Am J Physiol Regul Integr Comp Physiol 298: R1499–R1508, 2010.
First published March 24, 2010; doi:10.1152/ajpregu.00675.2009.

Expression of heat shock proteins in anoxic crucian carp (*Carassius carassius*): support for cold as a preparatory cue for anoxia

Kåre-Olav Stensløkken,[1,2,*] Stian Ellefsen,[3,*] Helene Kile Larsen,[1] Jarle Vaage,[2] and Göran E. Nilsson[1]

[1]*Department of Molecular Biosciences and* [2]*Faculty Division Ullevål University Hospital, University of Oslo, Oslo, Norway; and* [3]*Lillehammer University College, Lillehammer, Norway*

Submitted 14 October 2009; accepted in final form 23 March 2010

Stensløkken K-O, Ellefsen S, Larsen HK, Vaage J, Nilsson GE. Expression of heat shock proteins in anoxic crucian carp (*Carassius carassius*): support for cold as a preparatory cue for anoxia. *Am J Physiol Regul Integr Comp Physiol* 298: R1499–R1508, 2010. First published March 24, 2010; doi:10.1152/ajpregu.00675.2009.—The crucian carp (*Carassius carassius*) tolerates anoxia for days to months depending on temperature. During episodes of stress, heat shock proteins (HSPs) are important for limiting cellular damage, mainly by ensuring protein function. Accordingly, we hypothesized that anoxia would change the expression of HSPs and that this response would be temperature dependent. Real-time RT-PCR was used to investigate the effects of 1 and 7 days anoxia (A1 and A7) on the expression of HSP70a, HSP70b, HSC70, HSP90, and HSP30 in the brain and heart of 8°C- and 13°C-acclimated crucian carp. In general, the expression of all HSPs changed in response to anoxia, although varying in size and direction, and with organ and temperature. HSP70a expression increased drastically (~10-fold) in A7 brains and hearts at 13°C but not at 8°C. HSC70 and HSP90 expression decreased in A7 brains (by 60–70%), but not in A7 hearts. HSC70 expression increased in A1 brains and hearts at both temperatures (by 60–160%), and HSP30 expression decreased in A7 brains and hearts at both temperatures (by 50–80%). Notably, normoxic fish showed 7- and 11-fold higher HSP70a expression in the brain and heart at 8°C compared with 13°C. This difference disappeared during anoxia, suggesting that cold may function as a cue for preconditioning the crucian carp's HSP70a expression to the approaching anoxic winter period.

heart; brain; temperature; preconditioning; teleost

THE FRESHWATER FISH CRUCIAN carp (*Carassius carassius*) has exceptional abilities to handle physiological stress. It has evolved an extraordinary anoxia tolerance, allowing it to live in small lakes and ponds, which because of thick ice cover in the winter can be totally devoid of oxygen for long periods. Thus, it survives anoxia for months at low temperature and for days at room temperature (36). During periods of oxygen depletion, oxidative phosphorylation is not possible, and the crucian carp has to rely on glycolysis as the only route for ATP production. This yields much less ATP per unit nutrient substrate, implying severe metabolic stress. Still, the anoxic crucian carp remains active (38), and its cells continue to proliferate (50). It is thus likely to exhibit both energy-conserving and protective mechanisms, enabling maintenance of cellular function in anoxia.

Heat shock proteins (HSPs) constitute a large family of proteins known to protect vertebrate cells against a wide spectrum of stressors (27). HSPs has two main functions: *1*) as molecular chaperones, protecting newly synthesized proteins, and *2*) as templates that ensure proper folding of proteins (27). In mammals, HSPs are typically induced by stress, such as ischemia and anoxia (6, 28). HSPs are grouped into five subfamilies: the high-molecular-weight (100–110 kDa) family; the 83–90-kDa family (HSP90 family); the 66–78-kDa family (HSP70 family), the 60-kDa family, and the small HSPs (15–30 kDa). The HSP superfamily shows high degrees of homology throughout the animal kingdom. Indeed, HSP70 has been postulated to be the most conserved protein in evolution (8).

In mammalian cells, HSP90 displays selectivity toward its target proteins (42) and ensures proper structure and function of a variety of proteins, including the hypoxia-inducible factor 1α (HIF1α) (30). In contrast, members of the HSP70 family, such as the inducible HSP70 and the constitutive heat-shock cognate 70 (HSC70), display low specificity toward their target proteins. They are involved in tasks, such as folding of newly synthesized proteins, transport across membranes, and refolding of misfolded and aggregated proteins (8). HSP70 is also involved in protection against ischemia in the heart (20) and brain (6). Ischemic heart preconditioning, originally discovered by Murry et al. (34), is described as the most potent mechanism of cardiac protection (62), and HSP70 plays a role in the late phases of protection (63). While HSP70 is typically expressed at low levels and can be induced by stress, HSC70 is expressed at high levels in most tissues and is important for housekeeping of cell functions (8). In contrast to most heat-shock proteins, small HSPs such as HSP27, show low degrees of sequence conservation between species (41). Still, their functions seem to be well conserved, protecting the cell from protein aggregation (11).

Several types of stress can induce HSP expression in teleost fish (4). Most studies have been performed on heat shock and HSP expression, but cold shock can also induce expression in fish (1). In the crucian carp, HSP70/HSC70, and HSP90 protein expression have been shown to increase markedly in liver, heart, gills, and kidney in response to lowering of acclimation temperature (47). However, in rainbow trout blood, the expression of HSP70 is higher at 17°C than at 5°C. It has been suggested that a fall in temperature is an important cue for adapting to winter conditions and anoxic survival (19). Interestingly, several studies have indicated distinct summer and winter phenotypes of the crucian carp heart and brain, including myosin heavy chain composition (59), glycogen stores (58, 60), and brain lipid composition (25). However, little is known about HSP expression and oxygen deprivation in fish, particularly in combination with temperature.

We wanted to test whether members of the HSP family would show increased expression in crucian carp brain and heart in response to anoxia, and hypothesized that the changes would be less marked at 8°C than at 13°C. The rationale for the

* K.-O. Stensløkken and Stian Ellefsen contributed equally to this article.
Address for reprint requests and other correspondence: K.-O. Stensløkken, Dept. of Molecular Biosciences, PB 1041, N-0316 Oslo, Norway (e-mail: k.o.stenslokken@imbv.uio.no).

latter hypothesis is that a low water temperature will signal an approaching anoxic winter period, making cold a likely preconditioning cue for anoxia tolerance. Thus, cold fish probably have a pattern of gene expression better suited for anoxic survival, and fewer adjustments would be needed in response to anoxia due to a cold-induced preconditioning effect.

