Title: ***In vitro*** **study on the regulation of cellular mRNA levels by changes in transcription rate and transcript stability in fish red blood cells**

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Abstract

The interplay of transcriptional and posttranscriptional processes in the regulation of gene expression has been extensively studied in mammals but little is known in other vertebrates so far. Most non-mammalian vertebrates are faced with environmental cues and stressors distinct from those experienced by mammals and thus it is likely that the gene expression strategies differ from those of mammals. Here we performed experiments to study *in vitro* the various levels of gene expression regulation in nucleated fish red blood cells. Three critical environmental cues frequently experienced by fish were chosen: exposure to hypoxia (2.5%), ambient water temperature increase by 10°C (from 10°C to 20°C), and exposure to stress hormones (represented by the β-adrenergic agonist isoproterenol). We found that β-adrenergic stimulation increases the stability of the β_{3b}-adrenergic receptor (*β_{3b}-ar*) mRNA, suggesting that mRNA stability can play a role in the regulation of hormonal stress responses in fish. The *β_{3b}-ar* gene encodes a unique β-adrenergic receptor subtype in fish red blood cells which controls the β-Na^+/H^+ exchanger activity – an important component of responses to oxygen limitations. Our results furthermore show a yet undescribed link between the Hif1a signaling pathway and the β-adrenergic receptor response. After β-adrenergic stimulation, the transcription of *hif1a* was activated significantly after 4 hours of exposure. So far, such a response has only been described from mammalian species. This indicates that the β-AR is fundamental to the molecular and physiological responses to hypoxia and that Hif1a might have additional functions than those already known.

1. Introduction

Modulations of gene expression at the transcriptional and post-transcriptional level are central cellular strategies in response to any kind of stimulus and crucial for proper adaptation. Gene expression involves various steps, including transcription, conversion of nascent transcripts to mature mRNA, mRNA degradation, translation, and posttranslational modification of proteins, and all of them are tightly controlled and regulated. Changes on the transcriptional level include the activation or inhibition of gene expression, whereas important post-transcriptional events include degradation and/or stabilization of mRNA species. Cellular steady-state mRNA levels are determined by both transcription rate and mRNA decay. The cells' ability to regulate gene expression is important in the adaptation to environmental changes (López-Maury et al., 2008). To date, little is known about the link between transcriptional and post-transcriptional gene expression processes and their regulation in animals other than mammals (de Nadal et al., 2011). Likewise, the roles of the transcriptional and post-transcriptional processes in the cellular adaptation to environmental cues are not well understood in non-mammalian species. Although many control mechanisms show extensive conservation across eukaryotes, most non-mammalian vertebrates are faced with environmental challenges and stressors distinct from those experienced by mammals. Hence it might be misleading to assume that the responses are the same in mammals and for example fish.

Standard gene expression profiling methods, such as quantitative real-time PCR (qPCR), microarray, or RNA-seq, are giving good measures of cellular steady-state mRNA levels, but they do not determine changes in transcription rate or transcript stability (Hayles et al., 2010). With those methods it is thus not possible to determine whether alterations in steady-state levels are a result of changes in transcription rate or of post-transcriptional regulatory mechanisms. Only a few prior studies have so far addressed the interrelation between transcriptional changes and alterations of mRNA decay rates in fish (e.g. Sadar et al., 1996; Bemanian et al., 2004; Ehrmann et al., 2004; Bremer and Moyes, 2014) and many

mechanistic details are still unknown (de Nadal et al., 2011). In salmon hepatocytes Bemanian et al. (2004) found that environmental contaminants inhibited the activation of vitellogenin and estrogen receptor alpha gene transcription while they did not influence the stability of the respective transcripts. Flouriot et al. ((Flouriot et al., 1996)) showed that a 17β-estradiol-mediated increase in vitellogenin steady-state mRNA was the result both of an increased transcription rate and a stabilization of the transcript. Exposure to stress caused by other factors such as changes in temperature or in external pH, exposure to oxidative stress or changes in food availability also strongly affects gene expression (de Nadal et al., 2011). Temperature for example has clear influence on the decay rates of cytochrome c oxidase subunits, which is responsible for observed deviations from stoichiometry between subunit steady-state transcript levels in goldfish (Bremer and Moyes, 2014).

None of the studies so far has comparatively investigated the various levels of gene expression in genes of different regulatory pathways under the same environmental cues. Hence, the objective of our study was to reveal the contributions of transcriptional (synthesis) and post-transcriptional (decay) processes to cellular steady-state mRNA levels of selected genes under various environmental stresses. The products of the representative genes possess important functions in teleost red blood cells (RBCs, erythrocytes). We included genes encoding the oxygen carrier protein haemoglobin a-1 (*Hba-1*) and the membrane-bound oxygen binding protein globin x (*Gbx*), a hypoxia-inducible transcription factor (*hif1a*) and a structural protein (*β-actin*). Furthermore, we studied genes encoding the β_{3b}-adrenergic receptor (*β_{3b}-AR*)/ β-Na^+/H^+ exchanger (*β-NHE*) system. This fish RBC-specific system is induced by catecholamines which are secreted into the circulation as an acute response to a wide variety of severe stresses such as anaemia, hypoxia, exhaustive exercise (reviewed in (Perry and Bernier, 1999)). Fish RBCs are a very good *in vitro* model system to investigate transcriptional and post-transcriptional processes because they retain their nucleus, ribosomes and other organelles, which are necessary for all steps in gene expression. In contrast to their mammalian counterparts, they are transcriptionally and

translationally active; however, they lose important functions during their life span in the circulation (Lund et al., 2000; Speckner et al., 1989). In older RBCs, RNA concentration and general protein synthesis are decreased, resulting for example in a reduced ability to respond to heat stress (Lund et al., 2000). While young RBCs are able to respond to changes in their immediate environment effectively, older RBCs serve mainly as oxygen transporters.

In three experiments we exposed fish RBCs to stress stimuli representing critical environmental cues frequently experienced by fish in the wild. We then measured the transcription rate of genes by the nuclear run-on assay (NRO) and the changes in mRNA decay of the corresponding transcripts by actinomycin D (ActD) treatment. The results are linked to the cellular transcript levels of the respective genes. We examined the effects of oxygen limitation (hypoxia; 2.5 % O_2), increases in ambient water temperature (from 10°C to 20°C), and exposure to stress hormones (catecholamines). Our study gives important comparative insights into cellular stress response and gene expression strategies in fish.

2. Materials and methods

2.1. Animals and blood sampling procedures

Rainbow trout (*Oncorhynchus mykiss*, *N* = 12, weight 835.8 ± 302.6 g) were obtained from a commercial hatchery (Finnish Institute for Fisheries and Environment, Parainen, Finland). The fish were reared under natural photoperiod and under seasonal influences of water temperature since tank water is pumped from the nearby Baltic Sea. At the date of sampling (May, experiment 1; July, experiments 2 & 3), the water temperature was 10°C and 14°C respectively. In the different experiments, we used the same temperature that the fish were acclimated to in the hatchery to avoid temperature responses in cases they were not a part of the experimental protocol. All procedures were approved by the Finnish Animal Experiment Board (ESAVI/3705/04.10.07/2015).

128 The fish were killed by a blow on the head, their weight and length measured, and blood (~6
129 to 8 ml) sampled from the caudal vessel into heparinized syringes, transferred into sterile
130 falcon tubes, and stored on ice. Blood was washed three times in saline (128 mM NaCl, 3
131 mM KCl, 1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.6 (Nikinmaa and Jensen,
132 1992) to remove the buffy coat consisting of white blood cells. The red blood cells were re-
133 suspended in fresh saline at a hematocrit (Hct) of 18 - 20% and then stored well-aerated
134 overnight at 10°C (experiment 1) 14°C (experiments 2 & 3) in cell culture flasks (75 cm^2) with
135 open caps. The over-night storing was done to assure that the cells were not in a
136 catecholamine-stimulated condition (Cossins and Richardson, 1985).
137 Before the experiment, RBC samples of four individual trouts were washed again in saline
138 and each sample was divided into three or four subsamples (1.2 to 2 ml each). In the
139 experiments (1 to 3), RBC samples were exposed to normoxia (= control), hypoxia (2.5%
140 O_2), and β-adrenergic stimulation under hypoxia (2.5% O_2). Simultaneous exposure to
141 hypoxic conditions has been found to potentiate the β-NHE response of RBCs in rainbow
142 trout (Brauner et al., 2002). In experiment 1, one subsample was additionally subjected to a
143 short heat shock, done by linearly increasing water temperature by 10°C.
144 Normoxic conditions (control) were achieved by aerating the samples with humidified air
145 using an aquarium pump. Humidified gases for hypoxic conditions (2.5% O_2, 0.1% CO_2,
146 balanced with N_2) for both hypoxia and β-adrenergic stimulation experiments were provided
147 by a gas mixing flowmeter (Cameron Instrument Company, Texas, USA). The CO_2 values
148 are much lower than in blood and slightly higher than in air, but preliminary experiments
149 showed that this slightly higher CO_2 concentration compared to air had neither an effect on
150 the genes under study nor on the mechanisms investigated. In β-adrenergic stimulation
151 experiments, RBCs were stimulated by the addition of the β-adrenergic agonist
152 isoproterenol.
153
154 *2.2. Experimental procedures*

Two different experiments were performed to determine the transcription rate using the nuclear run-on assay (Sambrook and Russell, 2006). In the first experiment (Exp. 1), RBCs were exposed to different types of short-term stress, while in the second experiment (Exp. 2) the time-course of changes in transcription rates was examined for 4 h and 10 h under three different conditions. Furthermore we determined the stability of the transcripts (Exp. 3) using the unselective transcriptional inhibitor actinomycin D.

