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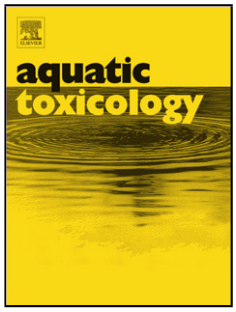

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Highlights

- Hypoxia causes trans-generational effects in the water fleas Daphnia.
- 124 genes were differentially expressed in Daphnia embryos under hypoxia.
- There were acclimatory changes of haemoglobin.
- There was suppression in vitellogenin gene family.
- The expressions of histone H2B, H3, H4 and HDAC4 were deregulated.

Transcriptomic alterations in *Daphnia magna* embryos from mothers exposed to hypoxia

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ABSTRACT

Hypoxia occurs when dissolved oxygen (DO) falls below 2.8 mg L^{-1} in aquatic environments. It can cause trans-generational effects not only in fish, but also in the water fleas *Daphnia*. In this study, transcriptome sequencing analysis was employed to identify transcriptomic alterations induced by hypoxia in embryos of *Daphnia magna*, with an aim to investigate the mechanism underlying the trans-generational effects caused by hypoxia in *Daphnia*. The embryos (F1) were collected from adults (F0) that were previously exposed to hypoxia (or normoxia) for their whole life. *De novo* transcriptome assembly identified 18270 transcripts that were matched to the UniProtKB/Swiss-Prot database and resulted in 7419 genes. Comparative transcriptome analysis showed 124 differentially expressed genes, including 70 up- and 54 down-regulated genes under hypoxia. Gene ontology analysis further highlighted three clusters of genes which revealed acclimatory changes of haemoglobin, suppression in vitellogenin gene family and histone modifications. Specifically, the expressions of histone H2B, H3, H4 and histone deacetylase 4 (HDAC4) were deregulated. This study suggested that trans-generational effects of hypoxia on *Daphnia* may be mediated through epigenetic regulations of histone modifications.

1. Introduction

In aquatic environments, hypoxia occurs when dissolved oxygen (DO) falls below 2.8 mg L^{-1}, at which level can lead to mass mortality of fish and macroinvertebrates (Gray et al., 2002; Díaz and Rosenberg, 2011). Substantial changes in ecosystem composition and function may also occur in affected estuaries, coastal waters and freshwater lakes. Although hypoxia occurs naturally in some areas, the duration, intensity and frequency of hypoxia are increasing worldwide. This is primarily due to eutrophication fuelled by the use of agricultural fertilizers and sewage discharges (Gilbert et al., 2010; Rabalais et al., 2010). Global warming may further exacerbate the problem, through an increase in freshwater outflow, influx of nutrients, water stratification and community metabolism coupled with a reduction in oxygen solubility (Zeis et al., 2009).

The freshwater planktonic crustacean (water fleas) *Daphnia magna* and conspecific species are frequently used as model organisms for ecology, ecotoxicology and evolutionary genomics studies due to their small size, short life cycle and amenability to culture in the laboratory (Guilhermino et al., 2000; Tatarazako and Oda, 2007; Zeis et al., 2009). When exposed to hypoxia, *Daphnia* demonstrated changes in fitness traits, including a smaller body size (Seidl et al., 2005), reduced body mass (Seidl et al., 2005; Andrewartha and Burggren, 2012), higher mortality rate (Lyu et al., 2014) and reduced fecundity (Homer and Waller, 1983) as well as altered phenotypes, such as an increase in concentration and oxygen affinity of haemoglobin and changing heart rate (Seidl et al., 2005). Remarkably, the effects may persist to filial generations. The first and second brood neonates (F1) from mothers previously exposed to chronic hypoxia were significantly smaller in body mass compared to those from mothers of normoxic control. These neonates (F1) in turn produced offspring (F2) that were much smaller during early development than F2 produced from neonates whose mothers were not exposed to hypoxia (Andrewartha and Burggren, 2012).

In this study, transcriptome sequencing analysis was employed to identify transcriptomic alterations induced by hypoxia in embryos of the freshwater crustacean *Daphnia magna*, with an aim

to investigate the mechanism underlying the trans-generational effects caused by hypoxia in *Daphnia*. The embryos (F1) were collected from adults (F0) that were previously exposed to hypoxia (or normoxia) for their whole life (i.e. from newly released neonates to gravid females; ten days). Comparative transcriptome analysis showed 124 differentially expressed genes, including 70 up- and 54 down-regulated genes under hypoxia. Gene ontology analysis highlighted three clusters of genes which revealed acclimatory changes of haemoglobin, suppression in vitellogenin gene family and histone modifications. qPCR analysis of haemoglobin (*Hb*), histone H3, H4, H2B and vitellogenin (*VTG*) was employed to verify the reliability of transcriptome sequencing analysis results. *De novo* transcriptome assembly further identified a subset of novel transcripts, which provides genetic information for further trans-generational study of hypoxia in *Daphnia*.

2. Materials and methods

2.1. Maintenance of Daphnia magna

Daphnia magna Straus cultures were purchased from Carolina® (Burlington, USA) and maintained in the laboratory in continuous parthenogenetic reproduction following OECD guideline 211 (1998). Briefly, *D. magna* were cultivated in artificial M4 medium (Elendt and Bias, 1990) at a density of 1 individual 10 mL^{-1} in plastic beakers (capacity: 500 mL) at 20 ± 1°C under a DO level >6 mg O_2 L^{-1} and a 16:8 h light:dark photoperiod. One-third of the medium was renewed twice a week. *D. magna* was fed with the unicellular freshwater green algae *Ankistrodesmus angustus* (Strain NIES-2192).