MATERIAL AND METHODS

Crucian carp (37 ± 13 g) were captured in Tjernsrud pond, Oslo municipality in June and kept in 750-liter holding tanks (12:12-h light-dark cycle) continuously supplied with aerated and dechlorinated tap water. Two experimental series were performed at different temperatures (13°C in August and 8°C in November). At each temperature, four exposures were performed; 7 days normoxia (N7), 1 day anoxia (A1), 7 days anoxia (A7), and 7 days anoxia followed by 3 or 7 days reoxygenation (R3 or R7). Experiments were performed in flow-through (2–4 liter/h) circular 25-liter tanks (16 fish in each), wherein fish were left to acclimate for 18 h in the dark prior to experimental onset. Tanks were then sealed with a tight lid, and the flow-through water was either bubbled with air (normoxia and reoxygenation) or nitrogen (anoxia). Oxygen concentrations and temperatures were continuously monitored using a galvanometric oxygen electrode, Oxi 340i (WTW, Weilheim, Germany), connected to a computer. Water with no detectable oxygen (<0.1 mg O_2/l) was considered anoxic.

The experimental protocol was approved and performed in adherence with the Norwegian Animal Health Authority and followed ethical guidelines of the National Animal Research Authority of Norway. Fish were killed by stunning them with a sharp blow to the head, before cutting the spinal cord and dorsal aorta, and removing the brain and the heart. Within 1 min of collecting the fish, the tissues were snap-frozen in liquid nitrogen. Tissues were stored at −80°C until use.

Obtaining Sequences for HSP Genes

For HSP70a, HSC70, and HSP30, no cloning was necessary, as their sequences were available for goldfish (*Carassius auratus*), a close relative to crucian carp (accession numbers are listed in Table 1). Gene-specific primers (GSPs) for cloning of HSP70b and HSP90 are listed in Table 1 and were designed from zebrafish (*Danio rerio*) sequences using Primer3 (http://frodo.wi.mit.edu/primer3/input.htm). Zebrafish belongs to the same family as crucian carp (Cyprinidae). Zebrafish sequences were retrieved using the National Center for Biotechnology Information home page (www.ncbi.nlm.nih.gov/) or the Ensembl Genome Browser (www.ensembl.org/index.html). Sequence alignments were performed using GeneDoc (ver. 2.6.0.2, www.psc.edu/biomed/genedoc/) and ClustalX (55) (ver. 1.81, ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/).

Total RNA was extracted from untreated crucian carp brain tissue using TRIzol reagent (Invitrogen, Carlsbad, CA), and whereas quality control was performed using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA), quantification was performed using a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Rockland, DE).

For partial cloning, 1 μg of total RNA was DNase I treated (Sigma-Aldrich, St. Louis, MO) and reverse transcribed using oligo$(dT)_{18}$ and Superscript III in reaction volumes of 20 μl (Invitrogen, Carlsbad, CA). PCR was performed on 1/25 dilutions of the resulting cDNA using Platinum Taq (Invitrogen). The following PCR program was used: *1*) 94°C for 10 min, *2*) 94°C for 30 s, *3*) 50°C for 1 min, *4*) 72°C for 1 min, *5*), repeating steps 2–4 44 times, *6*) 72°C for 10 min, and *7*) hold 4°C. The resulting PCR products were cloned using pGEM-T Easy Vector System I (Promega, Madison, WI) and $CaCl_2$-competent cells (TOP10 F′; Invitrogen). Positive clones were checked for inserts of correct size using PCR and were sequenced using T7 primers (ABI-Laboratory, University of Oslo, Oslo, Norway). All procedures were carried out according to manufacturer's protocol.

The resulting crucian carp sequences were submitted to The European Molecular Biology Laboratory Nucleotide Sequence Database (www.ebi.ac.uk/), and accession numbers are listed in Table 1. HSP70a and HSP70b represent two related paralogs and were thus labeled a and b.

RNA Extraction and cDNA Synthesis for Real-Time RT-PCR

Total RNA for real-time RT-PCR experiments was extracted from 42 mg brain tissue (8°C and 13°C) and 5 mg/17 mg heart tissue (8°C/13°C) of crucian carp using 15 μl TRIzol per mg. The extractions were performed in accordance with the detailed protocol outlined by Ellefsen et al. (14), adding 20 pg external RNA control gene (*mw2060*) per milligram tissue. The quality and quantity of the total RNA were assessed using 2100 Bioanalyzer and NanoDrop ND-1000 UV-Vis spectrophotometer.

Reverse transcription was performed on 1 μg total RNA using oligo$(dT)_{18}$ and Superscript III (with a reaction volume of 20 μl), and while total RNA from 8°C brains and 13°C brains and hearts were pretreated with DNase I, total RNA from 8°C hearts was not. This lack of DNase I treatment was warranted by the lack of genomic DNA, seen by performing real-time PCR on dilutions of total RNA. Such control reactions were carried out on all samples. Duplicate cDNA syntheses were performed on RNA from 8°C brains and hearts and 13°C brains, whereas a single cDNA synthesis was performed on RNA from 13°C hearts. Negative RT controls were performed as random checks on ~10% of the samples. cDNA reactions were

