3	0.0000266
	Secondary metabolism	78	5	0.00176
	Terpene metabolism	6	3	0.0000266
	Terpenoid metabolism	6	3	0.0000266
	Apocarotenoid metabolism	5	3	0.0000134
	Vitamin metabolism	38	3	0.00864
	Fat-soluble vitamin metabolism	15	3	0.000561
	Vitamin A metabolism	13	3	0.000359
	Retinol metabolism	7	3	0.0000461
	Retinal metabolism	5	3	1.34E−05
	Skeletal development	91	6	0.000524
	Limb morphogenesis	35	3	0.00687
	Forelimb morphogenesis	28	3	0.00364
	Pectoral fin morphogenesis	28	3	3.64E−03

Table 2
(*Continued*).

Type of comparison	Gene ontology category description	Genes in category	Genes in list in category	*p* value
	Digestive tract morphogenesis	10	3	0.000154
	Gut morphogenesis	10	3	0.000154
	Gut development	11	3	0.00021
Set 4: altered in both severe and moderate hypoxia – based on 58 differentially expressed probes				
	Electron transport	240	5	0.00412
	Porphyrin metabolism	77	3	0.00484
	Porphyrin biosynthesis	76	3	0.00467
	Steroid metabolism	27	2	0.00639
	Secretory pathway	98	3	0.00944
	Cofactor metabolism	221	5	0.00289
	Cofactor biosynthesis	173	4	0.00723

results of GO analyses performed for the following sets of differentially expressed genes: set 1 – all genes altered by hypoxia (gene list derived from the results of ANOVA where each tissue/time combination was analyzed separately and hypoxia was designated as the independent variable); Set 2 – genes altered by the severe hypoxia treatment (gene list derived from the results of Tukey's post hoc test comparing normoxia and severe hypoxia); Set 3 – genes altered by the moderate hypoxia treatment (gene list derived from the results of Tukey's post hoc test comparing normoxia and moderate hypoxia); Set 4 – genes that were altered by both moderate and severe hypoxia (gene list representing intersection of Venn diagrams of genes from Sets 2 and 3).

Comparison of differentially expressed genes from the present study and a list of 83 genes with known reproductive roles in the teleost HPG axis shows that a 4 d exposure to hypoxia exerted comparable effects in males and females (Table 5). In contrast, the 14 d exposure altered only one gene in males, whereas in the females expression of 11 of the 83 genes was affected (Table 5). Overall, the observed changes were indicative of downregulation of the HPG axis (76% of the genes were downregulated). Only a minority of HPG axis related genes were upregulated by hypoxia: *vg* in 4 d testis, *cyp17a1* in 14 d testis and activin receptor IIb (*acvr2b*), sult2st3 and 17β-HSD in 14 d ovary.

For the majority of genes the PCR data were consistent with the microarray data. The expression of *hif1a* was not significantly affected by hypoxia as determined by either PCR or microarray (Supplemental Fig. S1). The increased expression of *ldha* in male testis upon exposure to 4 d severe hypoxia was observed in males using both microarray and PCR analyses (Supplemental Fig. S2). The only significant effects of hypoxia on *aldoc* were observed in microarray data upon exposure to severe hypoxia for 4 d. The *aldo*c trend was the same for PCR data, but the difference was not statistically significant ($p = 0.09$) (Supplemental Fig. S3). For *tgf2b* the array and RT-PCR data matched well for females; no significant effects, but concentration-dependent increases were observed using both methods (Supplemental Fig. S4). The expression of *tgf2b* in males could not be adequately compared as most of the samples in microarray analyses did not pass the basic quality tests, so were flagged as absent and not included in the analyses. Similarly, *cyp11a1* could not be compared across two methods because it was flagged as absent in majority of the samples. The microarray analyses indicated a significant increase in *hsd3b* upon exposure to hypoxia, but the expression of *hsd3b* in females was not affected as measured by PCR (Supplemental Fig. S5). Nevertheless, the trends for both data sets were similar. In males 14 d exposure to hypoxia had no effects on *hsd3b* expression (as measured by either PCR or microarray). The trends for *hsd3b* expression upon 4 d exposure to hypoxia were similar, but the significant differences were detectable only with PCR (Supplemental Fig. S5).

4. Discussion

4.1. Effects of hypoxia on gene expression in zebrafish testes

4.1.1. 4 d testis

Examination of GO enriched categories shows that a variety of biological processes known to be involved in responses to hypoxia were altered in testes of zebrafish after 4 days. Indeed, one of the enriched GO categories was the "response to hypoxia". The onset of hypoxia normally leads to stabilization and accumulation of HIF1a, which receives signals from oxygen sensors and is considered to be an organizer of the response to hypoxia (Wenger et al., 2005). The majority of the genes responsible for enrichment of the "response to hypoxia" GO category in 4 d testis are known targets for HIF1a (e.g., phosphoglycerate kinase 1, lactate dehydrogenase A4, insulin-like growth factor binding protein 1; Table S1) (Wenger et al., 2005). All of the genes in this GO category were upregulated, which is consistent with coping with hypoxia via mechanisms such as such as upregulation of anaerobic metabolism, enhanced oxygen transport/storage (myoglobin) and suppression of growth, by reducing the levels of circulating growth factor (insulin-like growth factor binding protein 1). The main pathways of carbohydrate metabolism also were altered by hypoxia after 4 d. For example, approximately 20% of genes involved in glucose metabolism were affected. The genes altered reflected an enhancement of glycolysis (e.g., upregulation of enolase, which enhances glycolysis and glucose uptake) and a switch to anaerobic metabolism of carbohydrates to compensate for the lower levels of ATP production (Wu, 2002). The enrichment of GO processes related to amino acid metabolism (e.g., reduction of glutamine synthase and other genes related to amino acid metabolism) is indicative of suppression of protein synthesis, a well documented response to hypoxia, deployed to conserve energy expenditure (Nikinmaa, 2002; Nikinmaa and Rees, 2005).

