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RESEARCH ARTICLE

Hypoxia-induced mobilization of stored triglycerides in the euryoxic goby *Gillichthys mirabilis*

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SUMMARY

Environmental hypoxia is a common challenge that many aquatic organisms experience in their habitat. Responding to hypoxia requires metabolic reprogramming so that energy-demanding processes are regulated to match available energy reserves. In this study we explored the transcriptional control of metabolic reorganization in the liver of a hypoxia-tolerant burrow-dwelling goby, *Gillichthys mirabilis*. Gene expression data revealed that pathways associated with triglyceride hydrolysis were upregulated by hypoxia whereas pathways associated with triglyceride synthesis were downregulated. This finding was supported by tissue histology, which showed that the size of hepatic lipid droplets declined visibly during exposure to hypoxia. Proton nuclear magnetic resonance analysis confirmed the mobilization of hepatic triglycerides, which declined 2.7-fold after 5 days of hypoxia. The enzyme, adipose triglyceride lipase, was implicated in the mobilization of triglycerides because its expression increased at the level of both transcript and protein. This observation raises questions regarding the regulation of fat metabolism during hypoxia and the role played by the hypoxia-responsive gene leptin.

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Key words: hypoxia, fish, triglyceride, microarray, transcriptome, oxygen, leptin.

INTRODUCTION

For many aquatic organisms, conditions of low dissolved oxygen or hypoxia can occur regularly in their natural habitat. Episodes of hypoxia are responsible for many fish kills in enclosed freshwater and coastal areas, because of anthropogenic nutrient loading (Karim et al., 2003), and in offshore locations under certain upwelling conditions (Grantham et al., 2004). Furthermore, recent predictions indicate that increasing ocean temperatures and elevated CO_2 levels will exacerbate the effects of low oxygen on benthic ecosystems and will increase the frequency of hypoxia-related ecological catastrophes (Vaquer-Sunyer and Duarte, 2008). Other studies have implicated physiological hypoxia and a decline in oxygen delivery to tissues as one of the mechanisms by which some species will be rendered sensitive to the small increases in temperature such as those predicted to occur this century (Portner and Knust, 2007). Understanding the mechanisms that allow some species to tolerate hypoxia while others are sensitive to it will be crucial for predicting the effects that hypoxia will have on specific taxa and on the communities with which they interact.

The general physiological response to hypoxia is to reduce energy demands because hypoxia reduces mitochondrial oxidative phosphorylation, leading to a decline in the production of ATP; therefore, most organisms reduce ATP turnover to preserve dwindling ATP levels (Hochachka, 1997) and decrease ATP-demanding processes such as protein synthesis (Smith et al., 1996). At the same time, most organisms reorganize their metabolism to favor pathways that require less ATP and do not use oxygen. In most vertebrates, the key metabolic change is the switch towards substrate-level phosphorylation *via* the glycolytic pathway, in which glucose is metabolized through to pyruvate and then to lactate in the absence of oxygen, with the net production of two molecules of ATP. The switch to this pathway generally requires that an appropriate stored fuel is available, a requirement that is typically met by glucose stored as glycogen in tissues such as liver (Richards, 2011).

The changes in enzyme activity required for metabolic reorganization during hypoxia are often regulated by the differential expression of mRNA (Semenza et al., 1996). Here we apply microarray-based gene expression profiling to investigate the transcriptional control of metabolic reprogramming during hypoxia in the euryoxic fish *Gillichthys mirabilis*. This is a hypoxia-tolerant species of goby that inhabits estuaries in central and southern California, USA. The availability of microarrays for this species has made it a model to explore the transcriptional component of responses to hypoxia (Gracey et al., 2001), aerial emergence (Gracey, 2008), heat (Buckley et al., 2006; Logan and Somero, 2010; Logan and Somero, 2011) and osmotic stress (Evans and Somero, 2008). A previous gene expression screen of this organism's response to hypoxia reported that energy-requiring processes such as contraction in skeletal muscle and cell growth were downregulated, whereas pathways involved in anaerobic metabolism were enhanced (Gracey et al., 2001). The results presented here expand on these data and highlight the role that an unexpected metabolic pathway appears to play in the hypoxia response of *G. mirabilis*.

MATERIALS AND METHODS

Animal collection and hypoxia exposure

Gillichthys mirabilis Cooper 1864 were collected near Santa Barbara, CA, using baited minnow traps. Fish, ranging in length from 8.89 to 12.7 cm, were maintained in aerated aquaria with flowing seawater at 15°C and fed trout pellets *ad libitum*. Hypoxic time-course experiments were conducted as previously described (Gracey et al., 2001). Briefly, the fish were held in two identical aquaria at 15°C, with one aquarium serving as the control aerated habitat and the other as the hypoxia treatment. Physiological hypoxia was imposed by bubbling nitrogen gas into the aquarium and the desired P_{O_2} was controlled using an oxygen sensor (Point-Four Systems, Coquitiam, BC, Canada) coupled to a solenoid valve that regulated the flow of N_2. At the onset of the time course, the oxygen content of the tank was lowered from an aerated P_{O_2} of 100% (~8.0 mg l^{-1}) down to 10% (0.8 mg l^{-1}) over a 90 min period. Food was introduced into both aquariums on a daily basis, although the fish under hypoxia fed little and any uneaten food was removed 2 h after feeding. Fish in the hypoxic aquarium were sampled at 0, 6, 12, 24, 48, 72 and 120 h after the onset of the experiment. The hypoxia treatment was stopped at the 120 h time point and oxygen was introduced into the aquarium by vigorous aeration with atmospheric air; fish were sampled at 6 and 24 h following the initiation of reoxygenation. Fish were sampled in the control aerated aquarium at 6, 12, 24, 120 and 192 h. At each time point, five individual fish were killed by cranial concussion; their liver tissue was quickly dissected and frozen as aliquots in liquid nitrogen and then archived at –80°C for subsequent processing. In addition, a 5 mm^3 section of liver tissue from each fish was placed in 4% paraformaldehyde and fixed for histological analysis. All animal procedures were performed in accordance with Institutional Animal Care and Use Committee guidance.