Table 1. *Primer sequences used for cloning and real-time RT-PCR*

Gene	Accession Number(s)		Primers for Cloning		Primers for Real-Time RT-PCR	Priming Efficiencies
mw2060	DQ075244	F	TTTGACGGGGAAGGTAACAG	F	GTGCTGACCATCCGAG	E =1.88 ± 0.03
		R	GTTCTCCCAGTTGCCACATT	R	GCTTGTCCGGTATAACT	
GAPDH	AM701793	F	TGATGGTCAAGCCATCTCTG	F	TATGTAGTCGAGTCTACCG	E =1.88 ± 0.03
		R	ACTTGCCGTTAAGCTCAGGA	R	GTGTAGGCATGGACTG	
HSP70a	AB092839*	F		F	ACAAGCGACTAAAGACG	E =1.85 ± 0.03
		R		R	GTACGCCAACAGCTTC	
HSP70b	FM995213	F	ATCCTGACGATTGAGGATGG	F	CATCCTGATGGGCGAC	E =1.80 ± 0.04
		R	CGGCTGGTTATCGGAATATG	R	GGTTATCGGAATATGTGGAGA	
HSC70	AB092840*	F		F	GCTATTGCTTACGGTCTG	E =1.88 ± 0.02
		R		R	CCGCGAACTTGAGACA	
HSP90	FM995214	F	CGTAATAGGGTAGCCAATGAACT	F	GGAATCTTCCGCTGGAG	E =1,87 ± 0.04
		R	CGTAATAGGGTAGCCAATGAACT	R	CGAGTGCTTCTTGACGA	
HSP30	AB177389*	F		F	GACGCTGGACACTAAAG	E =1.88 ± 0.03
		R		R	ACTGCCGACTAAATGACC	

Values are expressed as means ± SD. F, forward primer; R, reverse primer. Priming efficiencies (E) are given in the rightmost column. *Goldfish sequences.

diluted to 1:25 using autoclaved MilliQ water. All procedures were carried out according to the manufacturer's protocol.

Real-Time RT-PCR

Real-time RT-PCR was performed on a LightCycler 2.0 (Roche Diagnostics, Basel, Switzerland). Calculations of normalized levels of gene expression were performed using the *Eq. 1*:

$$\frac{\mathrm{Tar}^{E^{Cp_{normoxia}}} \times \mathrm{Con}^{E^{Cp_{anoxia}}}}{\mathrm{Tar}^{E^{Cp_{anoxia}}} \times \mathrm{Con}^{E^{Cp_{normoxia}}}} = \text{expression of target gene in anoxia,}$$

where Tar is the target gene, Con is the control gene (*mw2060*), E is priming efficiency, and Cp is crossing point. *mw2060* was used as an RNA control gene in all real-time RT-PCR data sets. However, because of interexperimental variation in *mw2060* performance (14), *mw2060* could not be used for comparing expression levels between temperatures. For such analyses GAPDH was used. E values were calculated for each real-time RT-PCR reaction using LinRegPCR software (46), but in the final calculations, average priming efficiencies (E_{mean}) were used, calculated separately for each primer pair and based on all real-time RT-PCR reactions. Cp values were obtained for each reaction using the LightCycler 2.0 software and were generally defined as the second derivative maximum. An exception was made for HSP70b, which showed very low expression levels. Consequently, an alternative method for Cp value calculations had to be employed; the fit-point method (still using the LightCycler 2.0 software). Real-time RT-PCR primers were designed using Primer3 and are presented in Table 1.

All real-time RT-PCR reactions were performed with reaction volumes of 10 μl, using LightCycler Faststart DNA Master^{PLUS} SYBR Green I (Roche Diagnostics, Basel, Switzerland) and LightCycler Capillaries (Roche Diagnostics). Five-microliter 1:25 diluted cDNA was used as template (prepared as previously described), and the following real-time RT-PCR program was used: 94°C for 10 min, 94°C for 10 s, 60°C for 12 s, 72°C for 8 s, repeating steps 2–4 39 times. In general, two real-time RT-PCR reactions were performed on each gene for each cDNA synthesis. Since two cDNA syntheses were performed for each total RNA sample, a total of four real-time RT-PCR reactions were performed on each gene for each sample of total RNA. An exception was made for 13°C hearts, where one cDNA synthesis was performed for each total RNA sample and three real-time RT-PCR reactions were performed on each gene for each sample (note: this experiment contained more fish per exposure group). The mean value of all four/three reactions was used in the gene expression analyses. All real-time RT-PCR reactions were performed using primer concentrations of 100 nM, and all primer pairs were found to amplify the desired cDNA species. The latter was verified by melting curve analyses (LightCycler 2.0 software), gel electrophoresis, and cloning/sequencing (performed as previously described). Primer efficiencies and average Cp values for all primer pairs are summarized in Table 1. It should be noted that, to find primers that worked well with the outlined real-time RT-PCR protocol (primer concentration of 100 nM and annealing temperature of 60°C), three primer pairs were tested for each gene. The primer pairs that displayed distinct melting curves and the highest Cp values were chosen. This was done as an alternative to optimization of primer concentrations/annealing temperatures. Notably, HSP70b did not show detectable expression in heart tissue. All procedures were carried out according to the manufacturer's protocol.

Statistical analysis. For detection of differences in gene expression between different oxygen regimes, a one-way ANOVA with a Bonferroni post hoc test comparing all groups was performed. $P < 0.05$ was considered significant. If a Bartlett's test for equal variances showed significant differences between SD, a Kruskal-Wallis test with a Dunn post hoc test comparing all groups was performed. For detection of differences between temperatures and HSP expression, a two-way ANOVA, with temperature and anoxia as independent variables, was performed. Differences between normoxic HSC70 and HSP90 expression at 8°C and 13°C were done using a Student's *t*-test. All data are presented as means ± SD.

RESULTS

HSP70-Like Proteins

HSP70-like sequences. Two variants of the HSP70 gene were partially cloned from crucian carp. One of the variants (termed HSP70b) differed from the HSP70 gene available for goldfish (26) (termed HSP70a) (82% nucleotide homology; 93% amino acid homology, respectively). Since goldfish and crucian carp typically show nucleotide homologies that are much higher (97–98%, Ellefsen S. and Stensløkken KO, unpublished data), the two HSP70 sequences were reasoned to be paralogous variants, originating from an ancient duplication event (23). Compared with the human HSP70 gene, the a and b paralogs showed 81% and 82% nucleotide homology, respectively, corresponding to 90% and 92% amino acid homology. Furthermore, the third HSP70-like gene investigated in this study, goldfish HSC70, showed 93% amino acid homology with human HSC70.

Expression of HSP70a and HSP70b in anoxia. At 8°C, HSP70a expression showed significant but relatively small changes in brain and heart of crucian carp in response to different oxygen regimes (brain, $P = 0.035$; heart, $P < 0.0001$) (Fig. 1). Compared with normoxic values, being set to 1.0, expression varied from 0.6 to 1.7. Notably, no changes in HSP70a expression were observed between normoxia (N7) and 24 h of anoxia (A1) but increased after 7 days of anoxia (A7) in the heart ($P < 0.01$). In 8°C crucian carp, expression of HSP70b showed no anoxia-induced changes in brain ($P = 0.12$) and was not detectable in the heart (Fig. 1).