2.2.1. Experiment 1: Effect of stress conditions on transcription rate

Washed RBCs were counted using a hemocytometer before the start of the experiments. Prior to the start of the experiment, empty glass flasks were equilibrated to normoxic or hypoxic conditions (2.5% O_2, 0.1% CO_2, balanced with N_2) for 1 h. After that, individual RBC subsamples were filled in glass flasks and submerged into a water bath (10°C; Lauda, Germany). In hypoxia treatments, flasks were then exposed to 2.5 % O_2 (0.1% CO_2, balanced with N_2) for 1 h. In β-adrenergic stimulation treatments, RBCs were first pre-exposed to the same hypoxic conditions for 0.5 h before 10 µl (per mL of RBC sample) of isoproterenol (10^{-5} mol L^{-1}, final concentration; (Brauner et al., 2002; Tetens et al., 1988) was added. Then the adrenergically stimulated RBCs were maintained in the hypoxic conditions for another 10 min before sampling, because it has been shown that the adrenergic reaction peaks between 2 and 10 min after addition of the agonist in rainbow trout RBCs (Tetens et al., 1988). At the same time, 10 µl of saline was added to each flask of the normoxic control, hypoxia and heat treatment groups to maintain the same dilutions across all treatments. In the heat stress treatments, RBCs were exposed to normoxic conditions for 0.5 h before the temperature was increased linearly by 10°C in 90 min (1°C/9 min), i.e. to 20°C, and then kept at 20°C for 10 min before sampling. All flasks were regularly agitated throughout the incubation time. Aliquots corresponding to 5 x 10^7 cells were taken from each flask for nuclear run-on assay and analysis of steady-state RNA levels at the end of the respective exposure time. Isolation of nuclei was done as described below and samples for steady-

state levels were frozen at -80°C. Samples for determination of Hct were taken before and at the end of the experiments.

2.2.2. Experiment 2: Changes of transcription rate during long-term stress

In the long-term exposure experiment, RBC suspensions were exposed to control, hypoxia and β-adrenergic stimulation under hypoxia for up to 10 h at 14°C. Conditions (e.g. gases used, pre-treatment of flasks and washing of RBC samples) were the same as in Exp.1. Aliquots of RBC suspensions (corresponding to 5 x 10^7 cells) were removed from each flask after 0, 4, and 10 h for nuclear run-on assay and steady state RNA analysis. Isolation of nuclei was done as described below and samples for steady-state levels were frozen at -80°C. For determination of Hct, aliquots were taken at time points 0 h, 0.5 h, 4 h, and 10 h in β-adrenergic stimulation treatment, while in controls aliquots were only taken at 0 h and 10 h.

2.2.3. Experiment 3: Determining mRNA stability

Stability of mRNAs (transcript half-life) was examined over 12 h in normoxia, hypoxia, and β-adrenergic stimulation under hypoxia at 14°C. Synthesis of new transcripts was blocked by adding actinomycin D (final concentration 2.5 µg/ml). Prior to this experiment, we determined the appropriate concentration of actinomycin D (ActD) to inhibit transcription in rainbow trout RBCs efficiently for at least 12 hours (data not shown). The pre-experiment was performed under normoxic conditions with 2.5 µg/ml and 5 µg/ml (final concentration) of ActD which are within the range of widely used concentrations (1-10 µg/ml; e.g. (Bemanian et al., 2004; Bensaude, 2011; Sadar et al., 1996). Since the results indicated no difference in the trends of the individual transcripts between the two concentrations, we decided on the lower concentration (2.5 µg/ml) for the following experiment to reduce the physiological disturbance of the cells and to avoid potential toxic side effects caused by the ActD treatment (Lu et al., 2015; Soeiro and Amos, 1966).

In experiment 3, samples were taken every 4 hours over 12 hours after the addition of ActD and frozen at -80°C until RNA isolation. In β-adrenergic stimulation treatments, RBCs were first pre-exposed to hypoxia and stimulated by isoproterenol (10^{-5} mol L^{-1}, final concentration) for 30 min before addition of ActD. Hct samples were taken before and then every 4 h in β-adrenergic stimulation experiments and every 8 h in normoxic control samples. Results are expressed as percentage relative to time point zero (t = 0). Regression analysis was used to test the best fitting regression model (exponential or linear) for each transcript and revealed that the linear regression was the better fit for the data. The half-life of each transcript ($t_{1/2}$; Table 2) was then calculated from the linear estimation equation of each regression line.

2.3. Analytical procedures

2.3.1 Determination of Hematocrit

Activation of the β-NHE via the β-AR pathway by the addition of the β-adrenergic agonist isoproterenol results in a swelling of RBCs which can be followed by an increased Hct. The Hct of each blood sample in the experiments (1-3) was measured in duplicate in micro-hematocrit capillaries containing approximately 30 µL of blood after 10 and 30 min respectively. Capillaries were centrifuged at 13,000 rpm for 3 min in a Hettich Hematocrit centrifuge.

2.3.2. Preparation of nuclei

Nuclei were prepared from 5 x 10^7 red blood cells. Red blood cells were washed 3 times by re-suspension in ice-cold, sterile 1 x PBS (140 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and centrifugation at 500 xg for 5 min at 4°C. The washed pellet was then re-suspended in 4 ml ice-cold, sterile lysis buffer (10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 10 mM NaCl, 1% Nonidet® P-40), incubated 5 min on ice, and nuclei were collected by centrifugation (500 xg, 4°C). The nucleus pellet was gently washed with lysis buffer devoid of Nonidet® P-40 and nuclei were collected again by centrifugation. After centrifugation,

nuclei were re-suspended in 100 µl ice-cold glycerol storage buffer (50 mM Tris-HCl, pH 8.0, 30% glycerol, 2 mM $MgCl_2$, 0.1 mM EDTA) and stored at -80°C.

2.3.3. In vitro transcription and purification

The nuclear run-on assay (NRO) method was used to quantify transcriptional activity in RBCs with modifications to meet the physiological requirements of fish. Most protocols available are optimized for mammalian cell lines and typically use *in vitro* assay temperatures between 26°C and 37°C (Bemanian et al., 2004; Sambrook and Russell, 2006; Smale, 2009) even when studying temperate fish species (Sadar et al., 1996; Bemanian et al., 2004). But these conditions are inappropriate for many ectothermic animals, as they are outside the physiological range for example of salmonid species. In order to meet the specific temperature requirements of the species, we made adaptations to the protocol and lowered the labeling temperature during the *in vitro* transcription assay to 20°C. Further, we prolonged the labeling time to 1.5 hours to achieve sufficient incorporation of biotin-16-dUTP into the transcript at the low assay temperature. Transcription rate was measured using labelling with biotin-16-dUTP according to Patrone et al. (2000). Frozen nuclei were thawed and one volume of 2 x transcription buffer (10 mM Tris-Cl, pH 8.0, 5 mM $MgCl_2$, 300 mM KCl) containing 10 µl of a 10 x Biotin RNA labeling Mix (containing 3.5 mM biotin-16-UTP, 10 mM ATP, 10 mM CTP, 10 mM GTP; Roche Applied Science, Germany) was added. The mixture was incubated for 1.5 h at 20°C with gentle agitation. The reaction was stopped by adding 6 µl of 250 mM $CaCl_2$ and 6 units DNase I (Promega, Madison, USA), and incubated for another 15 min at 20°C. The nuclear run-on RNA was isolated using TriReagent LS (Molecular Research Center, Cincinnati, USA) according to the manufacturer's instructions. Biotin-labeled RNA was re-suspended in 40 µl of RNase-free water.

2.3.4. RNA binding to magnetic particles

Streptavidin magnetic particles (40 µL; Roche Applied Science, Germany) were washed 3 times in binding buffer (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 7.5) and then mixed

with an equal volume of biotin-labeled RNA. After incubation for 30 min at room temperature with gentle agitation, particles were washed 2 times in washing buffer (10 mM Tris-HCl, 1 mM EDTA, 1 M NaCl, pH 7.5) and then eluted in 30 µl of RNase-free water by incubating them for 10 min at 80°C. The concentration of the RNA samples was measured using a NanoDrop 2000 (Thermo Fisher Scientific) and nuclear run-on RNA was stored at -80°C.

2.3.5 RNA extraction and cDNA synthesis

mRNA was extracted using TriReagent (Molecular Research Center, Cincinnati, USA) according to the manufacturer's instructions from a separate red blood cell sample. Integrity of RNA was assessed by bleach gel analysis (Aranda et al., 2012), and concentration and quality was checked using a NanoDrop 2000 (Thermo Scientific). Only samples with $OD_{260/280}$ and $OD_{260/230}$ >1.8 were processed further. RNA was digested using DNase I (Promega, Madison, USA) and 500 ng were then reverse transcribed using the RevertAid First Strand Synthesis Kit and random hexamer primers (Thermo Fisher Scientific).

2.3.6 Genes studied and plasmid standards

In order to evaluate the effects of changes in transcription rate and transcript stability on the cellular steady-state mRNA levels we studied 6 genes, whose products possess important functions in teleost RBCs and which represent a variety of biological functions. The β_{3b}-adrenergic receptor (*β_{3b}-ar*) and the β-Na^+/H^+ exchanger (*β-nhe)* play important roles in the stress response of fish RBCs. Haemoglobin a-1 (*hba-1*) and GlobinX1 (*gbx*) are two members of the vertebrate globin superfamily. Hypoxia inducible factor 1a (*hif1a)* is a transcription factor that responds to changes in cellular oxygen levels. β-Actin (*β-actin*) is involved in cell structure, integrity and motility and is often used as a reference gene in qPCR studies because of its high stability. Recombinant plasmids for standard curve analysis in quantitative real-time PCR (qPCR) were obtained by amplification of the gene of interest using specific primers (Table 1) and by cloning the purified PCR product

(NucleoSpin gel and PCR clean up kit; Macherey-Nagel, Düren, Germany) into the pJET 1.2 cloning vector with the CloneJET PCR Cloning Kit (Thermo Fisher Scientific). All products were sequenced by a commercial service (GATC, Konstanz, Germany). Primers for qPCR (Table 1) were designed using Primer3 (Koressaar and Remm, 2007; Untergasser et al., 2012), and checked for secondary structures using Beacon Designer Software™ and mfold IDT (http://eu.idtdna.com/UNAFold). Each primer pair was blasted against the rainbow trout database (NCBI) to ensure specificity, and qPCR products were cloned and sequenced for verification. The optimal cDNA concentration for qPCR was tested using serial dilutions of pooled cDNAs and the efficiency of each primer pair was tested on 10-fold dilutions of respective plasmid standards. All primer pairs produced a single peak in the melting curve.