Gene expression analysis by microarray hybridization

Total RNA was extracted from the liver tissue of each individual fish using Trizol (Invitrogen, Carlsbad, CA, USA) and then poly(A)$^+$ mRNA was isolated from 500 g of total RNA using a commercial kit (Oligotex, Qiagen, Valencia, CA, USA). A common reference poly(A)+ mRNA sample was prepared from liver samples collected from 10 individual fish that were maintained under aerated conditions. Fluorescently labeled cDNA was synthesized from 1.5 g of poly(A)$^+$ RNA from each individual fish using amino-allyl adducts (Hughes et al., 2001) and labeled with a Cy5 fluorophore. Fluorescently labeled cDNA from the reference RNA was prepared similarly and labeled with the Cy3 fluorophore. Fluorescently labeled cDNA was prepared successfully from 22 fish that were sampled under control conditions (three fish at 12 h, four fish at 0 h, and five fish at 6, 120 and 196 h), 35 fish sampled under hypoxic conditions (five fish at 6, 12, 48, 72 and 120 h, and four fish at 24 h) and nine fish sampled after reoxygenation (4 fish at 24 h and 5 fish at 6 h). Gene expression was measured in each individual fish by hybridization to a *G. mirabilis* cDNA microarray using a common reference hybridization design in which the Cy5-labeled cDNA from each fish was competitively hybridized against Cy3-labeled cDNA derived from the common reference RNA sample on a single array. The cDNAs were resuspended in 40 l of hybridization buffer [3.5× saline-sodium citrate (SSC), 20 mmol l^{-1} Hepes pH 7.4], competitively hybridized to arrays for 18 h at 65°C, and then washed with 0.06× SSC at 20°C to remove unhybridized cDNA. The composition and annotation of the *G. mirabilis* array has been previously described (Gracey, 2008), and the array comprised 12,661 cDNA spots of which 3605 cDNAs had been sequenced from their 5′ end.

The washed microarrays were scanned on an Axon 4000B scanner to generate TIFF images (Molecular Devices, Sunnyvale, CA, USA) from which the median pixel intensity without background correction for each spot was determined using GenePix 4.0 software (Molecular Devices). Spatial and dye artifacts were corrected using a locally weighted intensity-dependent normalization step and the resulting normalized data were centered relative to the median expression of each gene in the control fish. We restricted our functional interpretation of the data to a subset of 3120 non-redundant genes. Differentially expressed genes were identified using an ANOVA model (GeneSpring GX 7.0, Agilent, Santa Clara, CA, USA) in which individual fish sampled within a time point were treated as biological replicates, with the exception of the 22 normoxic control samples, which were treated as replicates regardless of the time point at which they were collected, which provided an estimate of the natural biological variation that occurred over this time course. The *P*-values were corrected for false discovery rate (Benjamini and Hochberg, 2001) and genes for which $P<0.01$ were considered to be statistically significant. *K*-means clustering and visualization were implementing using the Cluster and TreeView software packages (EisenSoftware, UC Berkeley, Berkeley, CA, USA). Ingenuity Pathways Analysis (Ingenuity Systems, Redwood City, CA, USA) was used to investigate whether particular biological processes were enriched within the list of genes that were differentially expressed during hypoxia. Putative human orthologs of the 3120 non-redundant *G. mirabilis* genes were identified using BLASTX homology searches against the human protein collection in the SwissProt database. We assigned the *G. mirabilis* genes with a putative human gene identifier and used the lists of arrayed genes and hypoxia-regulated genes as the reference and query gene lists in the analysis. A right-tailed Fisher's exact test was used to calculate a *P*-value that determined the probability that an enriched biological function in the lists of hypoxia-regulated genes was due to chance alone.

The complete sequence of the adipose triglyceride lipase (ATGL) gene was generated by primer walking using custom primers designed against the 5′ and 3′ sequences of a cloned full-length cDNA of the gene.

Histological analysis

Liver pieces were fixed in 4% paraformaldehyde for 24 h and then the paraformaldehyde was changed and the tissues were fixed again for a further 24 h. Once fixed, the tissues were embedded in paraffin (Tissue Prep 2, Fisher Scientific, Pittsburgh, PA, USA) and sections were stained with hematoxylin and eosin. Glycogen was detected in stained sections by additional staining with periodic acid-Schiff reagent. For frozen histological analysis, a small section of liver tissue was dissected and placed in OCT embedding compound (Tissue-Tek, Torrance, CA, USA), frozen, and cryostat cut sections were stained with 1% osmium tetroxide.