By contrast, at 13°C, HSP70a expression showed large changes in brain and heart of crucian carp in response to different oxygen regimes (brain, $P = 0.0001$; heart, $P < 0.0001$) (Fig. 1). In brain, HSP70a expression changed from 1.0 in N7 to 3.7 in A1 and to 11.9 in A7 ($P < 0.05$ and $P < 0.001$), and in heart, HSP70a expression changed from 1.0 in N7 to 11.8 in A1 and 10.0 in A7 ($P < 0.001$ and $P < 0.01$). Moreover, in the brain at 13°C, HSP70b expression showed large changes in response to different oxygen regimes ($P = 0.002$), changing from 1.0 in N7 to 6.3 in A7 ($P < 0.01$). Again, HSP70b expression was not detectable in the heart. All observed anoxia-induced changes in HSP70a and HSP70b expression at 13°C were restored to normoxic levels after reoxygenation (R3).

Expression of HSC70. At both 8°C and 13°C, HSC70 expression showed changes in the brain and heart of crucian carp in response to different oxygen regimes (8°C brain, $P = 0.035$; 8°C heart, $P < 0.0001$; 13°C brain, $P < 0.0001$; 13°C heart, $P < 0.0001$) (Fig. 2, *A* and *B*). Notably, in 13°C hearts, expression increased from 1.0 in N7 to 2.6 in A1 ($P < 0.01$) (Fig. 2*B*). Similarly, in 13°C brains and 8°C hearts, expression increased in A1, going from 1.0 in N7 to 1.6 and 1.8, respectively ($P < 0.01$). In 8°C and 13°C brains, HSC70 expression decreased between N7 and A7, going from 1.0 to 0.4 and 0.3, respectively ($P < 0.05$ and $P < 0.001$). In the heart, HSC70 expression was reduced in A7 compared with A1 in 8°C and 13°C hearts ($P < 0.001$), but there was no difference between