Another GO category that was affected in the 4 d testis was iron homeostasis and transport. This category included a diverse group of differentially expressed genes: ferritin, globins (myoglobin and cytoglobin), cytochromes (CYPs) (P450s and CYP b5 (b5)), and nitric oxide (NO) synthase. Ferritin, which stores the iron in a soluble nontoxic form, was increased in response to hypoxia, probably in an attempt to increase iron supply in the gonad. The increases in myoglobin are used to enhance oxygen extraction and intracellular diffusion (Wittenberg (1992) as cited by Fordel et al. (2004)) and have also been shown to be involved in detoxification of NO which is increased by hypoxia (Fordel et al., 2004). Related to this we observed increased expression of NO synthase, which is thought to increase NO production, thereby inhibiting cellular respiration, and thus decreasing metabolism (Koivisto et al., 1999).

The cytoglobins are a novel group of globins (Burmester et al., 2000) and, while their function is not fully understood, there is

Table 3
Ovary gene ontology (GO) categories significantly enriched (Fisher's Test, $p < 0.01$) upon exposure to hypoxia for 4 days.

Type of comparison	Gene ontology category description	Genes in category	Genes in list in category	*p* value
Set 1: overall effect of hypoxia (ANOVA) – based on 480 differentially expressed probes				
	Adult behavior	5	2	0.00712
	Reproductive behavior	6	2	0.0105
	Oviposition	5	2	0.00712
	Protein kinase C activation	5	2	0.00712
	Reproduction	127	10	0.00257
	Male gamete generation	48	6	0.0019
	Spermatogenesis	48	6	0.0019
	Spermatid development	22	4	0.00276
	Telomere maintenance	4	2	0.00435
	Nucleotide-excision repair	10	3	0.00213
	Protein secretion	15	3	0.00731
	Hydrogen transport	70	7	0.00299
	Proton transport	69	7	0.00276
	Intracellular transport	340	22	0.000166
	Mitochondrial transport	42	5	0.00559
	Proton transport	69	7	0.00276
	Cofactor biosynthesis	173	11	0.00816
	Coenzyme biosynthesis	97	8	0.00513
	Group transfer coenzyme metabolism	70	8	0.000632
	Coenzyme biosynthesis	97	8	0.00513
	Modification-dependent protein catabolism	64	6	0.00805
	Ubiquitin-dependent protein catabolism	64	6	0.00805
	mRNA catabolism	6	2	0.0105
	Formaldehyde metabolism	3	3	2.05E−05
	Octanol metabolism	3	3	2.05E−05
	Phosphorylation	386	21	0.00218
	Aldehyde metabolism	10	3	0.00213
	Formaldehyde metabolism	3	3	2.05E−05
	Cofactor biosynthesis	173	11	0.00816
	Cellular localization	346	23	7.81 E−05
	Establishment of cellular localization	346	23	7.81 E−05
	Spermatid differentiation	23	4	0.00327
Set 2: normoxia vs. severe hypoxia (Tukey's test) – based on 249 differentially expressed probes				
	Adult behavior	5	2	0.00188
	Spermatid differentiation	23	4	0.000261
	Spermatid development	22	4	0.000218
	Reproduction	127	8	0.000388
	Sexual reproduction	109	6	0.00413
	Gametogenesis	99	6	0.00256
	Male gamete generation	48	5	0.000519
	Spermatogenesis	48	5	0.000519
	Cell organization and biogenesis	764	19	0.00949
	Telomere maintenance	4	2	0.00114
	Muscle contraction	44	4	0.00319
	Protein secretion	15	3	0.00107
	Mitochondrial transport	42	5	0.000276
	Protein targeting	86	5	0.00693
	Establishment of protein localization	304	10	0.00999
	Catabolism	299	10	0.00895
	Macromolecule catabolism	210	9	0.00265
	Cellular macromolecule catabolism	187	8	0.0046
	RNA catabolism	9	2	0.00653
	mRNA catabolism	6	2	0.0028
	Transcription from Pol II promoter	91	5	0.00876
	Deoxyuridine biosynthesis	340	16	1.93E−05
	Phosphorylation	386	13	0.00281
	Protein amino acid phosphorylation	307	11	0.00371
	Reproductive physiological process	24	3	0.00434
	Cellular localization	346	16	0.000024
	Establishment of cellular localization	346	16	0.000024
Set 3: normoxia vs. moderate hypoxia (Tukey's test) – based on 179 differentially expressed probes				
	Cofactor metabolism	221	7	0.00967
	Cofactor biosynthesis	173	7	0.00257
	Coenzyme biosynthesis	97	6	0.0006
	Coenzyme metabolism	138	6	0.00366
	Group transfer coenzyme metabolism	70	6	0.0001
	Folic acid and derivative metabolism	7	2	0.00232
	Phosphatidylserine metabolism	1	1	0.0108
	Phosphatidylserine biosynthesis	1	1	0.0108
	One-carbon compound metabolism	19	3	0.00103
	Formaldehyde metabolism	3	3	1.21E−06
	Octanol metabolism	3	3	1.21E−06

Table 3
(*Continued*).