2.2. Hypoxia exposure

Newly released neonates (F0) were reared under the same conditions as those described in above paragraph with the exception of dissolved oxygen (DO) level. Two levels of DO (hypoxia: 1.5 ± 0.2 mg O_2 L^{-1}; normoxia: 5.8 ± 0.2 mg O_2 L^{-1}) were set up and each level consisted twelve

replicate beakers. To achieve the desired DO of 1.5 ± 0.2 mg O_2 L^{-1}, a gas mixing tank was injected with nitrogen gas and air (the flow of nitrogen gas and air was regulated by a DO controller), following the design described in our previous study (Li and Chiu, 2013; Cheung et al., 2014). This gas mixing tank in turn supplied the replicate beakers (Cole-Parmer's 01972-00). Water for the normoxic control was bubbled with air only. After ten days of exposure, gravid females of *Daphnia* brooding embryos with black eye pigmentation from each replicate beaker were dissected and embryos carefully removed from brood chambers for RNA isolation.

2.3. RNA isolation and qualification

For cDNA library construction, there were two biological replicates for each of the normoxic and hypoxic groups. RNA extracted from embryos collected from ten individuals from the first six replicate beakers were pooled to obtain the first biological replicate, and those from the remaining six replicate beakers were pooled to obtain the second biological replicate (n=2). While for qRT-PCR analysis, there were twelve biological replicates for each of the normoxic and hypoxic groups. RNA extracted from embryos collected from three individuals from each of the twelve replicate beakers served as a biological replicate (n=12). Total RNA from *D. magna* was extracted using mirVanaTM RNA Isolation Kit (Applied Biosystems) and treated with DNase (Ambion) to remove contaminating genomic DNA. RNA quality was assessed using Agilent 2100 Bioanalyzer system and all samples that had an RNA Integrity Number (RIN) greater than nine were used for cRNA library construction.

2.4. cDNA library construction and strand-specific Illumina RNA sequencing

Four independent libraries (two from hypoxic group and two from normoxic group) were prepared for RNA sequencing as previously described (Gu et al., 2015; Hagenaars et al., 2013). Briefly, cDNA libraries were prepared using TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, USA) following the manufacturer's protocol. Index codes were ligated to identify individual samples.

mRNA was purified from the total RNA using poly-T oligo-attached magnetic beads (Illumina, San Diego, USA), and fragmented RNA was subjected to first and second strand cDNA syntheses using random oligonucleotides and SuperScript II, followed by DNA polymerase I and RNase H. After 3' end adenylation, Illumina PE adapter oligonucleotides were ligated to cDNA. DNA fragments that ligated with adaptor molecules were amplified using Illumina PCR Primer Cocktail in 15-cycle PCR. Products were purified using AMPure XP system and quantified using Agilent Bioanalyzer 2100 system. Before sequencing, the libraries were normalized and pooled together in a single lane on Illumina MiSeq platform. Paired-end reads, each of 150-bp read-length, were sequenced. Adapters and reads containing poly-N were trimmed and the resulting sequence-reads were in turn dynamically trimmed according to BWA's − q algorithm. Briefly, a running sum algorithm was executed in which a cumulative area-plot was plotted from 3'-end to the 5'-end of the sequence reads and where positions with a base-calling Phred quality <30 caused an increase of the area and *vice versa*. Such plot was built for each read individually and each read was trimmed from the 3'-end to the position where the area was greatest. Read-pairs were then synchronized and all read-pairs with sequence on both sides longer than 35 bp after quality trimming were retained. Singleton reads resulting from read trimming were removed. All downstream analyses were based on quality-trimmed reads (Lai et al., 2014; Li et al., 2014).

2.5. Differential expression of known genes and GO enrichment analysis

Quality-trimmed sequence reads were mapped to *Daphnia pulex* genome reference (*Daphnia_pulex*.GCA_000187875) obtained from Ensemble (Flicek et al., 2014) using BWA-MEM v.0.7.5a-r405. Read-counts of genes were quantified against Ensemble gene annotation (*Daphnia_pulex.GCA_000187875.1.24.gtf*) using HTSeq-count (Anders et al., 2015) with following parameters: --stranded=reverse for illumina dUTP strand specific sequencing; --mode=union; and, --type=exon. Read-count data were then subjected to differential expression analysis using edgeR package

(Li and Dewey, 2011). Genes with B&H corrected *p*-value<0.05 and log2 (fold change)>1 were considered to show statistically significant differential expression (Robinson et al., 2010).

2.6. De novo *transcriptome assembly, annotation and identification of novel transcripts*

Forward and reverse reads from all libraries/samples were pooled and subjected to transcriptome *de novo* assembly using Trinity (version r20140413p1) with ‘‘min_kmer_cov’’ set to 2; “SS_lib_type” set to RF, and all other parameters set to default (Grabherr et al., 2011). Coding sequences (open reading frames, ORF) were identified by Transdecoder (Haas et al., 2013) using following criteria: (1) the longest ORF was identified within each transcript; (2) from the longest ORFs extracted, a subset of the longest ones was identified and randomized to provide a sequence composition corresponding to non-coding sequences before being used to parameterize a Markov model based on hexamers; and, (3) all the longest ORFS were scored according to the Markov model to identify the highest scoring reading-frame out of six possible reading-frames. These ORF were then translated to protein sequences and subjected to (1) BLASTp search against UniProtKB/Swiss-Prot with a cut-off e-value (Ewen-Campen et al., 2011; Du et al., 2012) of 1 x 10^{-6}, (2) protein domain search *via* HMMScan, (3) transmembrane helicase prediction by TMHMM, and (4) signal peptide prediction by SignalP. Differential gene expression and TMM-normalized FPKM gene expression were calculated by RSEM pipeline using edgeR package. K-mer clustering of samples was performed with k=2 on differentially expressed genes (Li and Dewey, 2011). Transcripts in the resulting sub-clusters were BLASTn against *Daphnia pulex* transcripts with e-value cut off at 1 x 10^{-6}. Those without significant matches were considered as novel transcripts, and were subjected to manual BLASTx check to nr database with e-value cut off at 1 x 10^{-6}.