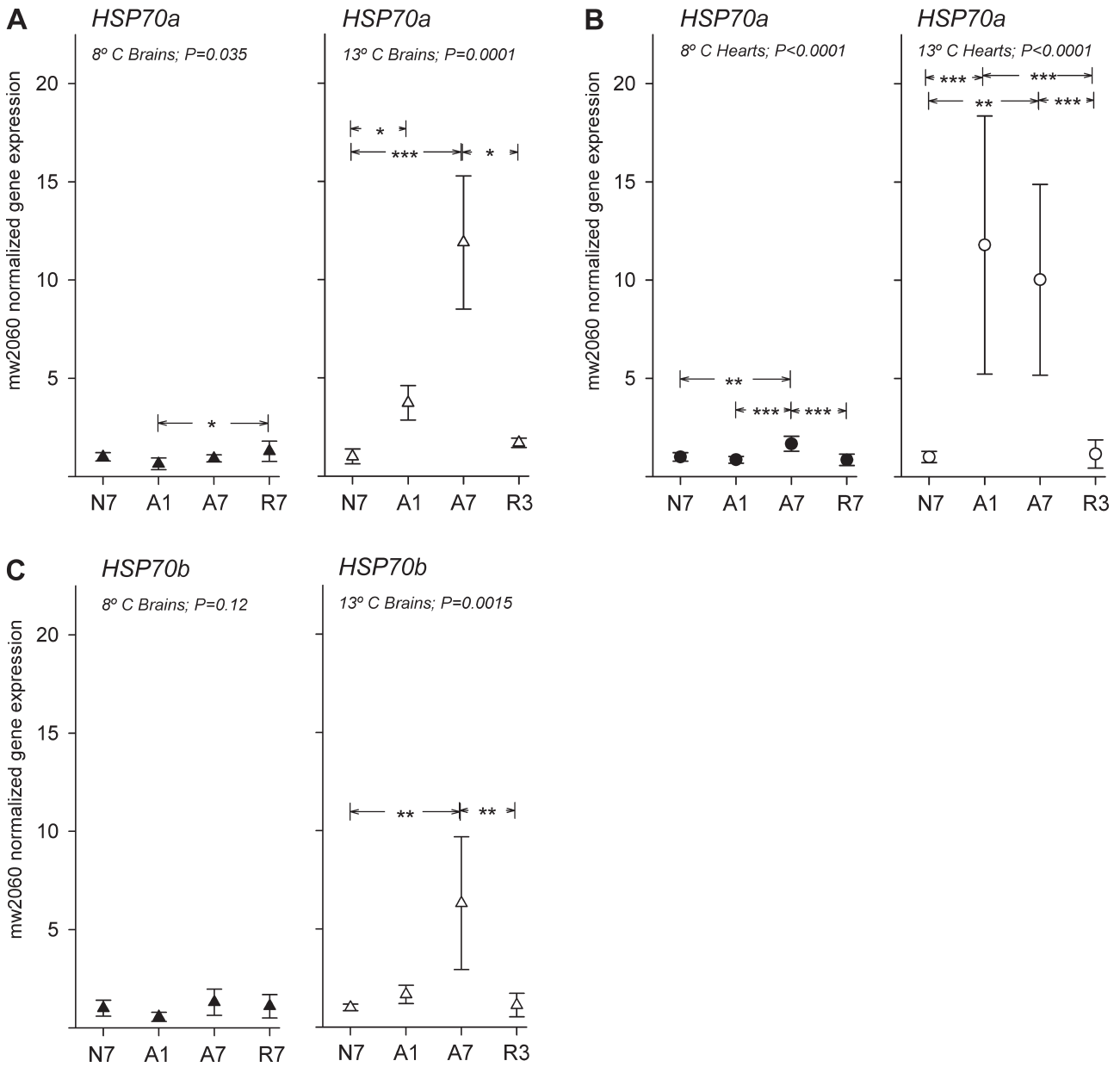

Fig. 1. Expression of heat shock protein 70 (HSP70) in brain (*A*, *C*) and heart (*B*) of 8°C and 13°C-acclimated crucian carp. Data sets were normalized to the external RNA control *mw2060* and were referenced to the control group N7. Values are expressed as means ± SD. N7, normoxia 7 days (8°C, brain $n = 5$, heart $n = 6$; 13°C, brain $n = 6$, heart $n = 10$); A1, anoxia 1 day (8°C, brain $n = 5$, heart $n = 6$; 13°C, brain $n = 6$, heart $n = 12$); A7, anoxia 7 days (8°C, brain $n = 6$, heart $n = 6$; 13° C, brain $n = 6$, heart $n = 9$); R7/R3, anoxia 7 days-reoxygenation 7/3 days (8°C, brain n=4, heart n=6; 13°C, brain n=6, heart n=10). The overall P value in the graphs is from an ANOVA (HSP70a brain) or a Kruskal-Wallis test on the remaining graphs. All groups were compared with a post hoc test (Bonferroni for the ANOVA and Dunn for the Kruskal-Wallis. The arrowheads indicate which group is statistically different: *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

N7 and A7 (Fig. 2*B*). Furthermore, in 8°C and 13°C hearts, HSC70 expression was higher in reoxygenation (R) compared with N7 and (8°C, R7; 13°C, R3), increasing from 1.0 to 2.0 and 2.2, respectively ($P < 0.01$ and $P < 0.05$).

HSP90

HSP90 sequence. The crucian carp HSP90 amino acid sequence showed 93% homology with the human HSP90 sequence and 96% homology with the zebrafish sequence.

Expression of HSP90. At both 8°C and 13°C, HSP90 expression changed in brain and heart of crucian carp in response to different oxygen regimes (8°C brain, $P = 0.002$; 8°C heart, $P = 0.0025$; 13°C brain, $P = 0.010$; 13°C heart, $P < 0.0001$) (Fig. 3). In general, HSP90 expression decreased between N7 to A7, falling from 1.0 in N7 to 0.4 in A7 8°C brains, to 0.3 in 8°C hearts, and to 0.4 in 13°C brains ($P < 0.05$). However, in 13°C hearts, no decrease was seen in HSP90 expression during anoxia, but rather an increase was seen, rising from 1.0 in N7 to 2.5 in A1 ($P < 0.01$).

HSP30

HSP30 sequence. Although the goldfish HSP30 sequence, which was utilized in this study, shows reasonable amino acid homologies with corresponding sequences from other

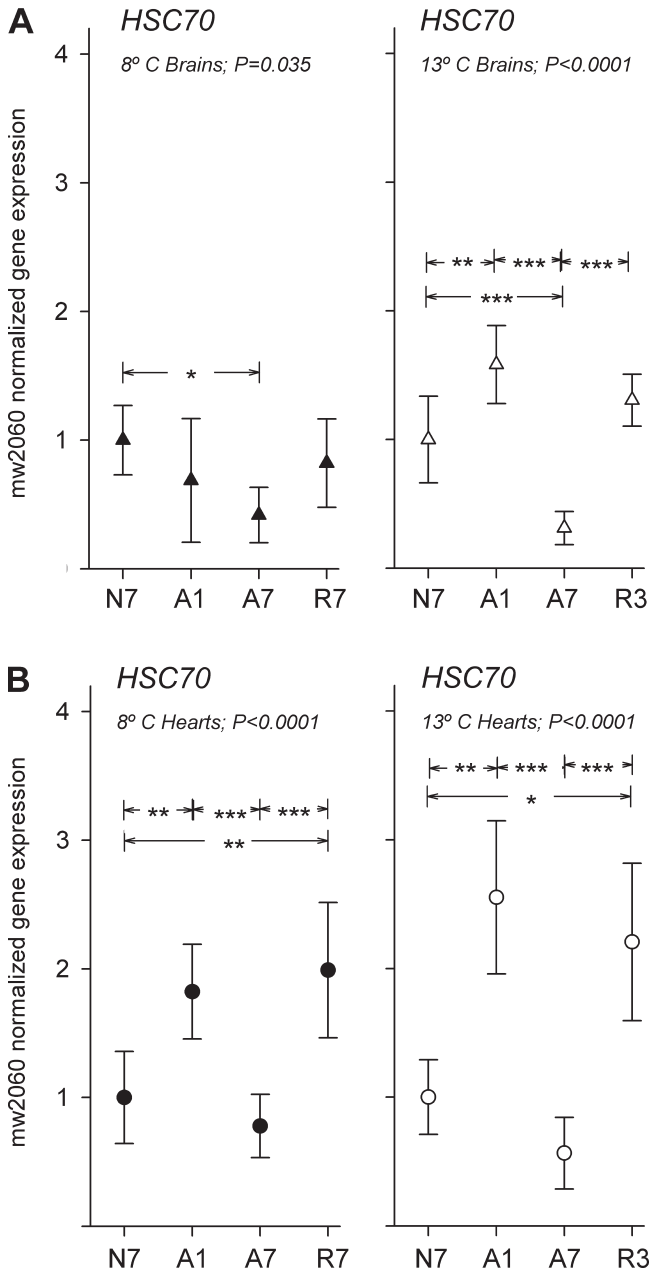

Fig. 2. Expression of heat-shock cognate 70 (HSC70) in brain (*A*) and heart (*B*) of 8°C- and 13°C-acclimated crucian carp. Data sets were normalized to the external RNA control *mw2060* and were referenced to the control group N7. Values are expressed as means ± SD. N7, normoxia 7 days (8°C, brain $n = 5$, heart $n = 6$; 13°C, brain $n = 6$, heart $n = 10$); A1, anoxia 1 day (8°C, brain $n = 5$, heart $n = 6$; 13°C, brain $n = 6$, heart $n = 12$); A7, anoxia 7 days (8°C, brain $n = 6$, heart $n = 6$; 13°C, brain $n = 6$, heart $n = 9$); R7/R3, anoxia 7 days-reoxygenation 7/3 days (8°C, brain $n = 4$, heart $n = 6$; 13°C, brain $n = 6$, heart $n = 10$). The overall P value in the graphs is from an ANOVA (all except brains 13°C) or a Kruskal-Wallis test (brains 13°C). All groups were compared with a post hoc test (Bonferroni for the ANOVA and Dunn for the Kruskal-Wallis. The arrows indicate which group is statistically different; $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

fish (77% similarity to *Salmo salar* and 74% to *Danio rerio*; using amino acids 85–155 in the goldfish sequence), it only shows 39% homology with the corresponding human sequence. The HSP30 gene seems to have been poorly conserved during evolution.