2.3.7 Quantitative real-time PCR

The cDNA products were amplified in triplicates using the KAPASYBR FAST qPCR Master Mix (KAPA Biosystems) on a QuantStudio™ 12K Flex Real-Time PCR System (Applied Biosystems, Darmstadt, Germany). Each 10 µL reaction mixture contained cDNA template corresponding to 0.45 to 5 ng RNA and the cycling protocol consisted of 20 s at 95°C followed by 40 cycles of 1 s at 95°C and 20 s at 60°C. In a final step, specificity of primer and amplification was evaluated using dissociation curves with a temperature range from 60°C to 95°C. Each qPCR plate contained non-template controls to detect potential contamination in reaction mixes. Data were analyzed with the QuantStudio 12 K Flex software version 1.2. Copy numbers per µg RNA were calculated using the standard curve method using a plasmid standard (10-fold dilutions). Standard curve reactions were performed in duplicate.

2.4. Statistical Analysis

Results are presented as means ± SD. All data of experiments 1 and 2 were tested for equal variances (Brown-Forsythe) and normality (Shapiro-Wilk). An one-way repeated measures ANOVA was conducted for between-group comparisons of experiment 1, and a two-way

repeated measures ANOVA was used to analyze the results of experiment 2. In experiment 2 we also tested for interaction effects of time and treatment. Posthoc analyses were done with the Holm-Sidak test. The statistical analyses were performed with SigmaPlot 13. Linear regressions of experiment 3 were compared using the Real Statistics Resource Pack software (Release 4.3; www.real-statistics.com; Copyright 2013-2015) in Excel 2010 (Zaiontz, 2015). *P* values < 0.05 were considered statistically significant.

3. Results

3.1 Changes in hematocrit (Hct)

In each experiment we determined the changes in Hct to verify the activation of the β-NHE via the β-AR pathway. In Exp. 1, the increase in Hct within the 10 min exposure time was not statistically significant ($P = 0.085$), while in Exp. 2 and 3 the Hct remained significantly elevated throughout the duration of the experiments ($P = 0.013$, Exp. 2; $P = 0.001$, Exp. 3).

3.2. High phenotypic transcriptional variation after short stress in teleost RBCs

To gain better insight into the regulation of transcription rates under various conditions, we exposed RBC subsamples of individual fish to short stress stimuli. Stress stimuli in experiment 1 (Fig. 1) included either a linear temperature increase from 10°C to 20°C over 90 min, exposure to hypoxia (1 h, 2.5 % O_2), or a β-adrenergic stimulation of the RBCs with the adrenergic agonist isoproterenol (ISO; 10^{-5} mol L^{-1}) for 10 min under hypoxia (2.5 % O_2). In contrast to the steady-state levels, the transcription rate revealed high variability between treatments and high heterogeneity among individuals (Fig.1), resulting in non-significant responses for all genes because of the small number of replications that could be done. We determined the highest transcript copy numbers for *haemoglobin a-1* (*hba-1*) in both the NRO and the steady–state analysis (Fig. 1B). In contrast to that, *globin x* (*gbx*) showed the lowest copy numbers of all genes in the steady-state analysis. We were not able to determine transcription rates for *gbx* (Fig. 1E; Fig. 2E) because of only sporadically seen amplifications in all treatments. This is probably caused by amplification close to the

detection limit of qPCR and high variation among individuals and treatments (Crawford and Oleksiak, 2007; Whitehead and Crawford, 2005).

3.3. β-adrenergic stimulation induced significantly decreases in β_{3b}-receptor mRNA

In experiment 2, we followed the alteration in transcription rate and cellular steady-state levels during exposure to hypoxia (2.5 % O_2), or after β-adrenergic stimulation (ISO; 10^{-5} mol L^{-1}) under hypoxia (2.5 % O_2) for 10 hours. We found significant effects in both steady-state mRNA levels and transcription rate in three transcripts (two-way repeated measures ANOVA, posthoc analysis with the Holm-Sidak method, $P < 0.05$). Clearly the most significant alterations in mRNA abundance were found after β-adrenergic stimulation (Fig. 2). *β_{3b}-adrenergic receptor* (*β_{3b}-ar*) mRNA steady-state levels significantly decreased within 4 h and were still lower after 10 hours of treatment ($P = 0.026$ and $P = 0.019$; Fig. 2D). Within the 4 h and 10 h time point the β-adrenergic stimulated groups had significantly lower means compared to the normoxic group ($P = 0.043$ and $P = 0.006$; Fig. 2D). Exposure to hypoxia significantly decreased transcript levels of *β-Na^+/H^+ exchanger* (*β-nhe*) after 10 h compared to t=0 ($P = 0.043$; Fig. 2C) and of *β_{3b}-ar* compared to the normoxic group ($P = 0.028$; Fig. 2D). *Hif1a* showed increases in transcription rate after 4 h after β-adrenergic stimulation ($P = 0.039$; Fig. 2F). Within the 4 h time point, the β-adrenergic stimulated group significantly differed from the normoxic and hypoxic group ($P = 0.028$ and $P = 0.025$ respectively; Fig. 2F). All other transcripts in Exp. 2 showed no changes in transcript levels as a result of any of the treatments. No significant interaction effects of time x treatment were found in any of the transcript ($P > 0.05$).

3.4. β-adrenergic stimulation increases transcript stability of the gene encoding the receptor (β_{3b}-ar)

To estimate the half-lives of the transcripts, we determined the mRNA decay by inhibition of transcription using actinomycin D (ActD). We measured mRNA levels at specified times after ActD addition, and then calculated transcript half-life from the mRNA level-time regression

line equations (Fig. 3 and Table 2). The *β_{3b}-ar* transcript showed a significant increase in stability in response to β-adrenergic stimulation (P = 0.0312; Fig. 3D), and the half-live increased 3-fold (Table 2). In *hif1a*, *β-actin* and *hba-1* transcripts the estimated half-lives increased up to 2.5-fold in both hypoxia and β-adrenergic stimulation although the increase remained insignificant (Fig 3A, B, F; Table 2). The half-life of *gbx* mRNA was the lowest of the genes studied (approximately 5 h) (Fig. 3; Table 2).

4. Discussion

Our results showed that mRNAs which encode highly abundant proteins in RBCs, *hba-1* and *β-actin*, showed high steady-state levels and stability (Figs. 1, 2, 3). Notably, genes with a longer transcript half-life (Table 2) showed more stable expressions across treatments (Fig. 1) and over time (Fig. 2) than those with shorter half-life. This response is explained by the fact that we could prove neither a significant increase in transcription rate nor a significant stabilisation of the transcripts under hypoxia for those genes. However, if there was an increase in the mRNA stability of *β-actin* and *β-nhe* (the mean value was increased, but the increase was non-significant) (Table 2), it could explain the increasing steady-state levels between 4 h and 10 h of exposure (Fig. 2A, C).

Differences between treatments in experiment 1 (Fig. 1) remained statistically non-significant probably because of high phenotypic transcriptional variation. Because of the magnitude of individual variation, much larger sample sizes than presently used would be needed to obtain statistical significances of the differences. High phenotypic variation can be an effective adaptive strategy of cells to respond to environmental challenges and facilitate evolution (López-Maury et al., 2008). Another possible explanation for the lack of statistical effects could be that the short exposure times might not have been sufficient to induce significant changes in the transcription rate.

In the present experiments we did not study the influence of cell age on transcription rate and stability of transcripts. Circulating RBCs are a population of cells of different age classes

and cell ageing is associated with serious changes in many aspects of RBC physiology (Speckner et al., 1989; Lund et al., 2000; Phillips et al., 2000). In particular the ability to transcribe and translate genes is impaired in older RBCs. Since, however, all experiments were done with similar mixed cell populations from rainbow trouts of the same age, any influence of RBC age on the results can be ruled out.

The β-adrenergic response is an acute rescue response in RBCs of many teleost fish species, especially under hypoxic conditions and exhaustive exercise, to increase intraerythrocytic pH and thereby oxygen affinity of haemoglobin (Hb) (Motais et al., 1987; Nikinmaa, 1983). It is caused by catecholamines which are released into the circulation during severe stress (Perry and Reid, 1992), and which bind to the β_{3b}-adrenergic receptor (Nickerson et al., 2003) activating the membrane-associated β-NHE via a cAMP-dependent pathway (Mahé et al., 1985). Immediately upon adrenergic stimulation the number of receptors on the cell surface increases as a result of recycling of internalized receptors (Reid and Perry, 1991). A prolonged exposure leads to a desensitization of β-adrenergic receptors, and a reduced responsiveness to further hormonal stimulation, because receptors are internalized from the cell surface (Gilmour et al., 1994). A reduction of the mRNA levels of *β_{3b}-ar* in response to hypoxia is not involved in β_{3b}-AR desensitization (Fig. 1; Fig. 2) (Nickerson et al., 2002). Our study indicates that stabilizing *β_{3b}-adrenergic receptor* transcripts can be a strategy of teleost RBCs to produce more receptor proteins in response to long-lasting hormonal stimulation (Huch and Nissan, 2014) to counteract the simultaneous receptor internalization. Post-transcriptional regulation is a common mechanism by which gene expression is controlled in β_3-ARs (el Hadri et al., 1996), occurring both in fish and mammals with different functions of the receptors. In contrast, transcript destabilization upon prolonged agonist stimulation occurs in β_1-AR and β_2-AR subtypes (Danner et al., 1998; Hadcock et al., 1989; Mitchusson et al., 1998). Notably, although *β_{3b}-ar* and *β-nhe* are involved in the same signaling pathway in teleost RBCs, their gene expression strategies are different (Figs. 2, 3; Table 2). Earlier studies have found that *β-nhe* mRNA level correlates