Metabolite analysis

Total lipid was extracted by pulverizing small pieces of liver of known mass in chloroform:methanol (2:1). Two rounds of extraction were performed and the resulting extract was dried under N_2 gas and stored in ampoules under N_2 at –80°C. Lipid quantification was performed by ^{1}H NMR using an 880 MHz instrument, and glycerol-type lipids were quantified using the deuterated chloroform $CDC1_3$ solvent peak as the internal standard (Fan and Lane, 2008). Stored glycogen was assayed by homogenizing small pieces of liver of known mass in 0.6 mol l^{-1} perchloric acid and measuring the change in glucose following incubation with amyloglucosidase as described

(Keppler and Decker, 1974). Statistically significant changes in metabolite levels were determined using a Student's *t*-test.

Western analysis

Western analysis was performed as previously described (Buckley et al., 2006). Briefly, an aliquot of tissue was homogenized in approximately 10 volumes of homogenization buffer [32 mmol l^{-1} Tris-HCl pH 6.8, 2% sodium dodecyl sulfalte (SDS)]. The homogenate was heated at 100°C for 5 min, centrifuged at 12,000 ***g*** for 10 min, and the concentration of solubilized protein in the supernatant was determined by Bradford total protein assay (Bio-Rad, Hercules, CA, USA). For each sample, 10 g of total protein were separated by SDS-PAGE on 10% mini gels (Bio-Rad Mini Protean). Following electrophoresis, the separated proteins in the gel were either stained with Coomassie Blue or transferred to nitrocellulose membranes by electroblotting (Bio-Rad Mini Trans-Blot) at 30 V at 4°C overnight. The high concentration of lipid in the tissue appeared to have an effect on the binding of dye in the Bradford assay and thus final quantification of the total protein in the samples was obtained by digital densitometry of the total protein detected by Coomassie staining of the gel following electrophoresis.

The membrane containing the transferred proteins was blocked for 1 h in 5% nonfat dry milk (Bio-Rad) in 1× phosphate buffered saline (PBS). Post-blocking, the membrane was washed 3× for 5 min in 1× PBS, 0.01% Tween 20. The membrane was then incubated with a primary antibody that was a rabbit anti-mouse adipose triglyceride lipase (ATGL) polyclonal antibody (raised against the complete amino acid sequence of the mouse ATGL protein and a gift from Dr Rudolf Zechner, University of Graz, Austria) diluted 1:10,000 in 5% nonfat dry milk in 1× PBS. The membrane was incubated with this antibody for 2 h and then washed 3× for 10 min in 1× PBS, 0.1% Tween 20. Primary antibody bound to the membrane was detected using Protein A–horseradish peroxidase conjugate (Bio-Rad) diluted 1:5000 in 5% nonfat dry milk in 1× PBS for 1 h and then washed 3× for 10 min in 1× PBS, 0.1% Tween 20. The membrane was incubated with a chemiluminescent detection agent (SuperSignal West Pico, Pierce, Rockford, IL, USA) for 5 min and then wrapped in plastic wrap and exposed to X-ray film (XOMATAR film, Kodak, Rochester, NY, USA). Densitometry of the Coomassie-strined gels and X-ray images were performed using the AlphaView software package (Cell Biosciences, Santa Clara, CA, USA).

RESULTS AND DISCUSSION

In this study we used microarray-based gene expression profiling to gain further insights into the transcriptional responses of *G. mirabilis* to environmental hypoxia. We employed a time course of hypoxia exposure that was demonstrated previously to induce broad changes in hepatic gene expression (Gracey et al., 2001). Environmental hypoxia was set at 0.8 mg l^{-1} O_2, which is below the critical oxygen tension for *G. mirabilis* at 15°C of 1.3 mg l^{-1} O_2 (Gracey et al., 2001). Our experimental design incorporated a large number of control animals (N=22), hypoxia-challenged animals (N=35) and animals recovering from hypoxia (N=9), which were collected across five, seven and two time points, respectively. Control fish were sampled at similar times of the day as the treatment animals to control for the presence of circadian or ultradian rhythms of gene expression. This large sample size allowed for the robust identification of genes whose differential expression was most likely attributed to environmental hypoxia and not to inter-individual or day-to-day variation in gene expression. We applied ANOVA to identify 650 genes that were differentially expressed during the time course of hypoxia and reoxygenation. Of these genes, 565 shared homology with annotated genes in the SwissProt database whereas the remaining 135 genes had no homology to annotated public sequences.