Type of comparison	Gene ontology category description	Genes in category	Genes in list in category	*p* value
	Methionine biosynthesis	1	1	0.0108
	Aldehyde metabolism	10	3	0.000137
	Formaldehyde metabolism	3	3	1.21E−06
	Folic acid and derivative metabolism	7	2	0.00232
	Negative regulation of Wnt receptor signaling pathway	9	2	0.00393
Set 4: altered in both severe and moderate hypoxia – based on 48 differentially expressed probes				
	Response to drug	8	1	0.0245
	DNA catabolism	3	1	0.00924
	Glycerophospholipid metabolism	14	1	0.0424
	Glycerophospholipid biosynthesis	13	1	0.0395
	Phosphatidylserine biosynthesis	1	1	0.00309
	DNA catabolism	3	1	0.00924
	Octanol metabolism	3	1	0.00924
	Aldehyde metabolism	10	1	0.0305
	Formaldehyde metabolism	3	1	0.00924

evidence that their expression is regulated by HIF1a (Fordel et al., 2004). Their hypothesized functions include: controlling oxygen supply, detoxification of reactive oxygen species and NO, facilitation of glycolytic production of ATP under semi-anaerobic conditions, and an oxygen sensor role (Fordel et al., 2004). The increased expression of cytoglobin upon exposure to hypoxia observed in the present study suggests that these are feasible hypotheses. The increased expression of *cypb5* in response to hypoxia has been documented by others, an observation consistent with its role as a redox protein involved in many physiological processes altered by hypoxia (e.g., fatty acid desaturation, catalyse methemoglobin reduction). In addition, the b5 is a functional module of b5 + b5 reductase flavohemoprotein – a candidate oxygen sensor (Zhu et al., 1999). Interestingly, b5 also colocalizes with cytochrome P450, family 17, subfamily A, polypeptide 1 (*cyp17a1*) in Leydig cells of mammalian testes and is thought to enhance 17,20-lyase activity (leading to production of androstenedione and testosterone (Dharia et al., 2004).

Modulation of CYPs by hypoxia has been reported by others and has been well documented for *cyp1*, *cyp2*, and *cyp3* isoforms in mammals (Kurdi et al., 1999; Yang et al., 2001). Interestingly, while *cyp1* and *cyp2* expression are suppressed by hypoxia, *cyp3a6* isoform is upregulated (Fradette and Du Souich, 2004), suggesting that expression of CYPs is differentially regulated. In the present study we observed an increase in the following CYPs: *cypa1*, *cyp26b* and cyp19. The increases in cyp1a and *cyp26b* may represent initial compensatory responses to hypoxia, but it is difficult to understand why *cyp19a1b* (aromatase), which catalyzes con-

Table 4
Ovary–gene ontology (GO) biological process categories significantly enriched (Fisher's test, $p < 0.01$) upon exposure to hypoxia for 14 days.

Type of comparison	Gene ontology category description	Genes in category	Genes in list in category	*p* value
Set 1: overall effect of hypoxia (ANOVA) – based on 1627 differentially expressed probes				
	Asymmetric protein localization involved in cell fate commitment	4	3	0.00394
	Positive regulation of actin filament polymerization	2	2	0.0105
	Innate immune response	5	3	0.0091
	Response to metal ion	21	7	0.00368
	Response to cadmium ion	17	7	0.000886
	Lipid transport	33	10	0.00123
	Lipoprotein metabolism	34	12	7.93E−05
	Sterol metabolism	9	4	0.00899
	Cysteinyl-tRNA aminoacylation	5	4	0.0005
	Protein amino acid methylation	9	4	0.00899
	Pentose-phosphate shunt, non-oxidative branch	2	2	0.0105
Set 2: normoxia vs. severe hypoxia (Tukey's test) – based on 1176 differentially expressed probes				
	Lipoprotein metabolism	34	10	0.000105
	Cysteinyl-tRNA aminoacylation	5	4	0.000133
	Lipid transport	33	9	0.000437
	Sterol metabolism	9	4	0.00263
	Pentose-phosphate shunt, non-oxidative branch	2	2	0.00532
	Gene silencing	11	4	0.00613
	Cell wall catabolism	7	3	0.0108
Set 3: normoxia vs. moderate hypoxia (Tukey's test) – based on 949 differentially expressed probes				
	Innate immune response	5	3	0.00178
	Pentose-phosphate shunt, non-oxidative branch	2	2	0.00337
	Fatty acid metabolism	39	7	0.00649
	Coenzyme A metabolism	8	3	0.00875
	Coenzyme A biosynthesis	8	3	0.00875
Set 4: altered in both severe and moderate hypoxia – based on 634 differentially expressed probes				
	Oocyte construction (sensu Insecta)	12	3	0.00908
	Oocyte axis determination (sensu Insecta)	12	3	0.00908
	Sterol metabolism	9	3	0.00377
	Fatty acid metabolism	39	6	0.00314
	Pentose-phosphate shunt, non-oxidative branch	2	2	0.00142

Table 5
The list of differentially expressed genes (upon exposure to hypoxia) from the present study that overlap with genes with known reproductive roles in the teleost HPG axis (nd – not determined).