433 with β-NHE activity but is independent of changes in *β_{3b}-ar* levels and number of β-ARs
434 (Koldkjaer et al., 2004).
435
436 The hypoxia inducible factor 1α (HIF1a) is the key regulator of the cellular response to
437 hypoxia. Previous studies in many species, including some fish, and cell types have reported
438 a mainly post-transcriptional regulation of HIF1a during hypoxia (Kaelin and Ratcliffe, 2008;
439 Soitamo et al., 2001). *Hif1a* transcription was surprisingly activated by β-adrenergic
440 stimulation under hypoxia but not under hypoxia exposure alone (Fig. 2F). The steady-state
441 mRNA level, however, remained unaffected (Fig. 2F). Prior studies suggested a link between
442 the hypoxia response and the β-adrenergic receptor (β-AR) signaling pathway, indicating
443 that the β-AR is fundamental to the molecular and physiological responses to hypoxia. The
444 β-agonist isoproterenol stabilized HIF1a and increased its levels in a mouse model (Cheong
445 et al., 2016) and the expression of known HIF-1a target genes was modulated by β-AR
446 agonists and independently of oxygen levels in human pancreatic cancer cell lines. The
447 authors furthermore showed that HIF-1a protein accumulation after stimulation of β-AR_1 and
448 β-AR_2 was not due to enhanced mRNA transcription, but rather to later events in gene
449 expression (Hu et al., 2009). Notably, hitherto most studies on the mechanisms and reasons
450 for hypoxia-inducible factor pathway have been studied in mammals, and it is clearly
451 possible that the Hif1a of fish has other or additional functions from those found in mammals.
452 For example, Hif1a is involved in temperature responses in fish (Rissanen et al., 2006).
453 Another link between hypoxia and β-AR was found in our study. The *β_{3b}-ar* steady-state
454 levels significantly changed during long-term exposure to hypoxia (Fig. 2D) but the
455 transcription was not affected. Hypoxia-response elements (HRE) have been identified in the
456 promotor regions of the gene sequences of several β-adrenergic receptor subtypes,
457 indicating a close link between the hypoxia response and the β-adrenergic pathway (Eckhart
458 et al., 1997; Semenza, 2001). Although this has not yet been studied in the β_{3b}-AR subtype,
459 it can be assumed that HREs are present in the promotor region of the receptor as well. The
460 β-AR/β-NHE response of RBCs in various fish species is potentiated by simultaneous

exposure to hypoxic conditions (Brauner et al., 2002). More studies need to be done to unravel the link between the β_{3b}-AR signaling pathway and HIF1a pathway in fish and to give insight into yet undescribed aspects of gene expression regulation.

Fish exhibit a remarkable multiplicity of Hbs with different functional properties, such as differences in O_2-affinity and sensitivity to allosteric regulators, which provide a molecular strategy for adapting to a changing environment (Weber, 1990). However, the proportions of different Hbs have not been shown to change during environmental adaptation. Our present results fit with this conclusion: the steady-state level, transcription or stability of the studied globin gene transcripts (*hba-1* and *gbx*) were not affected by any of the treatments (Fig.1, Fig. 2, 3; Table 2). Globin mRNAs are generally highly stable with reported half-lives between 10 and 24 h, which has been attributed to the fact that the mature mammalian red blood cell is lacking the nucleus (Peixeiro et al., 2011; Russell et al., 1997). In contrast to the conventional Hb genes, the half-life of the transcript of a recently discovered globin family member, *gbx,* was the lowest of the genes studied (approximately 5 h). Also, its transcription rate and cellular mRNA levels were the lowest (Figs. 1F, 2F, 3E) in the study. *Gbx* was only recently found to be transcribed in fish red blood cells (Götting and Nikinmaa, 2015; Corti et al., 2016), and both the regulation of its expression and its functions are currently unknown. Our findings that *gbx* has a very limited transcription, low steady-state level and reduced stability in a red blood cell population with mixed age classes would be best explained if *gbx* is being transcribed mainly in young red blood cells.

5. Conclusions

Our results show that gene expression strategies in teleost RBCs vary markedly between genes already before translation, probably associated with the distinct functions of the gene products. The impact of changes in transcript stability on mRNA steady-state levels and consequent protein production is underexplored and underestimated, although it may be a significant component in the regulation of gene expression. Ours is the first study giving a

comparative insight into various levels of regulation of transcription of selected genes in RBCs of teleost fish. Although several findings cannot be fully explained yet and need further investigations the results clearly show gene-specific differences in the regulation of the amount of mRNA in response to environmental cues. Our present study sought to investigate transcriptional mechanisms in the whole population of circulating RBCs and thus did not discriminate between different age classes of RBCs. Based on our results and those of earlier studies changes in transcription rate, steady-state mRNA levels and stability during maturation can be expected and requires further studies.

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Declaration of interest

Conflict of interest: none.

Author contributions

M.G. and M.N. wrote the manuscript and designed the trial; M.G. performed the experiments, analyzed the data and performed the statistical analysis.

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References

517 Aranda, P.S., LaJoie, D.M., Jorcyk, C.L., 2012. Bleach gel: a simple agarose gel for
518 analyzing RNA quality. Electrophoresis 33, 366–9. doi:10.1002/elps.201100335
519 Bemanian, V., Male, R., Goksøyr, A., 2004. The aryl hydrocarbon receptor-mediated
520 disruption of vitellogenin synthesis in the fish liver: Cross-talk between AHR- and
521 ERalpha-signalling pathways. Comp. Hepatol. 3, 2. doi:10.1186/1476-5926-3-2
522 Bensaude, O., 2011. Inhibiting eukaryotic transcription: Which compound to choose? How to
523 evaluate its activity? Transcription 2, 103–108. doi:10.4161/trns.2.3.16172
524 Brauner, C.J., Wang, T., Jensen, F.B., 2002. Influence of hyperosmotic shrinkage and beta-
525 adrenergic stimulation on red blood cell volume regulation and oxygen binding
526 properties in rainbow trout and carp. J. Comp. Physiol. B. 172, 251–262.
527 doi:10.1007/s00360-001-0249-5
528 Bremer, K., Moyes, C.D., 2014. mRNA degradation: an underestimated factor on steady-
529 state transcript levels of cytochrome c oxidase subunits? J. Exp. Biol. 4, 2212–2220.
530 doi:10.1242/jeb.100214
531 Cheong, H.I., Asosingh, K., Stephens, O.R., Queisser, K.A., Xu, W., Willard, B., Hu, B.,
532 Dermawan, J.K.T., Stark, G.R., Naga Prasad, S. V, Erzurum, S.C., 2016. Hypoxia
533 sensing through β-adrenergic receptors. JCI Insight 1, e90240.
534 doi:10.1172/jci.insight.90240
535 Corti, P., Xue, J., Tejero, J., Wajih, N., Sun, M., Stolz, D.B., Tsang, M., Kim-Shapiro, D.B.,
536 Gladwin, M.T., 2016. Globin X is a six-coordinate globin that reduces nitrite to nitric
537 oxide in fish red blood cells. Proc. Natl. Acad. Sci. U. S. A. 113, 8538–43.
538 doi:10.1073/pnas.1522670113
539 Cossins, A.R., Richardson, P.A., 1985. Adrenalin-Induced Na+/H+ Exchange in' Trout
540 Erythrocytes and its Effects Upon Oxygen-Carrying Capacity. J. Exp. Biol. 118, 229 LP-
541 246.
542 Crawford, D.L., Oleksiak, M.F., 2007. The biological importance of measuring individual
543 variation. J. Exp. Biol. 210, 1613–21. doi:10.1242/jeb.005454
544 Danner, S., Frank, M., Lohse, M.J., 1998. Agonist Regulation of Human β2-Adrenergic

545 Receptor mRNA Stability Occurs via a Specific AU-rich Element . J. Biol. Chem. 273,
546 3223–3229. doi:10.1074/jbc.273.6.3223
547 de Nadal, E., Ammerer, G., Posas, F., 2011. Controlling gene expression in response to
548 stress. Nat. Rev. Genet. 12, 833–45. doi:10.1038/nrg3055
549 Eckhart, A.D., Yang, N., Xin, X., Faber, J.E., 1997. Characterization of the α1B-adrenergic
550 receptor gene promoter region and hypoxia regulatory elements in vascular
551 smooth muscle. Proc. Natl. Acad. Sci. 94, 9487–9492.
552 el Hadri, K., Pairault, J., Fève, B., 1996. Triiodothyronine regulates beta 3-adrenoceptor
553 expression in 3T3-F442A differentiating adipocytes. Eur. J. Biochem. 239, 519–25.
554 Flouriot, G., Pakdel, F., Valotaire, Y., 1996. Transcriptional and post-transcriptional
555 regulation of rainbow trout estrogen receptor and vitellogenin gene expression. Mol.
556 Cell. Endocrinol. 124, 173–183. doi:https://doi.org/10.1016/S0303-7207(96)03960-3
557 Gilmour, KM; Didyk, NE; Reid, SG; Perry, S., 1994. Down-regulation of red blood cell beta-
558 adrenoreceptors in response to chronic elevation of plasma catecholamine levels in the
559 rainbow trout. J. Exp. Biol. 186, 309–314.
560 Götting, M., Nikinmaa, M., 2015. More than hemoglobin - the unexpected diversity of globins
561 in vertebrate red blood cells. Physiol. Rep. 3, 353–360. doi:10.14814/phy2.12284
562 Hadcock, J.R., Wang, H.Y., Malbon, C.C., 1989. Agonist-induced destabilization of beta-
563 adrenergic receptor mRNA. Attenuation of glucocorticoid-induced up-regulation of beta-
564 adrenergic receptors. J. Biol. Chem. 264, 19928–19933.
565 Hayles, B., Yellaboina, S., Wang, D., 2010. Comparing Transcription Rate and mRNA
566 Abundance as Parameters for Biochemical Pathway and Network Analysis. PLoS One
567 5, e9908. doi:10.1371/journal.pone.0009908
568 Hu, H., Ma, Q., Zhang, D., Shen, S., Han, L., Ma, Y., Li, R., Xie, K., 2009. HIF-1[alpha] links
569 [beta]-adrenoceptor agonists and pancreatic cancer cells under normoxic condition.
570 Acta Pharmacol Sin 31, 102–110.
571 Huch, S., Nissan, T., 2014. Interrelations between translation and general mRNA
572 degradation in yeast. Wiley Interdiscip. Rev. RNA 5, 747–763. doi:10.1002/wrna.1244