To help interpret the data, the genes were grouped into seven *K*-means clusters, which shared broad similarities in their pattern of temporal expression during hypoxia (Fig. 1A). The clusters reveal that the general regulatory response of liver to hypoxia was to induce gene expression, with transcript levels of 425 genes increasing, although transcripts of 225 genes declined. Furthermore, most genes showed a pattern of increasing or decreasing transcript level as a function of the duration of hypoxia, and the peak expression or repression of 67% of the genes fell on the 120 h final hypoxia time point (436/650 genes). Cluster 1 captured a set of 60 genes that were induced early in response to hypoxia and whose expression remained elevated even after 24 h of recovery in aerated seawater. Clusters 2 and 3 captured sets of 133 and 127 genes that were induced later during the time course of hypoxia exposure and whose expression generally declined during recovery. Cluster 4 included 48 genes whose transcript levels rose after 48 h of hypoxia and whose expression persisted during recovery. Cluster 5 comprised 57 genes whose expression was elevated during the first 72 h of hypoxia and was notable because it included 20 genes that encode subunits of the 60S ribosomal structure and six genes for subunits of the 40S ribosomal structure. Clusters 6 and 7 represented 113 and 112 genes whose expression declined in response to hypoxia. The complete list of differentially expressed genes with accession numbers is available as supplementary material Table S1.

The list of differentially expressed genes identified in this study included a number of genes involved in energy metabolism that had been identified in a previous gene expression screen of *G. mirabilis* liver during hypoxia (Gracey et al., 2001). For example, lactate dehydrogenase A, enolase, triosephosphate isomerase, glucose-6-phosphate dehydrogenase and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase were identified in both data sets and their induction is believed to play a role in the mobilization of glycogen stores and the breakdown of the released glucose by glycolysis. By employing a more comprehensive microarray (Gracey, 2008), the current data set extends the list of hypoxia-inducible glycolytic genes in liver to include hexokinase, glucose-6-phosphate isomerase, aldolase B, phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase. Furthermore, our data revealed that transcript levels of the SLC2 A1 glucose transporter 1 gene (Glut1) are elevated, suggesting that some of the mobilized glucose is also transported out of the liver during hypoxia. The transcriptional activation of glycolytic genes and glucose transporters during hypoxia is attributed to the binding of the transcription factor hypoxia-inducible factor 1 α (Hif1α) (Semenza et al., 1996), which accumulates during hypoxia (Jiang et al., 1996) because of the oxygen-dependent reduction in the rate at which it is degraded (Epstein et al., 2001). Transcript levels for Hif1α in *G. mirabilis* liver increased during the duration of the hypoxia exposure, suggesting that enhanced transcription of the gene might be an additional mechanism to elevate HIF1α levels during prolonged periods of hypoxia. The induction of HIF1α mRNA has been reported previously in a hypoxia-challenged species of bream fish (Shen et al., 2010). It was interesting to note that, for many genes, recovery of the fish in oxygenated water did not restore expression back to levels observed in normoxia, suggesting this expression program is not under the sole control of this oxygen-sensing pathway and that other signaling pathways remain active during recovery.