Expression of HSP30. At both 8°C and 13°C, HSP30 expression showed changes in brain and heart of crucian carp in response to different oxygen regimes (8°C brain, $P = 0.016$; 8°C heart, $P < 0.0001$; 13°C brain, $P < 0.001$;

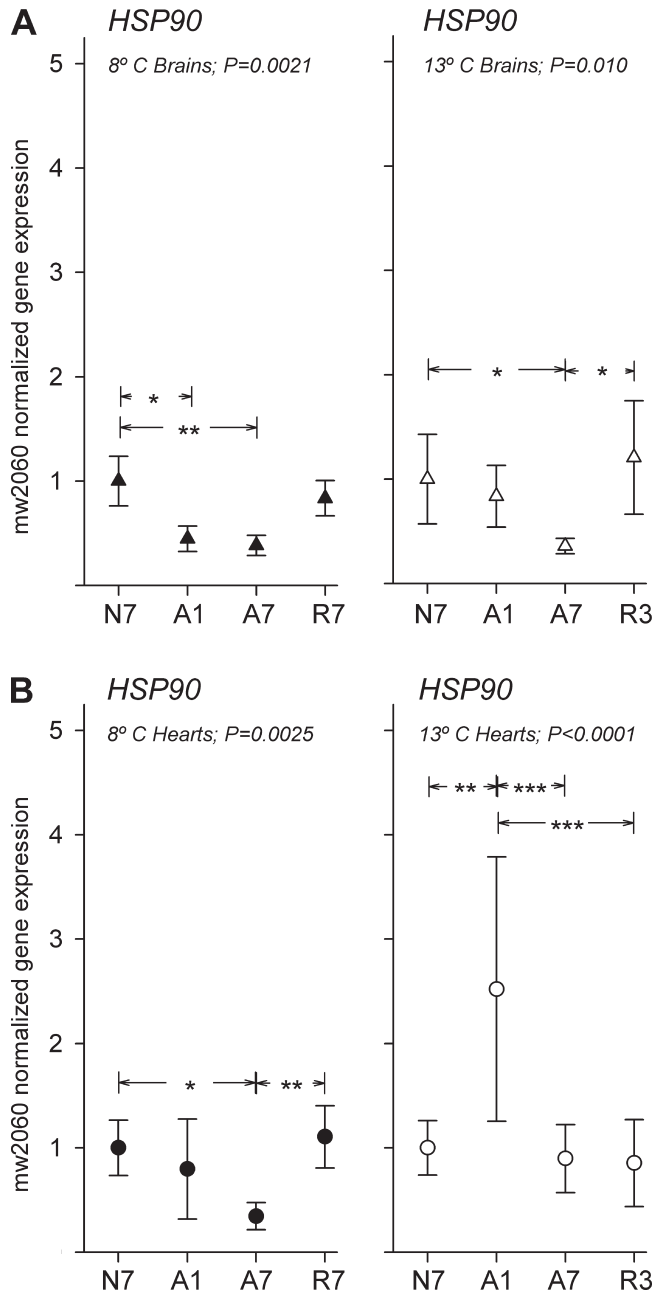

Fig. 3. Expression of HSP90 in brain (*A*) and heart (*B*) of 8°C- and 13°C-acclimated crucian carp. Data sets were normalized to the external RNA control mw2060 and were referenced to the control group N7. Values are expressed as means ± SD. N7, normoxia 7 days (8°C, brain $n = 5$, heart $n = 6$; 13°C, brain $n = 6$, heart $n = 10$); A1, anoxia 1 day (8°C, brain $n = 5$, heart $n = 6$; 13°C, brain $n = 6$, heart $n = 12$); A7, anoxia 7 days (8°C, brain $n = 6$, heart $n = 6$; 13°C, brain $n = 6$, heart $n = 9$); R7/R3, anoxia 7 days-reoxygenation 7/3 days (8°C, brain $n = 4$, heart $n = 6$; 13°C, brain $n = 6$, heart $n = 10$). The overall P value in the graphs is from an ANOVA (heart 8°C) or a Kruskal-Wallis test on the remaining graphs. All groups were compared with a post hoc test (Bonferroni for the ANOVA and Dunn for the Kruskal-Wallis. The arrows indicate which group is statistically different: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