573 Kaelin, W.G., Ratcliffe, P.J., 2008. Oxygen sensing by metazoans: the central role of the HIF
574 hydroxylase pathway. Mol. Cell 30, 393–402. doi:10.1016/j.molcel.2008.04.009
575 Koldkjaer, P., Pottinger, T.G., Perry, S.F., Cossins, A.R., 2004. Seasonality of the red blood
576 cell stress response in rainbow trout (Oncorhynchus mykiss). J. Exp. Biol. 207, 357–67.
577 doi:10.1242/jeb.00747
578 Koressaar, T., Remm, M., 2007. Enhancements and modifications of primer design program
579 Primer3. Bioinformatics 23, 1289–91. doi:10.1093/bioinformatics/btm091
580 López-Maury, L., Marguerat, S., Bähler, J., 2008. Tuning gene expression to changing
581 environments: from rapid responses to evolutionary adaptation. Nat. Rev. Genet. 9,
582 583–593. doi:10.1038/nrg2398
583 Lu, D.-F., Wang, Y.-S., Li, C., Wei, G.-J., Chen, R., Dong, D.-M., Yao, M., 2015. Actinomycin
584 D inhibits cell proliferations and promotes apoptosis in osteosarcoma cells. Int. J. Clin.
585 Exp. Med. 8, 1904–1911.
586 Lund, S.G., Phillips, M.C., Moyes, C.D., Tufts, B.L., 2000. The effects of cell ageing on
587 protein synthesis in rainbow trout (Oncorhynchus mykiss) red blood cells. J. Exp. Biol.
588 203, 2219–28.
589 Mahé, Y., Garcia-Romeu, F., Motais, R., 1985. Inhibition by amiloride of both adenylate
590 cyclase activity and the Na+/H+ antiporter in fish erythrocytes. Eur. J. Pharmacol. 116,
591 199–206.
592 Mitchusson, K.D., Blaxall, B.C., Pende, A., Port, J.D., 1998. Agonist-mediated destabilization
593 of human beta1-adrenergic receptor mRNA: role of the 3’ untranslated translated
594 region. Biochem. Biophys. Res. Commun. 252, 357–362. doi:10.1006/bbrc.1998.9598
595 Motais, R., Garcia-Romeu, F., Borgese, F., 1987. The control of Na+/H+ exchange by
596 molecular oxygen in trout erythrocytes. A possible role of hemoglobin as a transducer.
597 J. Gen. Physiol. 90, 197–207.
598 Nickerson, J.G., Drouin, G., Perry, S.F., Moon, T.W., 2002. In vitro Regulation of β-
599 adrenoceptor Signaling in the Rainbow Trout, Oncorhynchus mykiss. Fish Physiol.
600 Biochem. 27, 157–171. doi:10.1023/B:FISH.0000032723.78349.4e

Nickerson, J.G., Dugan, S.G., Drouin, G., Perry, S.F., Moon, T.W., 2003. Activity of the unique beta-adrenergic Na+/H+ exchanger in trout erythrocytes is controlled by a novel beta3-AR subtype. Am. J. Physiol. Regul. Integr. Comp. Physiol. 285, R526-35. doi:10.1152/ajpregu.00146.2003

Nikinmaa, M., 1983. Adrenergic regulation of haemoglobin oxygen affinity in rainbow trout red cells. J. Comp. Physiol. ? B 152, 67–72. doi:10.1007/BF00689729

Nikinmaa, M., Jensen, F., 1992. Inhibition of adrenergic proton extrusion in rainbow trout red cells by nitrite-induced methaemoglobinaemia. J. Comp. Physiol. B 162, 424–429. doi:10.1007/BF00258964

Patrone, G., Puppo, F., Cusano, R., Scaranari, M., Ceccherini, I., Puliti, A., Ravazzolo, R., 2000. Nuclear run-on assay using biotin labeling, magnetic bead capture and analysis by fluorescence-based RT-PCR. Biotechniques 29, 1012–4, 1016–7.

Peixeiro, I., Silva, A.L., Romão, L., 2011. Control of human β-globin mRNA stability and its impact on beta-thalassemia phenotype. Haematologica 96, 905–913. doi:10.3324/haematol.2010.039206

Perry, S.F., Bernier, N.J., 1999. The acute humoral adrenergic stress response in fish: facts and fiction. Aquaculture 177, 285–295. doi:http://dx.doi.org/10.1016/S0044-8486(99)00092-7

Perry, S.F., Reid, S.D., 1992. Relationship between blood O2 content and catecholamine levels during hypoxia in rainbow trout and American eel. Am. J. Physiol. 263, R240-9.

Phillips, M.C., Moyes, C.D., Tufts, B.L., 2000. The effects of cell ageing on metabolism in rainbow trout (Oncorhynchus mykiss) red blood cells. J. Exp. Biol. 203, 1039 LP-1045.

Reid, S.D., Perry, S.F., 1991. The effects and physiological consequences of raised levels of cortisol on rainbow trout (Oncorhynchus mykiss) erythrocyte beta-adrenoreceptors. J. Exp. Biol. 158, 217 LP-240.

Rissanen, E., Tranberg, H.K., Sollid, J., Nilsson, G.E., Nikinmaa, M., 2006. Temperature regulates hypoxia-inducible factor-1 (HIF-1) in a poikilothermic vertebrate, crucian carp (Carassius carassius). J. Exp. Biol. 209, 994 LP-1003.

629 Russell, J.E., Morales, J., Liebhaber, S.A., 1997. The role of mRNA stability in the control of
630 globin gene expression. Prog. Nucleic Acid Res. Mol. Biol. 57, 249–87.
631 Sadar, M.D., Ash, R., Sundqvist, J., Olsson, P.E., Andersson, T.B., 1996. Phenobarbital
632 induction of CYP1A1 gene expression in a primary culture of rainbow trout hepatocytes.
633 J. Biol. Chem. 271, 17635–43.
634 Sambrook, J., Russell, D.W., 2006. Transcriptional Run-on Assays. Cold Spring Harb.
635 Protoc. 2006, pdb.prot3956. doi:10.1101/pdb.prot3956
636 Semenza, G.L., 2001. HIF-1 and mechanisms of hypoxia sensing. Curr. Opin. Cell Biol. 13,
637 167–171.
638 Smale, S.T., 2009. Nuclear run-on assay. Cold Spring Harb. Protoc. 2009, pdb.prot5329.
639 doi:10.1101/pdb.prot5329
640 Soeiro, R., Amos, H., 1966. mRNA half-life measured by use of actinomycin D in animal
641 cells — A caution. Biochim. Biophys. Acta - Nucleic Acids Protein Synth. 129, 406–409.
642 doi:http://dx.doi.org/10.1016/0005-2787(66)90383-2
643 Soitamo, A.J., Rabergh, C.M., Gassmann, M., Sistonen, L., Nikinmaa, M., 2001.
644 Characterization of a hypoxia-inducible factor (HIF-1alpha) from rainbow trout.
645 Accumulation of protein occurs at normal venous oxygen tension. J. Biol. Chem. 276,
646 19699–705. doi:10.1074/jbc.M009057200
647 Speckner, W., Schindler, J.F., Albers, C., 1989. Age-dependent changes in volume and
648 haemoglobin content of erythrocytes in the carp (Cyprinus carpio L.). J. Exp. Biol. 141,
649 133–49.
650 Tetens, V., Lykkeboe, G., Christensen, N.J., 1988. Potency of adrenaline and noradrenaline
651 for beta-adrenergic proton extrusion from red cells of rainbow trout, Salmo gairdneri. J.
652 Exp. Biol. 134, 267 LP-280.
653 Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., Rozen,
654 S.G., 2012. Primer3--new capabilities and interfaces. Nucleic Acids Res. 40, e115.
655 doi:10.1093/nar/gks596
656 Weber, R., 1990. Functional significance and structural basis of multiple hemoglobins with

special reference to ectothermic vertebrates, in: Truchot, J., Lahlou, B. (Eds.), Animal Nutrition and Transport Processes Comparative and Environmental Aspects. Comp. Physiol. 6. Transport, Respiration and Excretion. Karger, Basel, pp. 58–75.

Whitehead, A., Crawford, D.L., 2005. Variation in tissue-specific gene expression among natural populations. Genome Biol. 6, R13. doi:10.1186/gb-2005-6-2-r13

Zaiontz, C., 2015. Real Statistics Using Excel.

664 Tables

665

666

667 **Table 1.** Primers used for cloning and qPCR.