Treatment	Symbol	Direction of change		Gene description
		Severe hypoxia	Moderate hypoxia	
4 d testis	vg1	up	up	Danio rerio vitellogenin 1 (vg1), mRNA [NM_170767]
	fst	down	down	STR00480 26 somite embryos Danio rerio cDNA clone CB446 5′ similar to SW:FSA_BRARE Follistatin precursor, mRNA sequence [BU670730]
	ENSDART00000008548	down	down	ADX_HUMAN (P10109) Adrenodoxin, mitochondrial precursor (Adrenal ferredoxin) (Hepatoredoxin) (Ferredoxin 1), partial (61%) [TC280394]
	esr1	down	down	Danio rerio estrogen receptor 1 (esr1), mRNA [NM 152959]
14 d testis	cyp17a1	up	up	Danio rerio cytochrome P450, family 17, subfamily A, polypeptide 1 (cyp17a1), mRNA [NM 212806]
4d ovary	TC283367	down	nd	Q8TDE4 (Q8TDE4) PGC-1-related estrogen receptor alpha coactivator short isoform, partial (7%) [TC283367]
	ENSDART00000034851	nd	down	FSL3_MOUSE (Q9EQC7) Follistatin-related protein 3 precursor (Follistatin-like 3) (Follistatin-related gene protein), partial (35%) [TC285335]
	hsd3b1	up	up	Danio rerio hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (hsd3b1), mRNA [NM 212797]
	tph1	down	up	tryptophan hydroxylase 1 (tryptophan 5-monooxygenase)
14 d ovary	hsd11b2	down	down	Danio rerio hydroxysteroid 11-beta dehydrogenase 2 (hsd11b2), mRNA [NM 212720]
	acvrl1	down	down	Danio rerio activin A receptor type II-like 1 (acvrl1), mRNA [NM 153643]
	esr2b	down	down	Danio rerio estrogen receptor 2b (esr2b), mRNA [NM 174862]
	acvr2b	up	up	Danio rerio activin receptor IIb (acvr2b), mRNA [NM 131210]
	fshr	nd	down	Danio rerio follicle stimulating hormone receptor (fshr), mRNA [NM 001001812]
	bambi	down	down	Danio rerio BMP and activin membrane-bound inhibitor (Xenopus laevis) homolog (bambi), mRNA [NM 131784]
	star	down	down	Danio rerio steroidogenic acute regulatory protein (star), mRNA [NM 131663]
	ddc	down	down	dopa decarboxylase
	hsd17b	up	up	Danio rerio hydroxysteroid 17-beta dehydrogenase 4 (hsd11b4)
	cyp19a	down	down	Danio rerio aromatase cyp 19a

version of testosterone to estradiol, would be increased in the testis where estrogens are not thought to play large role in reproduction. Putative (aromatase-induced) increased rates of estrogen production would, however, be in an agreement with the observed increase in gene expression of the two types of estrogen-dependent lipoproteins (vitellogenins *vg1* and *vg3*), and vigilin (*hdlbp*), which stabilizes vitellogenin mRNA. Whether aromatase expression is the cause of the observed *vg* expression remains unclear. Hypoxia can disregulate the metabolism of high density lipoprotein binding proteins (such as vigilin) independently and thus the observed increases in *vg* mRNA could be a result of increased stabilization of *vg* mRNA. However, before suggesting that hypoxia feminizes males (i.e., causes an inappropriate production of VG), it is important first to examine whether the microarray results from the present study can be confirmed with other techniques, and determine whether the changes in mRNA expression in fact translate to significant protein level changes. The manual examination of the genes suggests that the phenomenon is consistent for several individuals – therefore it is unlikely to be a result of sex misidentification. Furthermore, we detected a reduction in estrogen receptor (*esr1*) expression by hypoxia, which provides further support that the observed results are likely not due to the sex misidentification (if the increases in *vg* and aromatase were stemming from the presence of female tissue, the *esr1* mRNA levels should also be higher in this group).

Two additional GO categories affected in the testis by hypoxia were DNA and RNA metabolism. Both of these were characterized by downregulation of dozens of genes and are indicative of a slow-down of general metabolic processing. Similar effects have been documented by others studying gene expression response to hypoxia in fish (Ju et al., 2007a; Ton et al., 2003).

Examination of the GO categories enriched in moderate and severe hypoxia treatments indicated that the majority of the processes discussed above were altered only by severe hypoxia, and in the case of moderate hypoxia the processes of gametogenesis and apoptosis were affected. The suppression of gametogenesis even after exposure to moderate hypoxia has been documented, and is not surprising given that reproduction is an energetically expensive process which is not critical to an organism's survival. An increased rate of apoptosis is another well documented response to hypoxia (Nikinmaa, 2002) and is under control of HIF-1 in the mammalian cells (Goda et al., 2003). In any case, results of the present study indicate that the short-term exposure to moderate hypoxia is not sufficient to bring about an extensive metabolic reorganization in testes to the degree seen upon exposure to severe hypoxia. Overall, the processes altered by both moderate and severe hypoxia were limited largely to those related to hemopoesis, protein biosynthesis, carboxylic acid transport, and RNA fragmentation.