2.7. Quantitative PCR analysis

PCR primers of the target genes are listed in **Supplementary Table 1**. Each reaction

contained 1:25 diluted RT products, 1 x SYBR FAST qPCR Master Mix (Kapa Biosystems, Woburn, MA) and 200nM of each primers and was run at a profile including 3 min initial denaturation at 95°C, 40 cycles of 95°C for 5s and 60°C for 20s on StepOnePlus Real-Time PCR System (Life Technologies). The beta-actin housekeeping gene was used for internal normalization for each gene. A dissociation curve was constructed for each reaction to analyse the specificity of amplification. Data were checked for normality with Shapiro-Wilk's *W* test (Shapiro and Wilk, 1965). Student's *t*-test was used to test the differences in gene expression level.

2.8. Availability of supporting data

Sequencing data of this study were submitted to NCBI Sequence Read Archive (SRA) (http://www.ncbi.nlm.nih.gov/sra) under the accession number **SRP057045**.

3. Result

3.1. Sequencing summary

9.54 M and 11.18 M quality-trimmed reads were obtained from normoxia replicates, and 11.07 M and 10.10 M quality-trimmed reads were obtained from hypoxia replicates. 3.08 Gb of clean bases were obtained from normoxia samples, and 2.86 Gb from hypoxia samples (**Supplementary Table 2**). Integrated analysis was performed by combining reference based and *de novo* based transcriptome analyses (**Figure 1**).

3.2. Differential gene expression and GO enrichment analysis

Since *D. magna* genome and transcriptome reference were not available, the transcriptome sequencing data were mapped to the reference genome of the congeneric species *Daphnia pulex* (Colbourne et al., 2011). The mapping rate of cDNA libraries ranged from 63.56% to 67.97% (**Supplementary Table 2**). A total of 124 genes were identified to be differentially expressed under

hypoxia. These included 70 up- and 54 down-regulated genes in the hypoxic group compared to the normoxic group (**Table 1 and Table 2**). The deregulated genes under hypoxia could be classified into three clusters according to their functional annotation and gene ontology analysis (**Table 3**). Cluster I: haemoglobin-related genes were highly expressed under hypoxia treatment. Cluster II: *VTG1*, *VTG2C* and *VTG2N* of the vitellogenin gene family were suppressed under hypoxic condition. Cluster III: histone-related genes including histone H2B, H3, H4 and histone deacetylase 4 (HDAC4) were deregulated. Our results is concordant to the previous findings that hypoxia could induce the expression of haemoglobin, but cause a reduction of vitellogenin expression in different organisms (**Table 4**). Also, HDAC4 was reported to be associated with hypoxia-inducible factor 1 α (HIF1α). Five genes from these clusters were selected and validated by qRT-PCR analysis and the results agreed with the Illumina sequencing data (**Figure 2**).

3.3. De novo *transcriptome assembly and identification of novel transcripts*

Results of *de novo* transcriptome assembly were complimentary to those obtained by reference mapping. The *de novo* transcriptome was formed by 43835 contigs with an average contig length of 1216 bp (the shortest sequence was 201 bp and the longest was 23730 bp). 18270 transcripts were matched to the UniProtKB/Swiss-Prot database and resulted in 7419 genes (**Supplementary Table 3**). K-mean clustering based on expression profiles with k=2 further resulted in clusters of transcripts that were deregulated under hypoxia. The upregulated cluster contained 36 genes, of which 55.6% (20/36) could be matched to *D. pulex* gene sets and they were haemoglobin genes and known genes but unannotated in *D. pulex* (**Supplementary Table 4**). Unmatched transcripts were sulfate anion transporters, serpin peptidase inhibitor, cytoglobin-2 and platelet derived growth factor receptor. The downregulated cluster contained 42 genes and 52.4% (22/42) could be matched to *D. pulex* gene sets (**Supplementary Table 5**). They were vitellogenin, myosin, dystrophin, and Cubitus interruptus-like protein. Unmatched transcripts were trypsin, ARV1 like mRNA and Chitinase-3-like protein 2.

4. Discussion

Trans-generational effects may be mediated *via* maternal processes, epigenetic processes or a combination of both. Using comparative transcriptome analysis, this study revealed a dysregulation of histone expression, including histone H2B, H3, H4 and HDAC4 in *D. magna* embryos from mothers which was previously exposed to hypoxia. H2A, H2B, H3 and H4 are core histones, playing an important role in the maintenance of chromatin structure (Luger et al., 1997). Histone modification especially on histone H3 is one of the major epigenetic mechanisms controlling gene expression without alteration of DNA sequence (Colon-Caraballo et al., 2015). Histone deacetylation can be promoted by HDAC (histone deacetylase), which facilitates the removal of an acetyl group from histone, leading to a condensed nucleosome and transcriptional inactivation. The results in this study suggested that hypoxia could alter the expressions of histone as well as histone deacetylase, which may be linked to the trans-generational effects of hypoxia on *Daphnia* embryos. Trans-generational and epigenetic effects of hypoxia were also demonstrated in *Oryzias melastigma* fish in our previous study (Wang et al., accepted). When the parental fish (F0) were exposed to hypoxia, there were reproductive impairments, including retarded gonad development, decrease in sperm count and sperm motility in F1 and F2 generations associated with a differential methylation pattern of specific genes in sperm of both F0 and F2. Trans-generational effects of environmental hypoxia was not yet demonstrated in *in vivo* mammalian studies, but results from *in vitro* studies suggested that tissue hypoxia can alter histone modifications which often correlate with transcriptional response to hypoxia (Johnson et al., 2008; Ponnaluri et al., 2011; Hancock et al., 2015). For instance, hypoxia induced the expression of a number of histone lysine demethylases (KDMs) in retinal pigment epithelial cells, which influenced the expression of pro-angiogenic genes (ADM, GDF15, HMOX1, SERPE1 and SERPB8) (Ponnaluri et al., 2011). Nevertheless, these genes were not found in our *de novo* transcriptomic assembly database.The results in this study are concordant to previous findings which suggested that