668

Gene	Gene symbol	Primer for (5'-3') Primer rev (5'-3')	Amplicon size (bp)	
A. Primers for cloning				**Accession no**
β-actin	*β-actin*	ATGGAAGATGAAATCGCCGCAC TTAGAAGCATTTACGGTGGACG	1128	NM_001124235
haemoglobin a -1	*hba-1*	GTCTGACAGCAAAGGACAAATCT CTCAGCACCGACGACATCT	80	NM_001124551
globin x1	*gbx*	ATGGGCTGCGCTATATCAGGAC TCATCTGGGTGTGTTCCTCTTCTC	592	this study
β-Na^+/H^+-exchanger	*β-nhe*	ATGCCGGCATTCTCGTGTGCCT TTGTCCTCGATACATGAGATACTTG	2490	M94581
β_{3b}-adrenergic receptor	*β_{3b}-ar*	TCGTGTGGCTATGAGGAAGACT TGCCTGCTGTGACTTTATGACC	121	NM_001124452
hypoxia inducible factor 1a	*hif1a*	ATGGACACAGGAGTTGTCCCCG TCAGTTGACTTGGTCCAGGGCAC	2301	AF304864
B. Primers for qPCR				**Efficiency**
β-actin	*β-actin*	TCAACCCCAAAGCCAACAGG AGAGGCGTACAGGGACAACA	107	2.00
haemoglobin a -1	*hba-1*	GTCTGACAGCAAAGGACAAATCT CTCAGCACCGACGACATCT	80	1.95
globin x1	*gbx*	ACAACGCCCCACCCAAATAC TCCATGCCTCCTCAAGCTCA	105	2.00
β-Na^+/H^+-exchanger	*β-nhe*	AGGAGTTGAGGGTGTCTGTGG TTCACGTAGGTCTTGTTGAAGCG	80	2.00
β_{3b}-adrenergic receptor	*β_{3b}-ar*	TCGTGTGGCTATGAGGAAGACT TGCCTGCTGTGACTTTATGACC	121	1.95
hypoxia inducible factor 1a	*hif1a*	CAGCCCCAGTGTGTTGTGTG GCCTCATATCCTCCGTCTGCT	94	2.00

669

670

671 **Table 2.** Estimated transcript half-lives (in hours) determined from regression equation of
672 actinomycin D (ActD, 2.5 µg/ml) treatment (Fig. 3).
673

Gene	normoxia	hypoxia	β-adrenergic stimulation + hypoxia
β-actin	22.7	> 48	> 48
hba-1	41.8	> 48	> 48
gbx	4.7	4.6	5.2
β-nhe	8.2	10.9	11.4
β_{3b}-ar	7.0	7.0	22.6
hif1a	8.2	16.4	19.2

674

675 Figure legends

676

677 **Fig. 1** Transcription rate and steady state levels of mRNA (copy number per µg RNA) in fish
678 red blood cells during short- term stress (Exp. 1). Red blood cells were exposed to control
679 conditions (= normoxia, 21 % O_2), a temperature increase from 10°C to 20°C within 90 min,
680 hypoxia (2.5 % O_2; dark grey bars) or were stimulated with isoproterenol (ISO; 10^{-5} mol L^{-1})
681 under hypoxia (2.5 %). At the end of the experiments nuclei were isolated (nuclear run-on) or
682 total RNA was extracted and transcript levels were quantified by qPCR as described in
683 Material and methods. Gene abbreviations are *β-actin* (*β-actin*; A), *haemoglobin a-1* (*hba-1*;
684 B), *β-Na+/H+-exchanger* (*β-nhe*; C), *β_{3b}-adrenergic receptor* (*β_{3b}-ar*; D), *globin X* (*gbx*; E),
685 *hypoxia inducible factor 1a* (*hif1a*; F). Data are means ± SD (n = 3 – 4 individual fish). No
686 statistical differences were found between treatments ($P < 0.05$; one-way repeated
687 measures ANOVA). For *gbx* (E) no transcription rate could be determined.

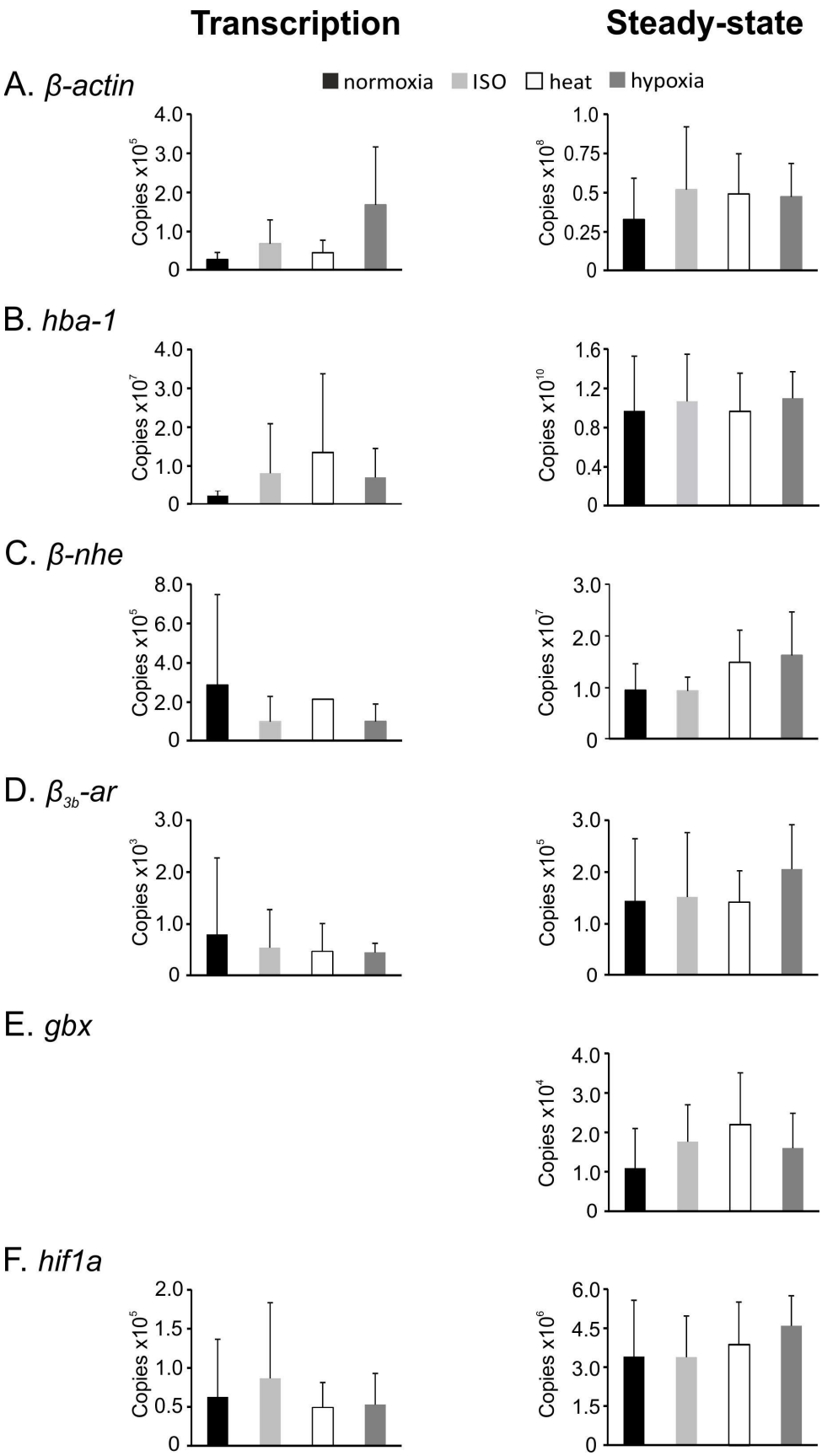
688

689

690 **Fig. 2** Time course of transcription rate and steady state levels of mRNA (copy number per
691 µg RNA) in fish red blood cells (Exp. 2). Red blood cells were exposed to normoxia (21 %
692 O_2), hypoxia (2.5 % O_2) or were stimulated with isoproterenol (ISO; 10^{-5} mol L^{-1}) under
693 hypoxia (2.5 %). Samples were collected at 0, 4, and 12 hours and nuclei were isolated
694 (nuclear run on) or total RNA was extracted and transcript levels were quantified by qPCR
695 as described in Material and methods. Gene abbreviations are *β-actin* (*β-actin*; A),
696 *haemoglobin a-1* (*hba-1*; B), *β-Na+/H+-exchanger* (*β-nhe*; C), *β_{3b}-adrenergic receptor* (*β_{3b}-*
697 *ar*; D), *globin X* (*gbx*; E), *hypoxia inducible factor 1a* (*hif1a*; F). Data are means ± SD (n = 3 –
698 4 individual fish). Uppercase letters indicate statistical significance ($P < 0.05$; two-way
699 repeated measures ANOVA) between treatment groups within each time point (4 h or 10 h);
700 lowercase letters indicate statistical significance between time points ($P < 0.05$; two-way
701 repeated measures ANOVA). Whenever two-way ANOVA identified statistically significant
702 differences ($p < 0.05$) posthoc testing (Holm-Sidak) was conducted.

Fig. 3 Transcript half-lives in fish red blood cells were determined using actinomycin D (ActD, 2.5 µg/ml) treatment. Red blood cells were exposed to normoxia (21 % O_2; black diamonds), hypoxia (2.5 % O_2; dark grey triangles) or were stimulated with isoproterenol (ISO; 10^{-5} mol L^{-1}; light grey squares) under hypoxia (2.5 %) at 14°C. Samples were collected at 0, 4, 8, and 12 hours after ActD addition and total RNA was extracted and transcript levels were quantified by qPCR as described in Material and Methods. Gene abbreviations are *β-actin* (*β-actin*; A), *haemoglobin a-1* (*hba-1*; B), *β-Na+/H+-exchanger* (*β-nhe*; C), *β_{3b}-adrenergic receptor* (*β_{3b}-ar*; D), *globin X* (*gbx*; E), *hypoxia inducible factor 1a* (*hif1a*; F). Data are means ± SD (n = 3 – 4 individual fish) and are expressed as percentage of the time point zero (t = 0). Data were fitted by linear regression and regression equation was used to calculate half-lives ($t_{1/2}$; Table 2). * indicates statistical significance between regression slopes ($P < 0.05$) compared to normoxia.