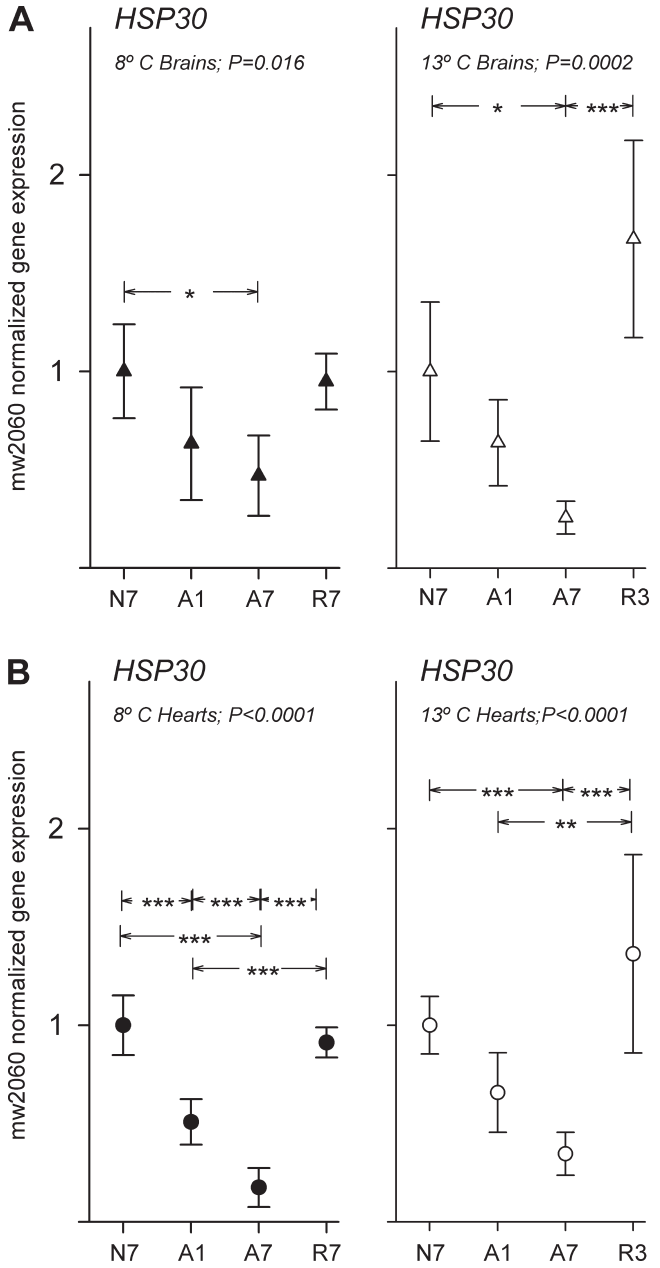

Fig. 4. Expression of HSP30 in brain (*A*)and heart (*B*) of 8°C- and 13°C-acclimated crucian carp. Data sets were normalized to the external RNA control *mw2060* and were referenced to the control group N7. Values are expressed as means ± SD. N7, normoxia 7 days (8°C, brain $n = 5$, heart $n = 6$; 13°C, brain $n = 6$, heart $n = 10$); A1, anoxia 1 day (8°C, brain $n = 5$, heart $n = 6$; 13°C, brain $n = 6$, heart $n = 12$); A7, anoxia 7 days (8°C, brain $n = 6$, heart $n = 6$; 13°C, brain $n = 6$, heart $n = 9$); R7/R3, anoxia 7 days reoxygenation 7/3 days (8°C, brain $n = 4$, heart $n = 6$; 13°C, brain $n = 6$, heart $n = 10$). The overall P value in the graphs is from an ANOVA (heart 8°C) or a Kruskal-Wallis test on the remaining graphs. All groups were compared with a post hoc test (Bonferroni for the ANOVA and Dunn for the Kruskal-Wallis; $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

13°C heart, $P < 0.0001$) (Fig. 4). In all experiments, HSP30 expression decreased from N7 to A7, falling from 1.0 to 0.5 in 8°C brains, to 0.2 in 8°C hearts, to 0.3 in 13°C brains and to 0.3 in 13°C hearts ($P < 0.05$ in brain, and $P < 0.001$ in heart).

Temperature Effects on HSP70 and HSP30 Expression Patterns

Normoxic HSP70a expression (normalized to GAPDH) in brain and heart of crucian carp was 7- and 11-fold higher at 8°C than at 13°C (Fig. 5*A*). This difference almost disappeared during anoxia (A7), and although HSP70a expression remained higher in 8°C brain and heart, it did so by a mere 1.1-fold and

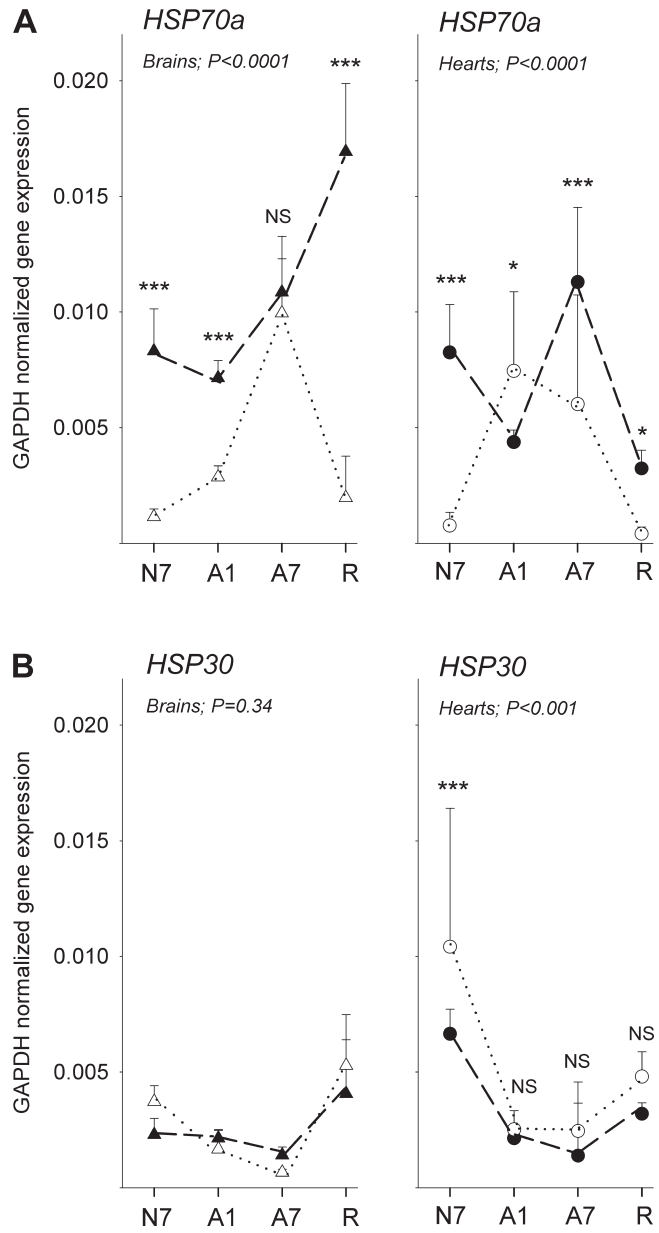

Fig. 5. Expression of HSP70 (*A*) and HSP30 (*B*) in brain and heart of 8°C- (● and ▲) and 13°C-acclimated (○ and △) crucian carp. Data sets were normalized to GAPDH. Values are expressed as means ± SD. N7, normoxia 7 days (8°C, brain $n = 5$, heart $n = 6$; 13°C, brain $n = 6$, heart $n = 10$); A1, anoxia 1 day (8°C, brain $n = 5$, heart $n = 6$; 13°C, brain $n = 6$, heart $n = 12$); A7, anoxia 7 days (8°C, brain $n = 6$, heart $n = 6$; 13°C, brain $n = 6$, heart $n = 9$); R, anoxia 7 days-reoxygenation 7/3 days (8°C, brain $n = 4$, heart $n = 6$; 13°C, brain $n = 6$, heart $n = 10$). P values in graph show the effect of temperature detected by the two-way ANOVA. *Temperature-related differences at each treatment group with a Bonferroni post hoc test. NS, nonsignificant. $^*P < 0.05$, and $^{***}P < 0.001$.