Daphnia can adapt to a low level of environmental oxygen through an up-regulation of haemoglobin expression (Lyu et al., 2014). Increased haemoglobin (Hb) levels may result in a higher haemolymph concentration and oxygen affinity (Zeis et al., 2009; Andrewartha and Burggren, 2012), and may confer significant ecological advantages, including refuge from predation, access to alternative food resources and better survival, growth and reproduction (Pirow et al., 2001). In *Daphnia*, hypoxia-induced expression of Hb genes were suggested to be mediated by hypoxia-inducible factor 1 (HIF-1) and play a key role to a central regulatory mechanism in face of hypoxia (Becker et al., 2011; Gerke et al., 2011). Our data did not suggest an increase or decrease in the expression level of HIF-1 (DAPPUDRAFT_347564) under hypoxic condition. Nevertheless, it has been suggested that HIF-1 stimulation may disappear under prolonged exposure to hypoxia (Uchida et al., 2004).

Results of current study further suggested that hypoxia can lead to a reduction in the expression of vitellogenin gene family in *D. magna*. Vitellogenin is a precursor of lipoproteins and phosphoproteins, which are main components of egg yolk in oviparous species including fish and most of the invertebrates (Robinson, 2008; Kim, 2011). Reduced vitellogenin gene expression may therefore, have adverse effects on embryonic development and carryover effects on larval development. *D. magna* neonates were much smaller in size when their mothers were previously exposed to chronic hypoxia (Andrewartha and Burggren, 2012). Interestingly, there is a vitellogenin and juvenile hormone feedback loop in invertebrates – juvenile hormones stimulate the transcription of the vitellogenin genes and subsequently, vitellogenin production (Wyatt and Davey, 1996; Engelmann, 2002). At the same time, juvenile hormones co-regulate the haemoglobin genes through a juvenoid signalling pathway (Gorr et al., 2006). This coincidence may suggest that the regulation of vitellogen and haemoglobin are related.

De novo transcriptome assembly produced a reference set of mRNA sequences for *D. magna* and facilitated the understanding of local adaptation, genome evolution and population genetics of *Daphnia*. This study also resulted in a number of hypoxia-induced novel transcripts, including sulfate anion transporters, serpin peptidase inhibitor, cytoglobin-2 and platelet-derived growth factor receptor, as well

as hypoxia-reduced novel transcripts, including trypsin, ARVI like mRNA and Chitinase-3-like protein 2. Serpin peptidase inhibitor, a potent inhibitor of angiogenesis, is a regulator of bone density (Ziff, et al., 2016). It is supressed in many types of tumors, such as breast and prostate cancers (Vecchi, et al., 2008; Teoh, et al., 2010). Cytoglobin-2 is a new member of vertebrate globin, which can act as a respiratory protein that stores and transports oxygen (Wawrowski et al., 2011). The elevation of serpin peptidase inhibitor and cytoglobin-2 might help the organism to cope with hypoxic stress. Sulfate anion transporters are responsible for the homeostasis of sulfate and oxalate (Markovich, et al., 2011), and sulfate itself plays an important role in growth, development, and cellular metabolism (Regeer, et al., 2003). Platelet-derived growth factor receptor is a cell surface tyrosine kinase receptor that binds to platelet-derived growth factor, linking MAPK and PI3K signalling pathways. These processes are critical for embryonic development, cell proliferation and angiogenesis (Demoulin and Essaghir, 2014). The induction of sulfate anion transporters and platelet-derived growth factor receptor suggested that hypoxia could affect the embryonic development of *Daphnia*.

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Figure Legends

Fig. 1. Schematic summary of sample preparation, Illumina sequencing and bioinformatic analyses.

Fig. 2. Quantitative PCR (qPCR) results of haemoglobin (*Hb*), histone H3, H4 and H2B and vitellogenin (*VTG*) expression in embryos of *Daphnia magna*. The embryos were collected from adults that were previously exposed to hypoxia (or normoxia) for the whole life (i.e. ten days). Data are presented as the means ± s.e.m.; n = 12, each with embryos collected from three females. *$p < 0.05$, student's *t*-test.

Fig. 1.