717 Figures



718

719 Figure 1.

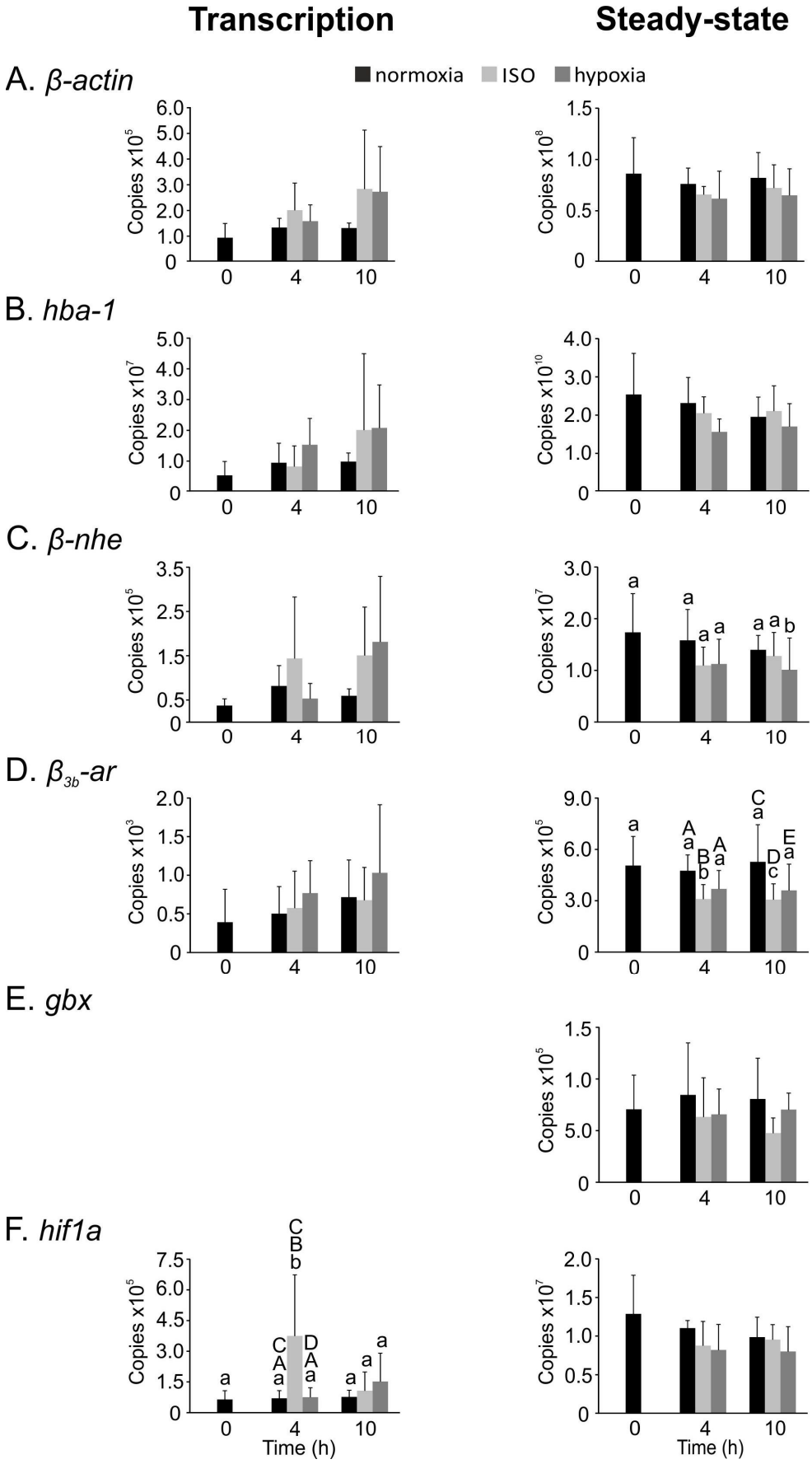
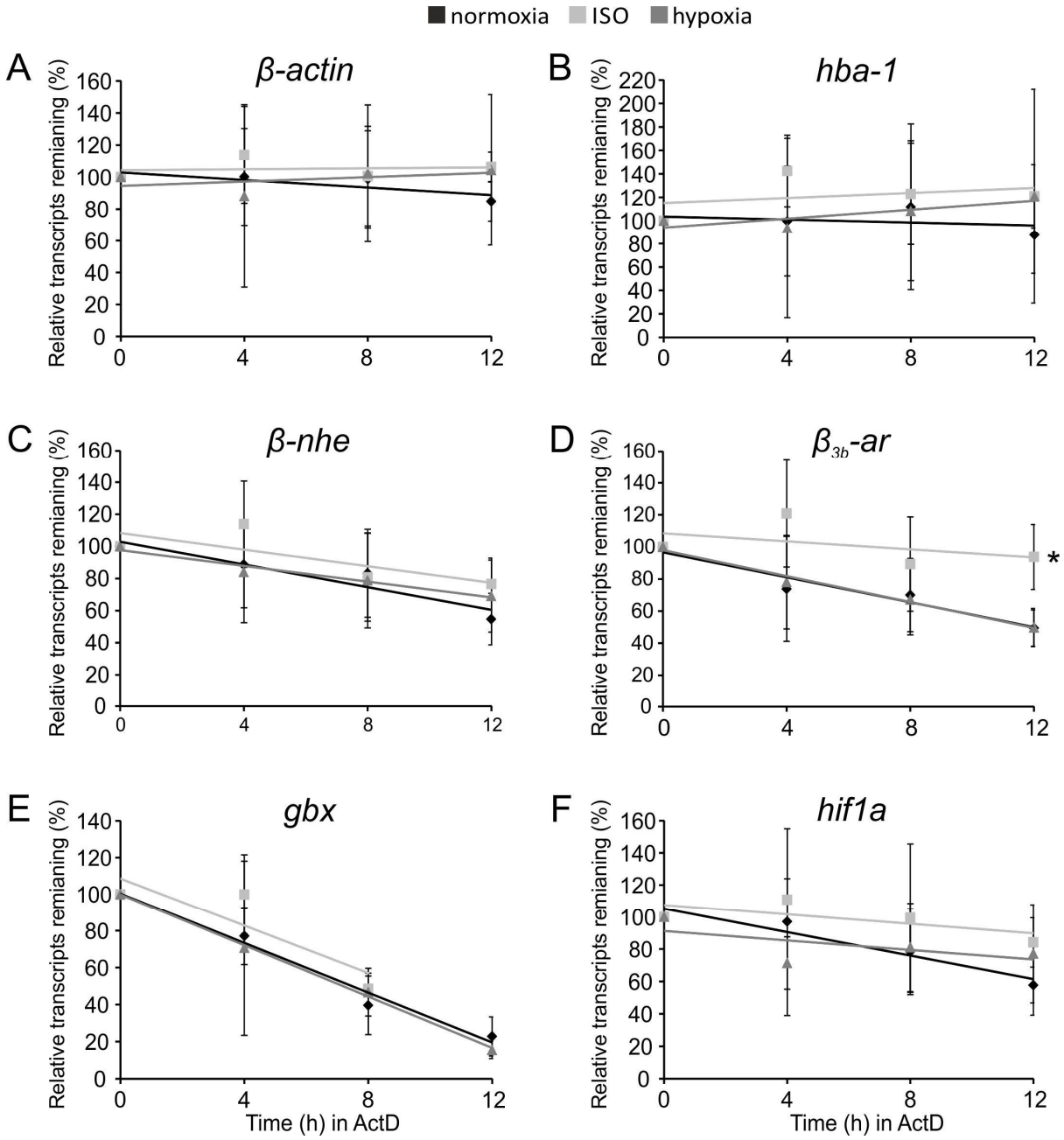

Transcription
Steady-state
normoxia
ISO
hypoxia
A. β-actin
B. hba-1
C. β-nhe
D. β3b-ar
E. gbx
F. hif1a
Copies x10^5
Copies x10^8
Copies x10^7
Copies x10^10
Copies x10^3
Time (h)
0
4
10