4.1.2. 14 d testis

In addition to the changes in carbohydrate and protein metabolism similar to those observed in 4 d testis, the most prominent effects of prolonged (14 d) exposure to hypoxia was a substantial reorganization of the metabolism of cellular lipids and steroid hormones (Table 2). In the hormone metabolism category, tryptophan hydroxylase 1 like (*tph1l*), hydroxysteroid-17ß-dehydrogenase 3 (*hsd17b3*) and retinol dehydrogenase 1 like were downregulated. Inhibition of TPH1, a rate limiting enzyme involved in biosynthesis of serotonin, upon hypoxia has been demonstrated by Thomas et al. (2007) in the Atlantic croaker. They suggested that hypoxia downregulates the serotonergic neuroendocrine pathway (and thus reproductive hormones) by inhibiting serotonin synthesis. It is uncertain, however, whether *tph1l* plays a similar role as *tph1* and/or whether the fish gonad could be a site of local serotonergic neuroendocrine regulation. Hydroxysteroid-17ß-dehydrogenase 3 catalyzes conversion of androstenedione to testosterone, so its downregulation is likely to result in reduced testosterone concentrations. Testosterone serves as the precursor for production of 11-ketotestosterone, a male-specific androgen in

teleosts, so it is likely that suppression of testosterone would lead to suppression of 11-ketotestosterone (Borg, 1994). Decreased circulating plasma concentrations of testosterone and ketotestosterone in males exposed to hypoxia have been documented previously (Thomas et al., 2006).

Changes in retinol dehydrogenase expression were largely responsible for enrichment of multiple GO categories (terpene metabolism and all subcategories, including vitamin A metabolism; hormone metabolism). The enrichment of pancreas development and gut morphogenesis GO categories was driven by changes in expression of retinol dehydrogenase1 like and *sox4b* genes which have well defined roles in pancreas development. While the role of *sox4b* in the testis of sexually mature fish is unknown, the function of retinol in testes is well studied. Vitamin A is an important factor in spermatogenesis and both Sertoli and germinal cells have a specific requirement for retinol (Griswold et al., 1989). Deficiency of vitamin A is known to lead to an arrest in spermatogenesis (Lee et al., 2004), and the observed alteration of this pathway by hypoxia suggests another putative mechanism of reproductive disruption.

Another gene that contributed to enrichment of multiple GO categories (cellular lipid metabolism, lipid biosynthesis) and has a role in reproduction is prostaglandin-endoperoxidase synthase 2 (*ptges2*), also known as cyclooxygenase (*cox-2*). It normally catalyzes conversion of arachidonic acid to prostaglandins and other eicosanoids (e.g., thromboxanes, prostacyclins) with diverse roles (stimulate inflammation, regulate blood flow, modulate synaptic transmission etc.; Stryer, 1998). There is evidence that *cox-2* is stimulated by hypoxia and that its overexpression can contribute to carcinogenesis. Prostaglandin E2 (PGE2), a major end product of COX2 catalyzed reactions, has been shown to potentiate HIF-1 expression and stabilization (Liu et al., 2002), which could explain its upregulation in the testis upon exposure to hypoxia. Prostaglandin E2 has been shown to stimulate testosterone production in goldfish testis (Wade and Van der Kraak, 1993). Thus, it is possible that the observed upregulation of ptges2 could be sufficient to counteract reduced production of testosterone due to *hsd17b* downregulation. In fact, Landry et al. (2007) did not detect effects on testosterone in Gulf killifish exposed to severe hypoxia for 1 month.

The comparison of processes and genes altered by moderate versus severe hypoxia shows that differential expression of 58 genes was common to both treatments. Just as in the 4 d testis, the overlap between severe hypoxia and moderate hypoxia was small, including changes in porphyrin metabolism, secretory pathway and, cofactor metabolism. Interestingly, steroid metabolism was altered upon prolonged exposure to either moderate or severe hypoxia, which suggests that the reproductive system is vulnerable to prolonged exposure to moderate hypoxia even in a hypoxia-tolerant species such as zebrafish. While we did not assess changes in males at the apical level in this study, the observed alterations in expression of multiple genes involved in reproduction are suggestive of reproductive impairment including a lag in gonadal maturation, suppressed sperm production and reduced fertility which is consistent with observations of others studying effects of hypoxia in male teleosts (Wu et al., 2003).