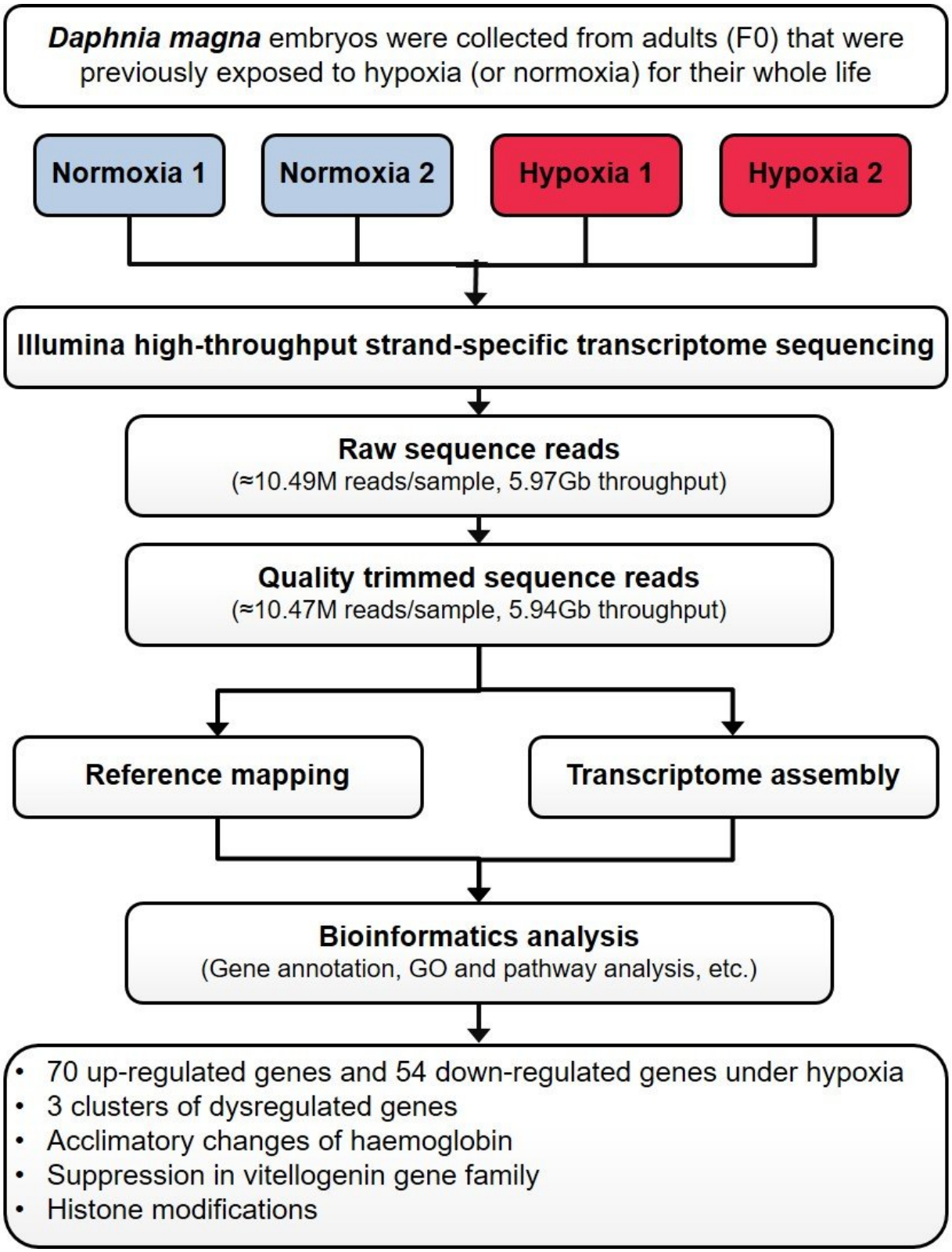

Fig. 2.

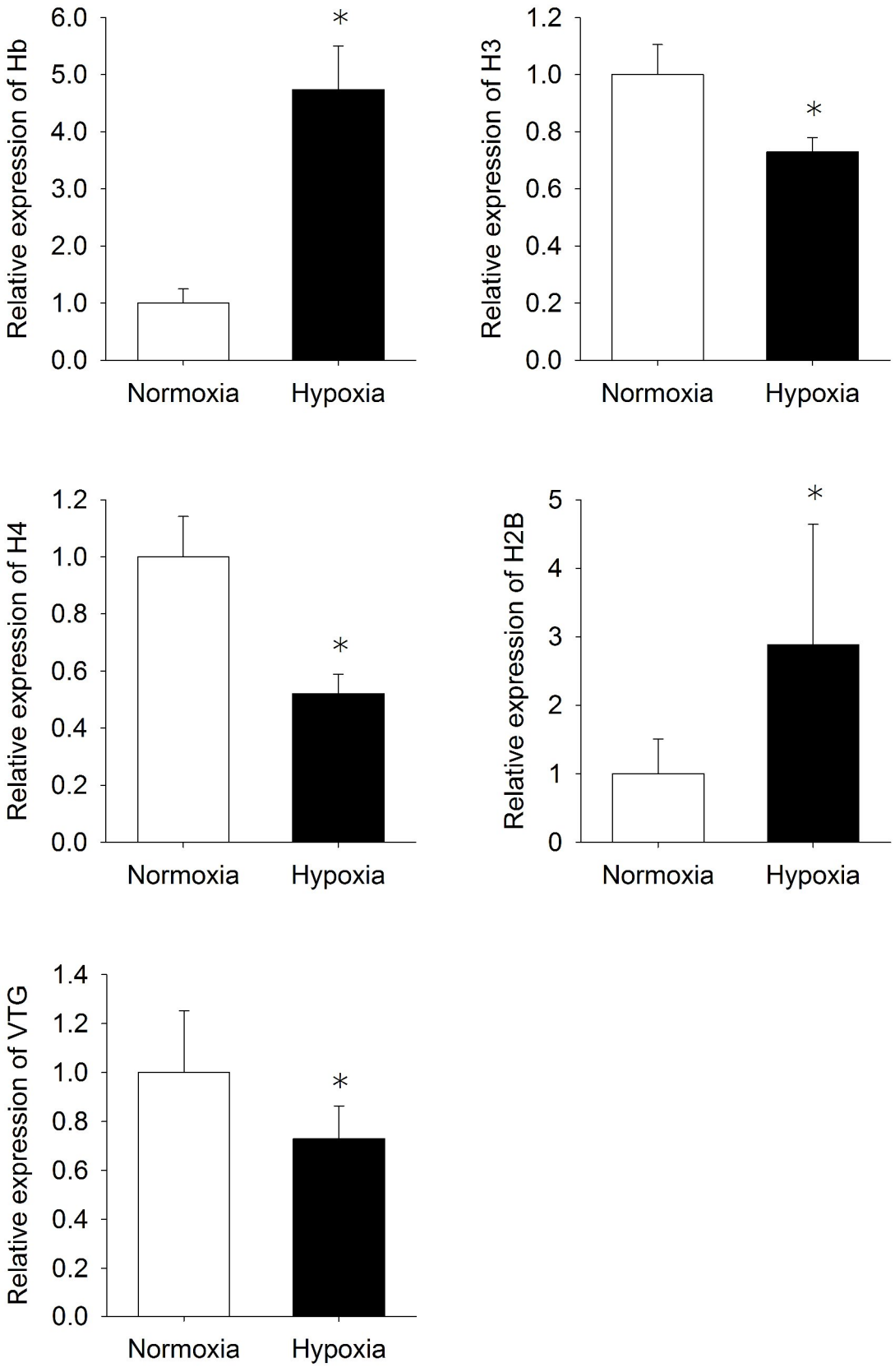

Table 1. 70 differentially up-regulated genes in *D. magna* under hypoxia.