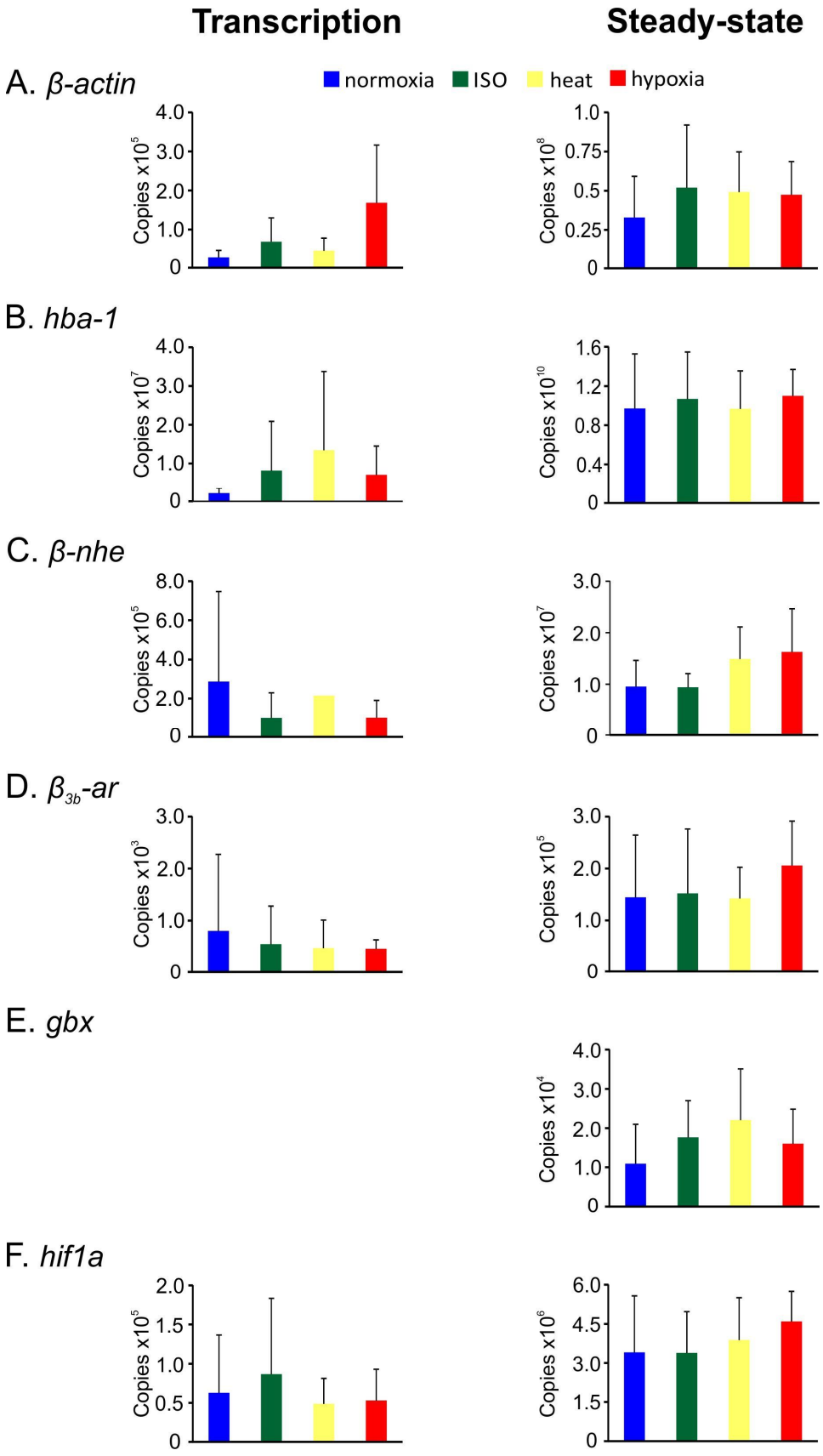
Figure 2.



722

723 Figure 3.

724 Colour figures for online version only.



725

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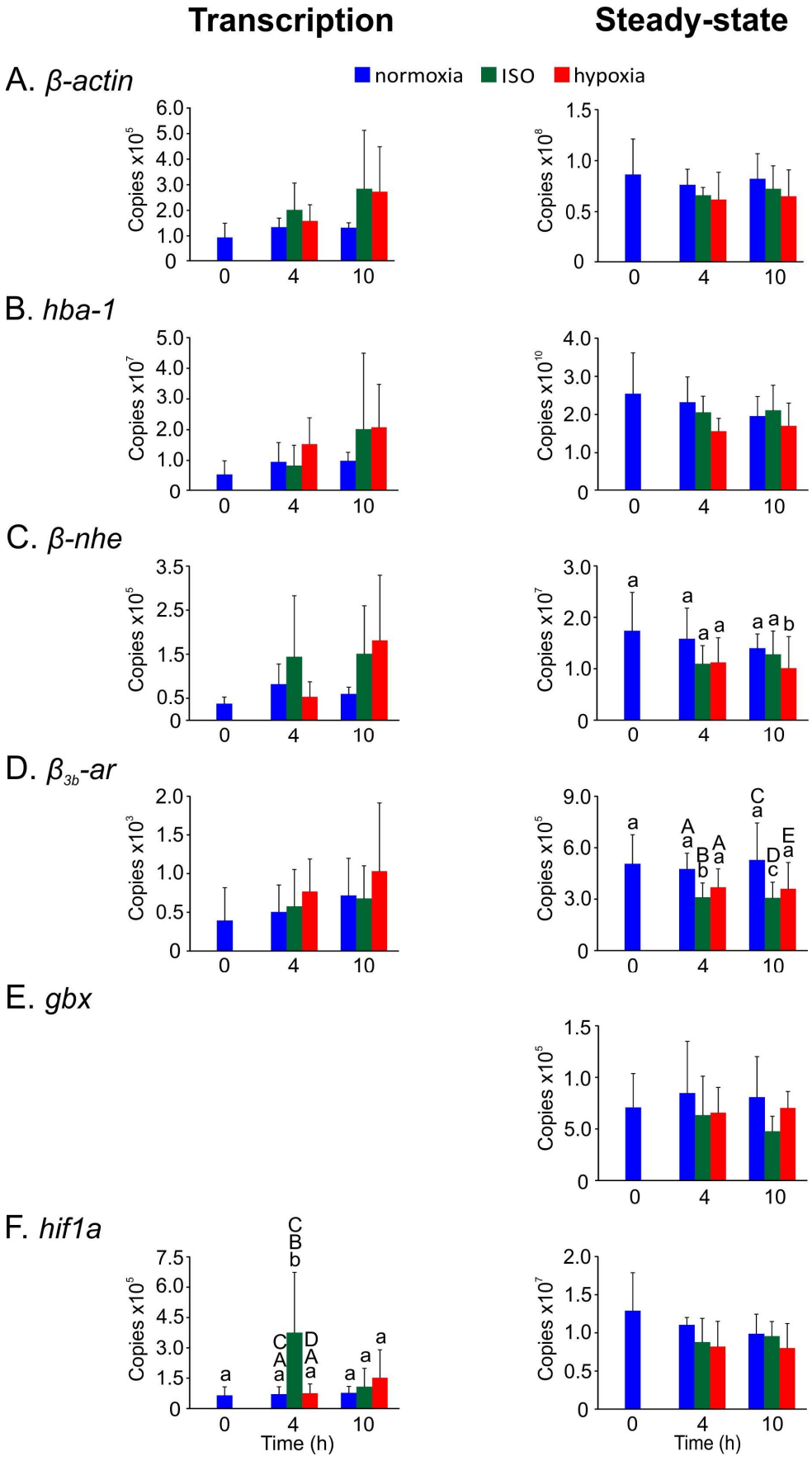

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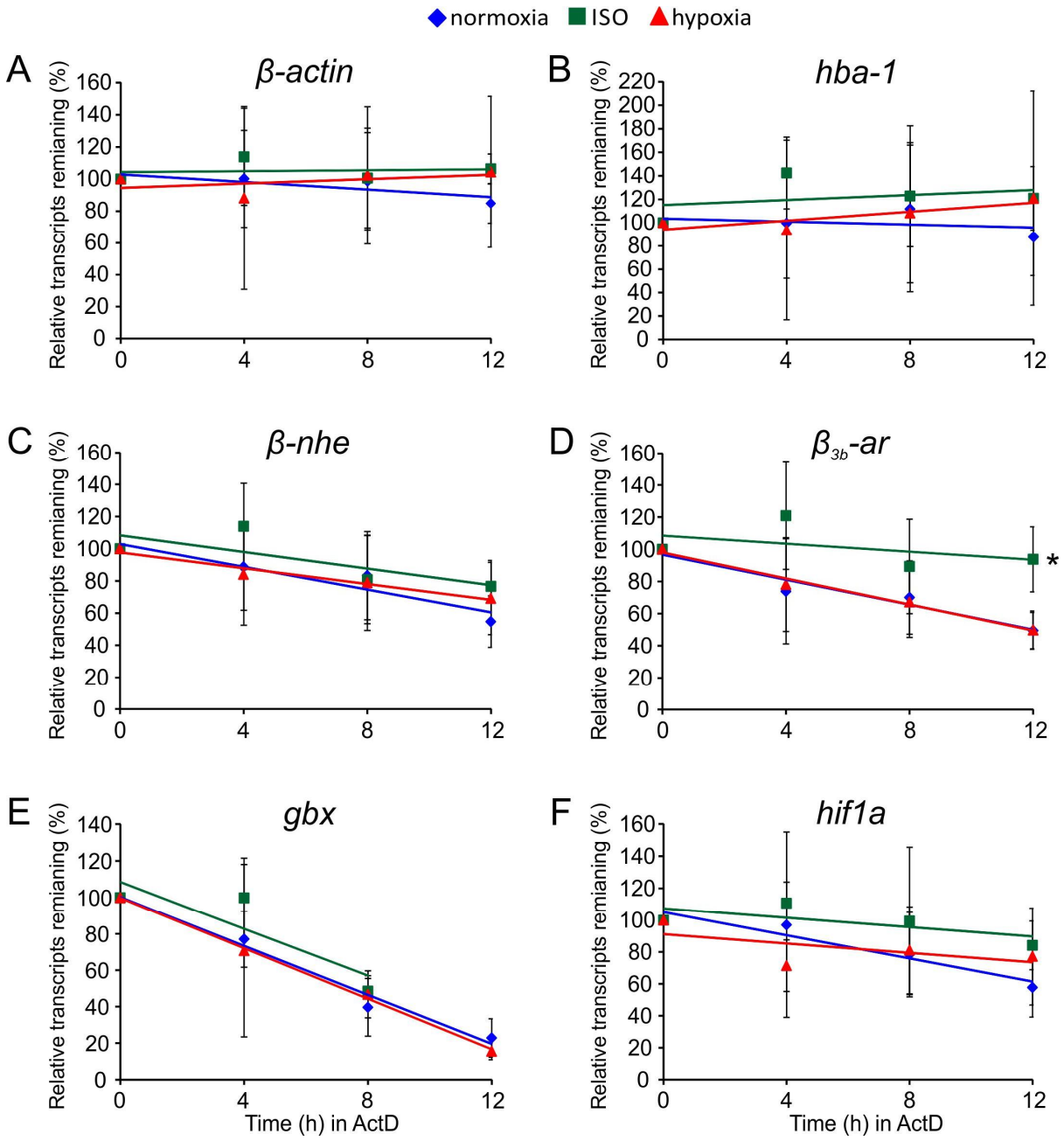
728 Figure 2.



729

730 Figure 3.