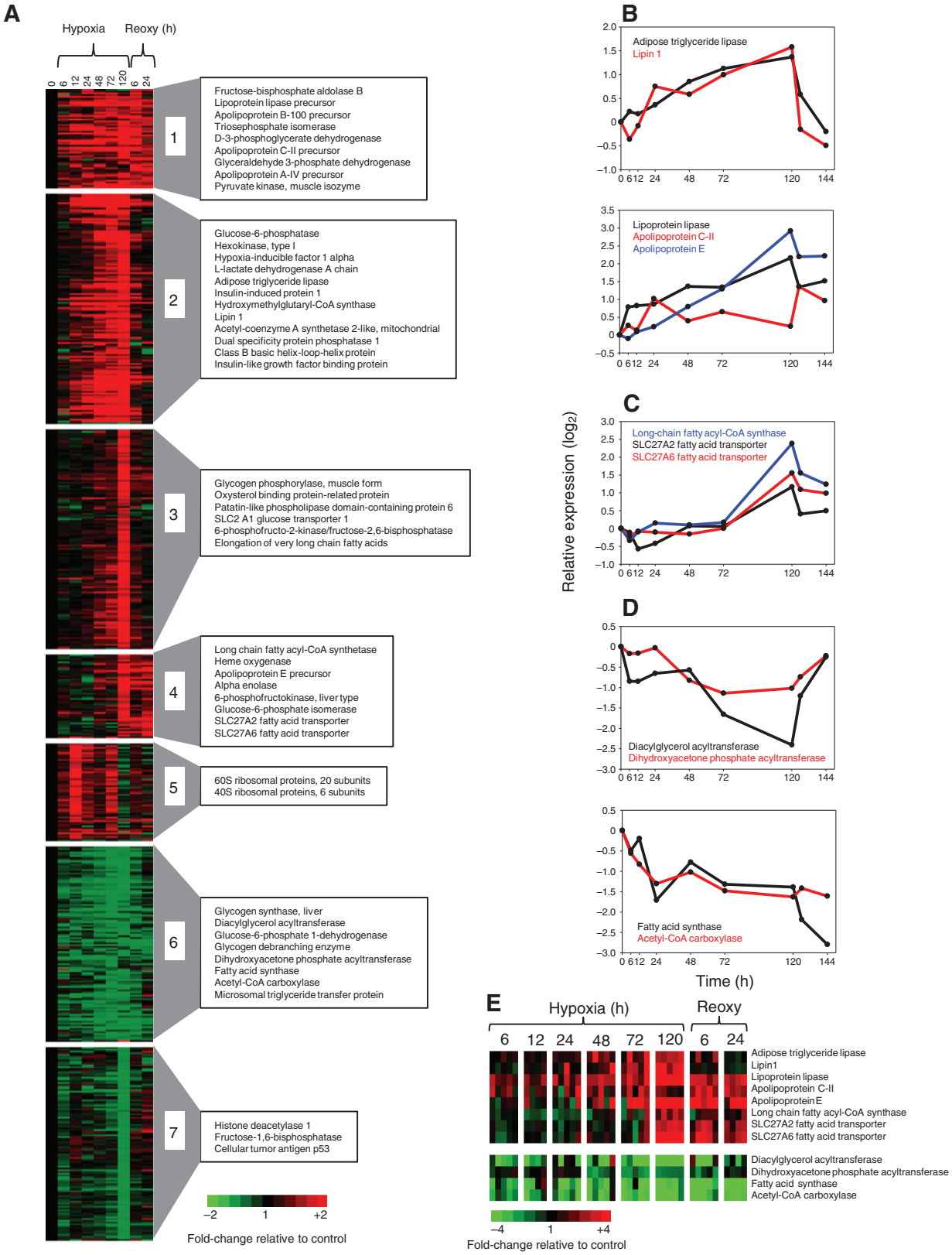

Fig. 1. Gene expression patterns observed in liver tissue of *Gillichthys mirabilis* challenged to hypoxia. (A) *K*-means clusters of genes that exhibited statistically significant differential expression in response to hypoxia. Each row corresponds to a single gene and each column corresponds to a particular time point in the experimental time course. The median expression of each gene at each time point is represented by a color, with red or green indicating that the gene is upregulated or downregulated, respectively, relative to the expression observed in normoxic control animals. Genes whose expression is particularly pertinent to hypoxia are indicated next to each cluster. The specific expression of selected genes with roles in lipolysis (B), fatty acid transport and activation (C), and triglyceride synthesis (D) are shown. (E) Heat map showing the expression levels of selected genes in individual fish sampled at each time point.

In order to obtain an objective interpretation of the functional significance of the observed gene expression changes, we used an enrichment analysis tool to search for functional groups of genes or pathways that were more represented in the lists of differentially expressed genes that would have been expected by chance alone. This analysis revealed that carbohydrate metabolism was the most significant metabolic pathway represented within the differentially expressed lists of genes (P=0.000002), a result that was consistent with the observed induction of genes involved in glycogen metabolism and glycolysis. The next most significantly enriched metabolic process was that of lipid metabolism (P=0.0003), with particular emphasis placed on genes associated with the metabolism of triacylglycerides. Fig. 1B–D illustrates the expression of the major genes that contributed to the enrichment of lipid metabolism. Central to this theme was the hypoxia-induction of the ATGL gene [alternative name patatin-like phospholipase domain containing 2 (PNPLA2); Fig. 1B], which catalyzes the initial step in triglyceride hydrolysis (Zimmermann et al., 2004) and has a central role in the degradation of lipid droplets, also known as adiposomes (Smirnova et al., 2006). A similar induction profile was observed for a key regulator of lipid metabolism, lipin 1 (Reue and Dwyer, 2009) (Fig. 1B). Environmental hypoxia also induced the expression of a second lipase, lipoprotein lipase 1, as well as apolipoproteins A-IV, CII, B-100 and E (Fig. 1A,B). Lipoprotein lipase 1 is localized to the endothelial cell surface and binds to circulating chylomicrons and lipoproteins, hydrolyzing the triglycerides they carry, while apoliproteins cooperate in this process and help to transport the lipids for uptake and use (Medh et al., 2000; Wang, B. et al., 2009). Hydrolysis of triglycerides releases both glycerol and fatty acids, which are then available for transport or metabolism. Our expression data suggests that fatty acid transport is elevated during hypoxia because we observed that the fatty acid transporter genes, SLC27 A2 and A6, followed a similar pattern of induction as that of ATGL (Fig. 1C), and these genes facilitate the translocation of fatty acids across the hepatocyte plasma membrane (Stahl, 2004). Interestingly, transcript levels for long-chain fatty acyl-CoA synthetase, the gene that catalyzes the activation of fatty acids to fatty acyl CoA, was upregulated and follows a similar profile to that of the fatty acid transporter genes (Fig. 1C). This is a somewhat surprising finding given that this reaction, a pre-step to the β-oxidation of fatty acids or their incorporation into phospholipids, requires ATP, which should be scarce and at a premium during hypoxia. The expression data did not offer any direct clues as to the fate of the released glycerol. Metabolism of glycerol requires that glycerol is first phosphorylated by glycerol kinase and then converted to dihydroxyacetone phosphate by glycerol-3-phosphate dehydrogenase. Both glycerol kinase and glycerol-3-phosphate dehydrogenase were present on our microarray but were not differentially expressed. Not every enzyme is regulated by altered gene expression and the absence of an induction of these genes at the transcriptional level does not preclude that glycerol is metabolized during hypoxia.