4.2. Effects of hypoxia on gene expression in zebrafish ovaries

4.2.1. 4 d ovary

The majority of the enriched categories in 4 d ovary comprised well-characterized responses to severe hypoxia (e.g., proton and mitochondrial transport, protein catabolism and secretion, mRNA catabolism, ubiquitin-dependent protein metabolism; Table 3). Regulation of ionic conductance and pumps, and protein synthesis commonly occurs in response to hypoxia to reduce the oxygen consumption. Ion pumping, protein synthesis and mitochondrial proton leak consume approximately 60% of the total ATP-coupled oxygen consumption (Bickler and Buck, 2007), so the observed alterations in these pathways represent an adaptive response to hypoxia. The substantial increase observed in genes regulating phosphorylation is in agreement with studies of other hypoxia-tolerant species, where AMP kinase, which regulates hypoxia-mediated decreases in protein synthesis, is regulated by differential phosphorylation and inactivated by hypoxia. Similarly, glycolytic control enzymes like phosphofructokinase and pyruvate kinase also show phosphorylation and decreased activities in hypoxic conditions (Bickler and Buck, 2007). The GO processes related to reproduction that were enriched in the ovary after 4 d of hypoxia included oviposition behavior and a few genes involved in the gamete generation.

Examination of the GO categories enriched in moderate vs. severe hypoxia treatments indicated that the majority of the processes discussed above were altered only by severe hypoxia. As with male gonads, the results indicate that the short-term exposure to moderate hypoxia is not sufficient to bring about an extensive metabolic reorganization in female gonads to the degree seen upon exposure to severe hypoxia.

4.2.2. 14 d ovary

Despite the large number of genes that were differentially regulated by hypoxia, only a small number of GO biological processes were enriched at a statistically significant level (Table 4). Examination of the enriched processes reveals that their nature in the 14 d ovary was substantially different than in the 4 d exposure. In fact, the processes altered were similar to those observed in the 14 d testis, and are indicative of substantial metabolic reorganization, and adverse effects on reproductive and immune function. The overlap between genes altered by moderate and severe hypoxia was substantial (approximately 50% of differentially expressed genes were common to both treatments) indicating that prolonged exposure even to moderate hypoxia has large impacts on global gene expression in ovary. While several processes indicative of hypoxia were altered in the 14 d exposure (e.g., response to cadmium ion, switch to a non-oxidative branch of pentose-phosphate shunt and processes involved in protein synthesis) below we mostly direct our attention to processes that are linked to reproductive function.

Examination of the GO category "asymmetric protein localization involved in cell fate commitment" reveals that both genes (*nanos* and *vasa*) responsible for enrichment of this category were upregulated by hypoxia. These genes are involved in germ cell proliferation and localized in the gonads (Castrillon et al., 2000). *Vasa* is upregulated in previtellogenic cells of fish, and it decreases in postvitellogenic cells (Kobayashi et al., 2000). *Nanos* has been shown to maintain oocyte production in adult zebrafish by maintaining self-renewal of germ line cells (Draper et al., 2007). Both genes are indicative of early stages of oogenesis, suggesting that hypoxia may be shifting the ovary towards an immature state. These gene expression changes correlate with our findings of reduced ovulation rates, as well as with those of Thomas et al. (2006), who observed impairment of ovarian growth and decreased production of fully grown oocytes in females exposed to moderate and severe hypoxia.

The enrichment of lipoprotein and lipid transport GO categories was largely driven by the fact that multiple oligonucleotides coding for apolipoprotein E (APOE) were downregulated. The gene coding for the precursor of APOE was also downregulated (approximately 25-fold in response to severe hypoxia). APOE is a plasma protein that serves as a ligand for low density lipoprotein receptors and, through its interaction with these receptors, participates in the

transport of cholesterol and other lipids among various cells of the body (Mahley, 1988). In many species, lipoproteins like APOE (rather than de novo synthesis from acetate) contribute the majority of cholesterol used for steroid production (Grummer and Carroll, 1988). *Apoe* is expressed by theca cells in mammalian follicles, and is thought to exert an important regulatory role in follicular development (Zerbinatti et al., 2001). At lower concentrations, APOE stimulates thecal androgen production, while high concentrations of APOE can inhibit androgen production. If too much androgen is produced by theca cells, the follicle becomes atretic as granulosa cells are eliminated by androgen-mediated apoptosis (Zerbinatti et al., 2001). The role of APOE in follicular atresia of fish is unknown, but if it is similar to mammals (Mahley, 1988), it is likely that the hypoxia-induced reduction of APOE observed in the present study could stimulate androgen production and promote ovarian atresia. The APOE-induced increase in androgens could have been exacerbated by an increase in testosterone due to the lack of conversion to estradiol, caused by hypoxia-related downregulation of aromatase mRNA expression. APOE also appears to be involved in the repair response to tissue injury, immunoregulation and modulation of cell growth and differentiation (Mahley, 1988) and thus the observed downregulation is of concern. Interestingly, the genes responsible for the "enrichment of sterol metabolism" GO category are involved in cholesterol biosynthesis and were upregulated, potentially to counteract cholesterol deficiency due to its decreased transport by APOE. Nevertheless, it is unlikely that these changes could have counteracted overt decreases in synthesis (e.g., reduced 7-dehydrocholesterol reductase (*dhcr*) expression) and transport of cholesterol (e.g., *apoe*, steroidogenic acute regulatory protein (*star*)).