Gene ID	log2 fold change (Normoxia/Hypoxia)	p-value	Gene description
DAPPUDRAFT_316317	-8.60	<0.01	Haemoglobin
DAPPUDRAFT_230333	-6.40	<0.01	*Daphnia magna* dhb2 mrna for complete cds
DAPPUDRAFT_234838	-5.97	<0.01	Haemoglobin
DAPPUDRAFT_93831	-5.51	<0.01	Haemoglobin
DAPPUDRAFT_234836	-4.89	<0.01	Haemoglobin
DAPPUDRAFT_315134	-4.50	<0.05	*Strongylocentrotus purpuratus* glycoprotein-n-acetylgalactosamine 3-beta-galactosyltransferase 1-like mrna
DAPPUDRAFT_104360	-4.44	<0.05	*Daphnia pulex* hsp90 complete cds
EMDPUG00000003016	-4.44	<0.05	tRNA-Thr for anticodon CGU
DAPPUDRAFT_314292	-4.44	<0.05	Transporter
DAPPUDRAFT_125764	-4.19	<0.05	*Pseudomonas brassicacearum* strain complete genome
DAPPUDRAFT_60881	-4.15	<0.05	Innexin
DAPPUDRAFT_65647	-4.15	<0.05	*Daphnia magna* partial mrna for protein (pairediiib gene) isolate embryonic allele 2
DAPPUDRAFT_327264	-4.11	<0.05	*Drosophila yakuba* ge18986 (dyak\ge18986) mrna
DAPPUDRAFT_311662	-4.01	<0.01	dmu67067 *Daphnia magna* haemoglobin complete cds
DAPPUDRAFT_92880	-3.99	<0.01	Haemoglobin
DAPPUDRAFT_105344	-3.74	<0.05	Septin-4-like protein
DAPPUDRAFT_43976	-3.74	<0.05	Histone H2B
DAPPUDRAFT_442640	-3.74	<0.05	Synaptotagmin 15
EMDPUG00000004510	-3.74	<0.05	tRNA-Leu for anticodon UAG
DAPPUDRAFT_107399	-3.74	<0.05	*Astyanax mexicanus* homeobox protein six3-like mrna
DAPPUDRAFT_23898	-3.74	<0.05	*Drosophila mojavensis* gi24543 (dmoj\gi24543) mrna
DAPPUDRAFT_311388	-3.74	<0.05	*Drosophila willistoni* gk16044 (dwil\gk16044) mrna
DAPPUDRAFT_234837	-3.42	<0.01	Haemoglobin
DAPPUDRAFT_318327	-3.38	<0.01	Na(+)/Pi cotransporter
DAPPUDRAFT_299574	-3.27	<0.01	Uroporphyrinogen decarboxylase
DAPPUDRAFT_234839	-2.88	<0.01	Haemoglobin
DAPPUDRAFT_65174	-2.86	<0.05	*Daphnia magna* ap1 mrna for apterous partial cds
DAPPUDRAFT_290567	-2.83	<0.05	Putative transcription factor odd-paired
DAPPUDRAFT_347625	-2.62	<0.01	Endoglucanase
DAPPUDRAFT_347308	-2.60	<0.05	Transcriptional factor scalloped, isoform 2
DAPPUDRAFT_264436	-2.54	<0.05	*Daphnia pulex* clone d11 retrotransposon dirs reverse transcriptase complete cds
DAPPUDRAFT_14338	-2.54	<0.05	DpSIX
DAPPUDRAFT_96715	-2.48	<0.01	Thromboxane A synthase-like protein
DAPPUDRAFT_128379	-2.43	<0.05	Ferrochelatase
DAPPUDRAFT_347244	-2.38	<0.05	BTB3
DAPPUDRAFT_59164	-2.36	<0.05	Protein Wnt
DAPPUDRAFT_303260	-2.33	<0.05	*Tupaia chinensis* srsf protein kinase 3 mrna
DAPPUDRAFT_318553	-2.31	<0.05	*Daphnia pulex* clone op11 dappu_318553-like protein partial cds