In contrast to the pattern of induction observed for genes involved in lipolysis, genes involved in triglyceride synthesis were downregulated by hypoxia. Hypoxia exposure resulted in the reduced expression of dihydroxyacetone phosphate acyltransferase, which catalyzes the addition of acyl-CoA chains to dihydroxyacetone phosphate, as well as diacylglycerol acyltransferase, which catalyzes the terminal step in the transfer of acyl-CoA chains into triglycerides (Casaschi et al., 2005) (Fig. 1D). Similarly, two key genes involved in the synthesis of fatty acids, acetyl-CoA carboxylase and fatty acid synthase, were observed to be downregulated during hypoxia (Fig. 1D). Although these fish were wild caught and out-bred, the individual animals sampled at each time point exhibited similar patterns of differential expression of these lipid metabolic genes (Fig. 1E). Together, the observed pattern of differential expression suggests that pathways involved in the mobilization of liver triglycerides stores and blood triglyceride–lipoprotein complexes are upregulated by hypoxia, whereas pathways involved in the synthesis of fatty acids and storage of fatty acids are downregulated.

These findings led us to search for further evidence for changes in triglyceride metabolism in the hypoxia-challenged fish. Histological inspection of liver tissue from control normoxic and fish exposed to 120 h of hypoxia revealed gross morphological differences in tissue structure. Under normoxic conditions, the liver tissue contains large polygonal vacuoles and the nucleated hepatocytes are compressed around the periphery of these vacuoles (Fig. 2A). In hypoxic fish, the vacuoles were observed to be shrunken and took on a more spherical appearance (Fig. 2B) (larger histological images are available as supplementary material Fig. S1). This change was observed consistently in all five individual fish sampled at the hypoxic 120 h time point. To identify the compound that was present in these vacuoles, frozen sections of liver tissue from control fish were prepared and stained with osmium tetroxide, which binds to double bonds in unsaturated lipids and imparts a brownish black color to lipid-dense areas of the stained tissue. The contents of the vacuoles were darkly stained, indicating that the vacuoles contained lipids and were probably droplets of triglyceride. To quantify the change in stored lipid during hypoxia, ^{1}H NMR spectroscopy was used to quantify the total triglyceride in lipid extracts of tissue from control and 120 h hypoxic animals. These data support the histological evidence and confirmed that triglyceride levels declined 2.7-fold during the course of hypoxia exposure, falling from 75.65 mol l^{-1} g^{-1} tissue triglyceride in control fish to 27.72 mol l^{-1} g^{-1} in hypoxic fish (P=0.002; Fig. 2D).

Glycogen stores are considered the primary source of stored energy for organisms that experience hypoxia in their natural environment, with hypoxia-tolerant fish such as the crucian carp (*Carassius carassius*) containing the highest levels of stored glycogen of any vertebrate (Hyvarinen et al., 1985). Quantification of hepatic glycogen in these liver samples revealed that glycogen stores were significantly depleted 40-fold after 72 h of hypoxia (P=0.004) and remained depleted even after 4 days of recovery in oxygenated water (P=0.009) (Fig. 2E). Similarly, staining of histological sections with period acid-Schiff reagent revealed a dramatic drop in the number of glycogen storage granules present in the liver after 5 days of hypoxia (supplementary material Fig. S2).

To investigate the molecular mechanism responsible for the decline in stored triglycerides, we measured the level of ATGL protein in control and 120 h hypoxic fish using western blot analysis. The rabbit anti-mouse ATGL polyclonal antibody detected a single immunoreactive band on western blots of ~53 kDa, which agrees well with the 466-amino-acid length of the *G. mirabilis* ATGL gene (NCBI accession number ACY30627.1). Densitometric analysis of the western blot data revealed that ATGL protein levels were 3.18-fold elevated in the livers of hypoxic fish (P=0.00005) compared with those of control fish maintained under normoxic conditions (Fig. 2F). ATGL was our top candidate gene for the mobilization of triglyceride because it has been implicated as playing a key role in lipolysis in a variety of organisms, including *Drosophila* (Gronke et al., 2005), *Caenorhabditis elegans* (Narbonne and Roy, 2009) and birds (Nie et al., 2010). Our results support previous evidence