Examination of 14 d ovary genes involved in regulation of the HPG axis shows a substantial alteration in functioning of the HPG axis which surpasses changes observed in any other sex/time treatment combination. The majority of the genes affected were downregulated by exposure to hypoxia, suggesting an overall suppression of reproductive function. The suppression of *star* is of special importance because *star* is responsible for transport of cholesterol, a rate limiting enzyme for steroidogenesis. Several other genes that catalyze steroid production also were downregulated (e.g., *hsd11b*, *cyp19a1b*). The suppression of *cyp19a1b*, which catalyzes a rate-limiting step in synthesis of estradiol, as well as an observed downregulation of the *esr* indicate a disruption of reproductive function likely to result in fertility loss. Similarly, the downregulation of dopamine decarboxylase and the follicle-stimulating hormone (FSH) receptor mRNAs provide further evidence for dysregulation of the HPG axis. Only two HPG-related genes were upregulated by prolonged exposure to hypoxia: *hsd17b*, which catalyzes conversion of androstenedione to testosterone, and activin receptor IIb (*acvr2b*). Effects on genes involved in the activin-inhibin pathway (upregulation of *acvr2b* and downregulation of *acvrl1*, *bambi* and *smad1*) are of concern because they play important roles in ovary and follicle growth, oocyte maturation and ovulation in zebrafish (Wang and Ge, 2004). The transforming growth factor receptor beta type 2 (*tbr2*), which is a crucial component of transforming growth factor ß signaling and interacts with activin pathway (Kohli et al., 2003), was upregulated in 14 d hypoxia treated ovaries. This is potentially important because *tbr2* can physically associate with cyclin B (a subunit of maturation-promoting factor) and inactivate *cdc-2* (a catalytic unit of maturation promoting factor). This then can stop oocyte maturation by preventing cells from entering G2/M phase and thus final developmental stages (Liu and Yang, 1999). This is in agreement with findings of Padilla and Roth (2001) who demonstrated that the G2 phase of the cell cycle in zebrafish embryonic development was arrested under hypoxia.

In addition to genes identified by GO enrichment and comparisons to the teleost HPG model, we manually identified several more genes potentially involved in reproduction that were altered by hypoxia. The most prominent groups of genes altered by hypoxia included: heat shock proteins, prostaglandin synthases, selenoproteins, and a group of genes that are involved in ovarian contractile processes.

Heat shock proteins (HSPs) serve as chaperones (intercellular housekeeping proteins) under normal physiological conditions and are rapidly induced by a variety of stressors including hypoxia (Ton et al., 2002). In the present experiment, we observed alterations of the multiple HSP mRNAs in the ovary. In the context of reproduction, HSPs have been hypothesized to play a role in ovulation and maintenance of the postovulatory metabolic activity and oocyte survival (Neuer et al., 2000). Interestingly, *hsp70* was downregulated by hypoxia, in contrast to usually observed induction of *hsp70* in the other tissues (e.g., Ton et al., 2002). However, changes in this gene in response to hypoxia appear to be cell/tissue specific (Oehler et al., 2000; Airaksinen et al., 1998; Currie et al., 1999; Gamperl et al., 1998). In any case, we propose that the alterations of HSPs by hypoxia could represent a mechanism by which low oxygen could disrupt reproduction.

Consistent with the male findings, upregulation of genes involved in prostaglandin synthesis was observed in female gonads. Prostaglandins play an exceptionally important role in the reproductive biology of female fish. Prostaglandins stimulate ovulation, modulate ovarian T production (Van der Kraak and Chang, 1990), control ovarian blood flow (Labhsetwar and Watson, 1974), induce and synchronize of spawning behavior and ovulation, and serve as sex pheromones (Sorensen, 1996; Sorensen et al., 1988; Sorensen and Scott, 1994). The observed dysregulation of prostaglandins by hypoxia could result in multiple reproductive impairments ranging from dissociation of spawning behavior and ovulation, to altered steroid synthesis.

Selenoprotein P and W mRNAs were consistently downregulated in females exposed to prolonged hypoxia; this could result in the reduced transport of selenium and thus reproductive impairment. Selenoproteins have been shown to influence several areas of cell biochemistry related to antioxidant function and redox status. It is therefore biologically consistent that hypoxia would alter expression of genes that code for selenoproteins; in fact some selenoproteins have binding sites for HIF-1 (Bierl et al., 2004). Selenoproteins also play an important role in reproduction and have been shown to affect fertility, particularly in males (Foresta et al., 2002). In females, the possible role of selenoproteins in reproduction is not as well understood, but there is evidence for selenium-stimulated proliferation of follicles, and augmented stimulatory effects of gonadotropins on follicles and E2 production (Basini and Tamanini, 2000).

Interestingly, the selenoproteins are also involved in modulating APOE metabolism (Sengupta et al., 2008). Furthermore, transport by selenoprotein P is thought to be mediated by APOE receptor, which is classified as an LDL receptor (Olson et al., 2007). In the present study, hypoxia-upregulated expression of ldl like receptor mRNA, which is indicative of a potential compensatory mechanism to low APOE and selenoprotein levels. In addition to the potential of hypoxia to modulate reproductive endocrine system by altering selenoprotein homeostasis, it is possible that it also impacts thyroid hormone metabolism. In fact, we have observed alterations in several genes involved in thyroid metabolism (e.g., thioredoxin interacting proteins, thyroid receptor interacting protein 11, thioredoxin-related transmembrane protein).