DAPPUDRAFT_309533	-2.24	<0.01	Phospholipase-like protein A2, group
DAPPUDRAFT_312948	-2.20	<0.05	ABC protein, subfamily ABCG
DAPPUDRAFT_442468	-2.17	<0.05	Alpha-carbonic anhydrase
DAPPUDRAFT_64722	-2.15	<0.01	*Ornithodoros coriaceus* clone oc-568 hypoxia-inducible factor prolyl hydroxylase 2 partial cds
DAPPUDRAFT_301963	-2.07	<0.01	Protein kinase C
DAPPUDRAFT_299576	-2.07	<0.05	Pyruvate carboxylase
DAPPUDRAFT_232076	-2.02	<0.01	*Daphnia arenata* isolate cc4 chitinase 15 partial cds
DAPPUDRAFT_249991	-2.00	<0.05	Eyeless
DAPPUDRAFT_240551	-1.93	<0.01	Daphnia pulex clone 1793392:1 ferritin 3-like protein e complete cds
DAPPUDRAFT_242072	-1.89	<0.05	*Elephantulus edwardii* light intermediate chain 1 mrna
DAPPUDRAFT_332183	-1.89	<0.05	ABC protein, subfamily ABCH
DAPPUDRAFT_18594	-1.86	<0.01	*Chrysemys picta* bellii ccaat enhancer binding protein (c ebp) epsilon mrna
DAPPUDRAFT_225386	-1.85	<0.05	5-aminolevulinate synthase
DAPPUDRAFT_290511	-1.84	<0.05	Ferritin 2 light chain-like protein
DAPPUDRAFT_108870	-1.82	<0.05	*Capsaspora owczarzaki* ATCC 30864 hypothetical protein (CAOG_05347) mRNA, complete cds
DAPPUDRAFT_301873	-1.81	<0.05	*Entamoeba nuttalli* p19 membrane complex biogenesis family protein partial mrna
DAPPUDRAFT_320395	-1.75	<0.05	5-aminolevulinate synthase
DAPPUDRAFT_313427	-1.75	<0.05	Putative cyclooxygenase
DAPPUDRAFT_302410	-1.74	<0.05	*Daphnia magna* hairy enhancer of split-like 2 partial cds
DAPPUDRAFT_442645	-1.71	<0.05	Glutathione S-transferase
DAPPUDRAFT_42690	-1.69	<0.05	Transporter
DAPPUDRAFT_64675	-1.65	<0.05	Protein Wnt
DAPPUDRAFT_98433	-1.58	<0.05	*Drosophila erecta* gg23047 (dere\gg23047) mrna
DAPPUDRAFT_111909	-1.54	<0.05	Putative allatotropin
DAPPUDRAFT_42745	-1.52	<0.05	*Daphnia magna* clone b039 microsatellite sequence
DAPPUDRAFT_442646	-1.47	<0.05	Glutathione S-transferase
DAPPUDRAFT_308636	-1.46	<0.05	*Daphnia pulex* clone jgiazsn- complete sequence
DAPPUDRAFT_233847	-1.46	<0.05	Uricase
DAPPUDRAFT_442802	-1.42	<0.05	Ecdysone-induced protein
DAPPUDRAFT_442965	-1.31	<0.05	Histone deacetylase HDAC4 protein-like protein
DAPPUDRAFT_347609	-1.29	<0.05	Endoglucanase-1,4-beta-glucanase
DAPPUDRAFT_310801	-1.26	<0.05	Glutathione peroxidase

Table 2. 54 differentially down-regulated genes in *D. magna* under hypoxia.

Gene ID	log2 fold change (Normoxia/Hypoxia)	p-value	Gene description
DAPPUDRAFT_347667	9.66	<0.01	Vitellogenin fused with superoxide dismutase
DAPPUDRAFT_58854	5.76	<0.01	mrna for alpha-like subunit of the nicotinic acetylcholine receptor
DAPPUDRAFT_64669	5.37	<0.01	Putative centromeric histone CENP-A
DAPPUDRAFT_313994	5.00	<0.01	*Daphnia pulicaria* clone wfms0000345 microsatellite marker dp334 sequence
DAPPUDRAFT_212927	4.86	<0.01	*Antheraea godmani* dopa decarboxylase partial cds
DAPPUDRAFT_347678	4.75	<0.01	Vitellogenin fused with superoxide dismutase
DAPPUDRAFT_47227	4.51	<0.01	*Daphnia pulex* clone jgiazsn- complete sequence
DAPPUDRAFT_299677	4.46	<0.01	Vitellogenin fused with superoxide dismutase
DAPPUDRAFT_226762	4.44	<0.05	*Daphnia pulex* clone jgiazsn- complete sequence
DAPPUDRAFT_299508	4.12	<0.01	G2/mitotic-specific cyclin B, copy E-like protein
DAPPUDRAFT_311736	4.08	<0.05	Lipoxygenase-like protein
DAPPUDRAFT_239293	4.07	<0.05	*Drosophila melanogaster* chromosome 3l
DAPPUDRAFT_47503	3.86	<0.01	*Daphnia pulex* clone jgiazsn- complete sequence
DAPPUDRAFT_241731	3.21	<0.01	*Poecilia reticulata* fibroblast growth factor 20-like partial mrna
DAPPUDRAFT_53927	2.77	<0.05	Zinc finger-like protein
DAPPUDRAFT_312949	2.67	<0.05	ABC protein, subfamily ABCG
DAPPUDRAFT_442486	2.59	<0.01	Alpha-carbonic anhydrase
DAPPUDRAFT_248107	2.58	<0.05	Superoxide dismutase
DAPPUDRAFT_43920	2.54	<0.01	Histone H4
DAPPUDRAFT_327753	2.47	<0.05	*Drosophila erecta* gg13378 (dere\gg13378) mrna
DAPPUDRAFT_347757	2.30	<0.01	Chymotrypsin-like protein
DAPPUDRAFT_63051	2.28	<0.01	Delta-like protein
DAPPUDRAFT_442485	2.22	<0.05	Alpha-carbonic anhydrase
DAPPUDRAFT_97116	2.18	<0.05	*Daphnia pulicaria* clone wfms0000203 microsatellite marker dp197 sequence
DAPPUDRAFT_306533	2.15	<0.05	*Musca domestica* cytochrome p450 4d8-like mrna
DAPPUDRAFT_347377	2.11	<0.01	ABC protein, subfamily ABCG
DAPPUDRAFT_442806	2.10	<0.05	Glycolipid-transport protein
DAPPUDRAFT_315707	2.06	<0.05	ABC protein, subfamily ABCG
DAPPUDRAFT_347230	2.05	<0.05	Putative transcriptional factor Ocelliless/Orthodenticle protein
DAPPUDRAFT_304645	2.00	<0.01	Histone H3
DAPPUDRAFT_223595	1.94	<0.01	Coronin
DAPPUDRAFT_301902	1.94	<0.05	*Cylicostephanus goldi* genome assembly c_goldi_cheshire cgoc_contig0032236
DAPPUDRAFT_347295	1.88	<0.05	ABC protein, subfamily ABCC
DAPPUDRAFT_347772	1.86	<0.01	Trypsin
DAPPUDRAFT_329544	1.84	<0.05	*Daphnia arenata* isolate cc1 gram-negative binding protein 6 partial cds
DAPPUDRAFT_196131	1.84	<0.05	*Eptesicus fuscus* leucyl cystinyl aminopeptidase mrna
DAPPUDRAFT_330188	1.74	<0.05	Putative UDP-glucuronosyltransferase