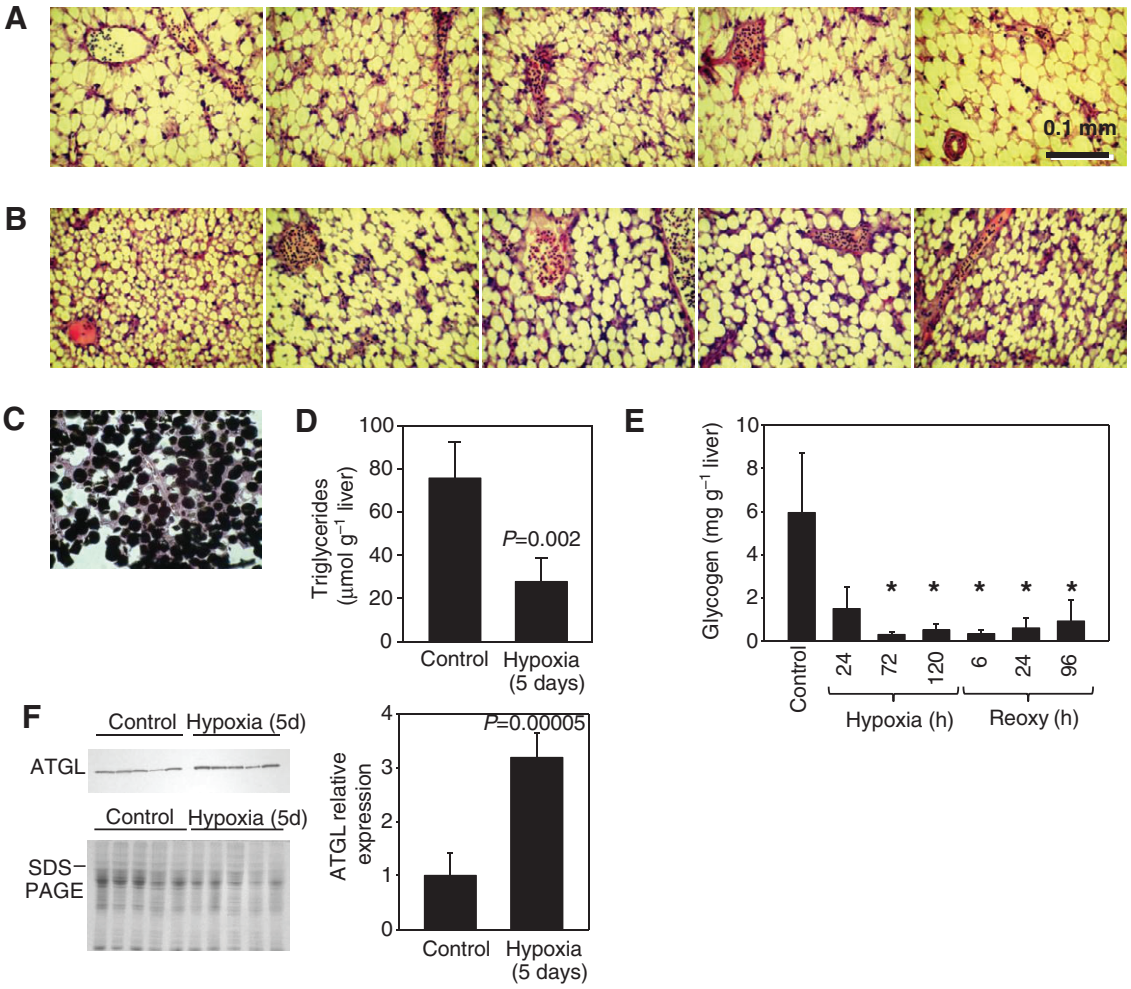

Fig. 2. Triglyceride stores are mobilized in the liver of hypoxia-challenged *Gillichthys mirabilis*. Representative sections of liver tissue sampled from five individual fish under control normoxic conditions (A) and after 120 h of hypoxia (B), stained with hematoxylin and eosin. The images are all taken at the same magnification (larger images of these sections are available as supplementary material Fig. S1). (C) A section of frozen tissue stained for triglycerides using osmium tetroxide. (D) Triglyceride levels measured by ^{1}H NMR in corresponding liver samples under conditions of normoxia or 120 h hypoxia (same individual fish as shown in A and B). Values are means ± s.d. (N=5). (E) Glycogen levels declined during hypoxia and recovery. Values are means ± s.d. (normoxia N=6, hypoxia and reoxygenation N=4). Asterisks indicate that glycogen levels are significantly different from normoxic samples (P<0.01). (F) Western blot analysis of the levels of ATGL protein in liver samples under conditions of normoxia or 120 h hypoxia (same individual fish as shown in A and B). Immunopositive bands (upper panel) and a corresponding Coomassie-stained gel (lower panel) are shown. The western blot and gel image were quantified densitometrically, and the pixel intensity for each western band was normalized to the total pixel intensity of the corresponding lane in the gel image. Values are means ± s.d. (N=5).

that showed that ATGL protein abundance is principally regulated by differential mRNA expression (Villena et al., 2004).

Our findings indicate that coordinated changes in triglyceride metabolism occurred in the liver of *G. mirabilis* during hypoxia exposure. The transcriptome data suggest that these changes are brought about by means of the transcriptional control of a number of metabolic enzymes and that their differential expression occurs late during hypoxia. Triglycerides are stored predominantly in the liver and muscle of fish, with additional stores in abdominal adipose tissue of certain species (Henderson and Tocher, 1987). Previous studies on lipid metabolism during hypoxia in fish have reported contrasting responses, with some studies indicating that lipolytic activity decreases in hypoxia-tolerant fish such as carp (Vanraaij et al., 1994; Vanraaij et al., 1996) but increases in more sensitive species such as trout (Haman et al., 1997). Most of this evidence is derived from quantification of fatty acids in plasma and it is unclear whether these changes reflected altered metabolism of triglycerides or membrane phospholipids (Van den Thillart et al., 2002). Overall, the emerging picture regarding hypoxia and lipid metabolism in fish is that lipolysis is relatively independent of the lipolysis-stimulating effects of hypoxia-induced catecholamine release (Van den Thillart et al., 2002). This is in contrast to the effects of hypoxia on mammals, where a frequent hallmark of hypoxia or ischemia is a rise in glycerol and free fatty acid levels because hypoxia stimulates lipolysis in some tissues (Yin et al., 2009) while at the same time fatty acids accumulate because the β-oxidation of fatty acids is inhibited by the absence of oxygen (Gimm et al., 2010). Interestingly, recent evidence in *Drosophila* has implicated a role for lipid metabolism in adaptation to severe chronic hypoxia, and adapted flies were found to express elevated transcript levels of lipid genes including Brummer lipase, the fly ortholog of ATGL (Azad et al., 2009).