Previous studies with fish have shown that hypoxia downregulates expression of genes that code for the contractile proteins tropomyosin, actin, and desmin (Gracey et al., 2001), which is in

agreement with our findings. The reduced expression of contractile elements has been observed in skeletal muscle, likely representing an important energy-saving strategy because contractile proteins are abundant in muscle (Gracey et al., 2001). The contractile proteins desmin and α-actin have been localized in the ovaries of zebrafish (Van Nassauw et al., 1991). Smooth muscle cells are located in theca interna, where their contraction results in follicular constriction, which increases follicular tension and pressure and culminates in rupture at the apex where tensile force is the weakest due to the lack of smooth muscle (Ko et al., 2006). In addition to suppression of several contractile elements, we also observed suppression of endothelin, which is produced in granulosa cells, and has been shown to diffuse to theca externa where smooth muscle cells are located to induce their contraction (Ko et al., 2006). We therefore hypothesize that hypoxia could affect ovulation by disrupting follicle rupture.

The prolonged (14 d) exposure to hypoxia reduced percent of ovulating females in a concentration-dependent manner, culminating in a complete absence of ovulating fish in the severe hypoxia treatment. This failure of females to reproduce agrees with the gene expression findings. The severe hypoxia treatment altered expression of 1176 genes, 227 genes more than the moderate hypoxia. The genes uniquely altered in the severe hypoxia treatment belong to functional categories relevant to reproduction including: lipoprotein metabolism, lipid transport and sterol metabolism, which supports our findings that severe hypoxia had more extensive effects on reproduction than moderate hypoxia. Unlike prolonged exposure to hypoxia, the 4 d exposure did not cause a prominent reduction in ovulation. Nevertheless, there was a trend toward reduced ovulation rates in the hypoxic treatments (approximately 60% of control fish were ovulating vs. 25% of fish from the hypoxic treatments). These apical findings agree with the gene expression results where a much smaller number of genes involved in reproduction was altered by the short-term exposure to hypoxia. Nevertheless, just as the ovulation data suggest, the exposure was not without impact on reproductive system. Multiple categories linked to reproductive process were altered, even in a 4 d exposure, but the extent of the alteration was much smaller. The apical ovulation data are also well supported by the analyses of HPG axis-related genes, where a 4 d exposure affected few genes, but the 14 d exposure consistently downregulated a large proportion (approximately 15%) of genes involved in HPG function.

5. Conclusion

In summary, hypoxia causes a major reorganization of metabolism in the gonad of mature fish. While the initial changes in metabolism are mostly adaptive and suitable for coping with the short-term stress of hypoxia, alterations induced by prolonged exposure seem to be maladaptive. Ovaries seem to be more susceptible to hypoxia than testis, and gene expression GO analyses suggest that reproductive processes in ovaries are affected even by short-term exposure to moderate hypoxia. The results of conditional clustering support this, showing that ovaries experience a higher level of reorganization than testes (Fig. 2). The evaluation of phenotypic endpoints indicative of reproductive condition (ovulation occurrence in females) supports the gene expression data and agrees with conditional clustering analyses. Multiple genes involved in steroidogenesis and regulation of reproduction were downregulated. The effects of 4 d exposure were less prominent that those of 14 d exposure (at both gene expression and apical level). The severity of effects culminated in females exposed to severe hypoxia for 14 d; these females experienced most extensive gene expression changes and were not ovulating at the time of sample collection.

Our results demonstrate that reproduction is likely affected by hypoxia via multiple mechanisms of action. These include already proposed mechanisms, such as inhibition of steroidogenesis via modulation of expression of steroidogenic genes (Shang et al., 2006) and by downregulation of the serotonogenic pathway (Thomas et al., 2007). In addition, we propose that there are multiple other points of disruption of reproductive system function linked to reorganization of lipid transport and other mechanisms involved in responding to hypoxia (e.g., changes in lipid metabolism, HSP alterations, downregulation of contractile elements and selenoproteins, etc.). Specifically, we hypothesize that dysregulation of lipoprotein-based cholesterol transport in the gonad may lead to cholesterolanemia and thus to suppressed steroidogenesis. We also speculate that dysregulation of hypoxia-responsive selenoproteins may be interconnected with the altered apolipoprotein metabolism. Finally, we hypothesize that hypoxia-driven reorganization of the metabolism of fatty acids (eicosanoids in particular) could contribute to disassociation of sex steroid and prostaglandin profiles. This would then lead to discrepancy between actual reproductive condition and reproductive behaviors and pheromonal signaling, which are synchronized by prostaglandins. Further proteomic and metabolomic work focusing on apical responses is needed to explore these hypotheses.

In the future, we plan to further examine data presented in this manuscript to determine whether gene expression signatures associated with a general stressor like hypoxia can be distinguished from those of endocrine disrupting chemicals with modes of action that directly target components of the HPG axis (e.g., estrogens and androgen receptor agonists, anti-estrogens and androgens, steroidogenesis inhibitors, etc.). This has important implications for biomarker identification (particularly, if the goal is to identify biomarkers diagnostic of specific chemical exposure, as opposed to biomarkers predictive of reproductive dysfunction) because hypoxic conditions often co-occur with endocrine disrupting chemicals and have been reported to have similar gene expression signatures.

Acknowledgements

We thank Robin Sternberg, Xiaowei Zhang and the anonymous reviewers for their helpful comments on the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2008.08.021.

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