DAPPUDRAFT_442498	1.73	<0.05	Alpha-carbonic anhydrase
DAPPUDRAFT_127273	1.70	<0.01	*Apis dorsata* uncharacterized loc102673834 transcript variant mrna
DAPPUDRAFT_308251	1.67	<0.05	*Strongyloides papillosus* genome assembly s_papillosus_lin spal_contig0000015
DAPPUDRAFT_222925	1.66	<0.05	Putative cyclin B, copy D
DAPPUDRAFT_347474	1.63	<0.05	ABC protein, subfamily ABCH
DAPPUDRAFT_55141	1.58	<0.05	Transporter
DAPPUDRAFT_53622	1.53	<0.05	*Canis lupus* familiaris ribonuclease t2 transcript variant mrna
DAPPUDRAFT_306471	1.45	<0.05	RFS1
DAPPUDRAFT_318905	1.41	<0.05	*Helicoverpa armigera* clone 5s01584 glucosidase partial cds
DAPPUDRAFT_317254	1.38	<0.05	*Arthroderma benhamiae* cbs 112371 leukotriene a4 mrna
DAPPUDRAFT_306271	1.38	<0.05	*Helobdella robusta* hypothetical protein partial mRNA
DAPPUDRAFT_305892	1.35	<0.05	*Naegleria gruberi* rab family small mrna
DAPPUDRAFT_225264	1.35	<0.05	Doublesex and mab-3 related transcription factor-like protein 1
DAPPUDRAFT_210571	1.34	<0.05	DpGSTS1
DAPPUDRAFT_347623	1.27	<0.05	Astacin-like protease
DAPPUDRAFT_226800	1.24	<0.05	*Drosophila ananassae* gf22409 (dana\gf22409) mrna
DAPPUDRAFT_299672	1.23	<0.05	Putative oxidoreductase

Table 3. Classification of genes from *D. magna* based on functional annotation.

Gene name (gene symbol)	Gene ID
Cluster I: Haemoglobin-related	
Haemoglobin	DAPPUDRAFT_316317
Haemoglobin	DAPPUDRAFT_234838
Haemoglobin	DAPPUDRAFT_93831
Haemoglobin	DAPPUDRAFT_234836
Haemoglobin complete cds	DAPPUDRAFT_311662
Haemoglobin	DAPPUDRAFT_92880
Haemoglobin	DAPPUDRAFT_234837
Cluster II: Embryonic development	
Vitellogenin fused with superoxide dismutase (*VTG1*)	DAPPUDRAFT_299677
Vitellogenin fused with superoxide dismutase (*VTG2C*)	DAPPUDRAFT_347678
Vitellogenin fused with superoxide dismutase (*VTG2N*)	DAPPUDRAFT_347667
Cluster III: Histone modification	
Histone H2B	DAPPUDRAFT_43976
Histone deacetylase HDAC4 protein-like protein	DAPPUDRAFT_442965
Histone H3	DAPPUDRAFT_304645
Histone H4	DAPPUDRAFT_43920

Table 4. Effects of hypoxia on the gene expression of haemoglobin (*Hb*), vitellogenin and histone deacetylase 4 in different organisms.

Gene	Cell / Organisms	Findings	Reference
Haemoglobin	Murine ATII cell line MLE-15	Hb mRNA and protein were up-regulated during hypoxic exposure.	Grek et al., 2011
	Daphnia magna	The haem-based Hb concentration increased by 266% as a consequence of hypoxia acclimation. Significant differences between both acclimation groups occurred in all generation (F0-2).	Seidel et al., 2005
	Gadus morhua	Level of Hb was significantly elevated by about 15% in the hypoxia-exposed group.	Petersen and Gamperl, 2011
	Oryzias latipes	Significant increase in Hb mRNA level was found during hypoxia.	Wawrowski et al., 2011
	Danio rerio	Down-regulation of embryonic Hb mRNA was observed in embryos exposed in hypoxia for 24h.	Ton et al., 2003
	Sparus aurata	Hypoxia significantly increased blood haemoglobin content by 35%.	Bermejo-Nogales et al., 2014
Vitellogenin	*Spartina alterniflora*	Vitellogene-1 was significantly down-regulated in the wild-caught shrimp exposed to long-term cyclic hypoxia, suggesting a decrease in yolk provisioning of oocytes in grass shrimp.	Brown-Peterson et al., 2011
	Micropogonias undulatus	The production of mature oocytes and sperm (gametogenesis), as well as vitellogenin levels in the blood, were significantly lower in croaker from the hypoxic sites in East Bay compared to the values in fish collected from the adjoining normoxic Pensacola Bay.	Thomas et al., 2007.
	Micropogonias undulatus	Ovarian dysfunction was associated with significant decreases in vitellogenin, the yolk protein precursor sequestered by the growing oocytes under hypoxia.	Thomas et al., 2006
	Danio rerio	A marked downregulation in both vitellogenin-1 and vitellogenin-2 was observed in zebrafish exposed to hypoxia.	Lu et al., 2014
	Palaemonetes pugio	Vitellogenin was significantly down-regulated in shrimp from the cyclic hypoxic location. This inhibition might be related to the significant reduction in reproduction as demonstrated by lower percentage of ovigerous females and the lower relative fecundity at hypoxic site.	Li and Brouwer, 2013
Histone deacetylase 4	von Hippel-Lindau (VHL)-null kidney cancer cell lines	HDAC4 regulates HIF1α protein acetylation and stability.	Geng et al., 2011