Histological inspection of *G. mirabilis* liver indicated that a substantial portion of the liver volume is given over to stored triglyceride and externally their livers have a cream coloration, presumably due to the presence of lipid. Similar large vacuoles of triglyceride are observed in human patients with fatty liver disease, which is caused by the abnormal retention of lipids and is linked to a variety of metabolic syndromes (Hebbard and George, 2011). We have observed cream-colored livers in wild and laboratory-cultured *G. mirabilis*, suggesting that this is a natural phenomenon and not a peculiarity of their laboratory diet. Regardless, our data presents overwhelming evidence that triglycerides are mobilized during hypoxia in a concerted coordinated manner, suggesting that *G. mirabilis* retains the regulatory capacity to reorganize its metabolism throughout the time course of hypoxia. Interestingly, the differential expression of lipolysis genes occurred at the later time points of hypoxia, suggesting that the mobilization of lipid is perhaps a second phase response to hypoxia. Stores of glycogen were significantly depleted after 48 h of hypoxia and it is tempting to speculate that there might be a relationship in the timing of the mobilization of these two energy stores.

The fate of the glycerol and fatty acids released during hypoxia is unclear. Glycerol can be converted to glyceraldehye-3-phosphate and enter the glycolytic pathway with the same ATP cost as the conversion from glucose to glyceraldehyde-3-phosphate. However, the conversion yields FADH2, which requires a terminal electron acceptor, a role that is normally assumed by oxygen. It should be noted that *G. mirabilis* does respire during hypoxia, and opercular ventilation is exaggerated and rapid. The survival time of *G. mirabilis* at 15°C under anoxia is <1 h, and <24 h at 0.4 mg l^{-1} O_2 (A.Y.G., personal observation), indicating that their metabolism is not strictly anaerobic during hypoxia and that some oxygen is required for survival. Therefore, we cannot exclude the possibility that some oxidative phosphorylation does occur during hypoxia and that oxygen serves as an electron acceptor for FADH2. Nevertheless, the fish still faces the challenge of what to do with the released fatty acids. Under normoxic conditions, fatty acids are metabolized by β-oxidation in the mitochondria, but this requires oxygen, which will be scarce during hypoxia, and all available data indicate that fatty-acid β-oxidation is inhibited during cellular hypoxia (Gimm et al., 2010). Largely owing to their hydrophobic properties, fatty acids can exert harmful effects, causing cellular injury (Katz and Messineo, 1981; Wang, Y. et al., 2009), and cytotoxicity is reported in certain syndromes in which β-oxidation is reduced. This suggests that there might be a limit to the extent to which stored triglyceride can be hydrolyzed before the effects of elevated fatty acids becomes deleterious or that *G. mirabilis* is tolerant of high levels of free fatty acids.

In many organisms, lipid metabolism is controlled by the endocrine system and the principle regulatory molecule is the peptide leptin (Zhang et al., 1994). Leptin is expressed in adipocytes and acts as a regulator of lipid stores by altering appetite and changing the rate of lipolysis (Reidy and Weber, 2000). Leptin is reported to regulate a number of the key genes that were identified in our study. Elevated levels of leptin stimulates the breakdown of stored triglyceride and *in vitro* studies have confirmed that ATGL is a leptin target, with ATGL mRNA and protein levels increasing with administration of leptin (Li et al., 2010). Similarly, leptin inhibits triglyceride synthesis by inhibiting the expression of acetyl-CoA carboxylase, a key gene involved in triglyceride synthesis (Bai et al., 1996). Furthermore, recent studies have indicated a link between leptin and hypoxia, with hypoxia inducing the expression of leptin in cultured adipocytes (Wang et al., 2008), and evidence that Hif1 binds to the leptin gene promoter during hypoxia and stimulates its transcription (Grosfeld et al., 2002). Similarly, expression of the receptor for leptin also increases during hypoxia (Klaffenbach et al., 2011). Leptin expression is also elevated in zebrafish under chronic hypoxia and was correlated with the Hif1 expression (Chu et al., 2010). Together, the reported links between hypoxia, leptin and ATGL expression make leptin a candidate regulator of the hypoxic induction of lipolysis in *G. mirabilis* liver.

CONCLUSIONS

In summary, we explored transcriptional changes in the liver during a time course of hypoxia exposure and correlated a subset of the expression pattern to phenotypic changes in the liver. Data collected at the levels of transcript and protein expression, tissue morphology and biochemistry identified triglycerides as an energy reserve that is mobilized during exposure to prolonged environmental hypoxia. Detailed metabolomic and metabolic labeling experiments will be required to resolve the precise fate of the glycerol and fatty-acid products of triglyceride hydrolysis, but we hypothesize that the glycerol enters that glycolytic pathway and helps to sustain glycolysis during extended exposure to hypoxia. Finally, the role of leptin in the hypoxia response is not fully understood and induction of triglyceride hydrolysis in *G. mirabilis* may represent a new model to investigate the regulation and functional significance of leptin in hypoxia acclimation.

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