1.9-fold. During reoxygenation, the normoxic difference in HSP70 expression between the two temperatures were reestablished, although the 8°C brain expression did not return to normoxic values (Fig. 5*A*). Brain and heart expressed similar levels of HSP70. For comparison, the pattern of brain HSP30 expression was not significantly different between 8°C and 13°C, and in the heart, only the normoxic value was significantly different at the two temperatures (0.6-fold reduced in 8°C, $P < 0.001$, Fig. 5*B*). The two-way ANOVA detected significant interactions between anoxia and temperatures for brain and hearts HSP70 ($P < 0.001$) and heart HSP30 ($P = 0.018$). The P value for HSP30 brain was 0.0667.

Notably, normoxic HSC70 and HSP90 expression was higher at colder temperature: brain HSC70, 3.3-fold ($P < 0.0001$); heart HSC70 2.6-fold ($P < 0.0001$); HSP90 brain 2.5-fold ($P < 0.001$); and heart HSP90, 1.7-fold ($P = 0.028$) (data not shown). For HSP30, the 8°C groups was 0.6-fold lower than the 13°C groups ($P = 0.004$ and $P = 0.017$ for brain and heart, respectively).

DISCUSSION

In previous studies, the normoxic crucian carp has been suggested to exhibit gene expression patterns that set the stage for anoxia tolerance (12, 13). The HSP70 response seen in the data set of this study gives further support to such constitutive anoxic adaptation and may also suggest that gene expression could be preconditioned by low temperatures. Indeed, temperature would be an excellent cue for preparing crucian carp to an approaching anoxic winter period, and seasonal variation in the ability to withstand anoxia has been observed (43). Other seasonal adaptations in the heart and brain of the crucian carp have also been found (25, 56, 58–60). Moreover, the physiological mechanisms utilized by anoxia-tolerant organisms to protect against anoxia seem similar to those adopted by cold-tolerant organisms to protect against hypothermia (19). In nature, the crucian carp is not known to encounter anoxia at high temperatures, and anoxic periods in its habitat are only known to occur when the water becomes ice covered (37).

Hypothermia-induced regulation of gene expression has been suggested to be important for hypoxia tolerance in other fish, as cold exposure influenced the expression of over 3,000 genes in the common carp (16). Similarly, acclimation to lower temperatures increased the activity of hypoxia-inducible factor-1 (HIF-1), an important factor in coping with hypoxia in mammals, in multiple tissues of crucian carp (47). In the same study, they also reported that decreasing normoxic temperatures from 26°C to 8°C resulted in marked increases in HSP70/HSC70 and HSP90 protein expression. In trout, the mRNA expression in cold is lower than in warm water for HSP70 and HSP30 (7). Similarly, combined protein expression of HSP70/HSC70 in the liver of the goby (*Gillichthys mirabilis*) is lower at low temperature (31). In silver sea bream (*Sparus sarba*) liver, HSP70 increased and HSC70 mRNA expression decreased with a decrease in acclimation temperature (9). Our data indicate that the HSPs that show a large increase in expression during anoxia are also increased by a fall in temperature. A similar change, but in the inverse direction, was seen with HSP30, which tended to decrease with anoxia, as well as with falling temperature. It is, therefore, tempting to speculate that these combined expression patterns for temperature and anoxia are adaptive responses.

HSP70

Together, the hypothermia-induced increase in HSP70 expression in heart and brain of crucian carp and the observed anoxia-induced HSP70 expression at 13°C could suggest that a particular level of HSP70 expression is required during anoxia. The level of anoxic HSP70 expression does not seem to differ between different temperatures. Indeed, it is tempting to speculate that with HSP70 expression, crucian carp show specific winter and summer phenotypes, with the winter phenotype being prepared for anoxia.

Although we have assessed HSPs at the mRNA level rather than at the protein level, previous studies have reported HSP activity to be controlled at the transcription level (2, 17). Moreover, in studies assessing closely related genes, such as HSP70 and HSC70, and in studies assessing genes with low expression, such as HSP70 and HSP30, current approaches for protein expression analyses do not show the necessary sensitivity or resolution. In our hands, antibodies specific for HSP70 seemed not to separate between HSP70 and HSC70 in the crucian carp (data not shown). The same problem is also evident from the literature, and many studies report a combined HSP70 and HSC70 protein expression (31, 47).

In mammals, it is well established that HSP70 plays an essential physiological role during stress situations such as ischemia in the brain (6) and in the heart (28). In fish, less is known about oxygen deprivation and HSP expression. It has been shown that hypoxia at 25°C induced the combined protein expression of HSP70/HSC70 in brain and head kidney of juvenile Nile tilapia (*Oreochromis niloticus*) after hypoxia at 25°C (10). In contrast, hypoxia at 15°C did not result in increased HSP70/HSC70 expression in rainbow trout myocardium (15). In the anoxia-tolerant turtle brain, oxygen deprivation at 25°C induced a transient increase in HSP70 protein expression, while HSC70 expression remained elevated until the end of the experiment (12 h) (44). In contrast, our data show that anoxia at 13°C, but not at 8°C, induced a sustained increase in HSP70 expression in crucian carp brain (7 days). In the crucian carp heart, there was a small but significant increase at 8°C and a large increase at 13°C, which has also been found in the turtle heart at 17°C (45). Although the physiological function of HSP70 during oxygen-deprivation remains somewhat elusive, it involves chaperone control of protein conformation and protects the organism against cell death (5). In general, the crucian carp HSP70 genes were expressed at low levels compared with HSC70 (25- to 250-fold differences in expression levels, depending on tissue and temperature). However, the large increase in HSP70 expression seen in response to anoxia and temperature supports an inducible role for HSP70 in the crucian carp.

HSC70

The observed increase in HSC70 expression in heart of crucian carp after 24 h of anoxia, and the subsequent return to preanoxic levels after a further 6 days in anoxia, indicates a role for HSC70 at the early stages of anoxic adaptation. Interestingly, this early HSC70 boost was present at both temperatures, but was more pronounced at the higher temper-

ature. The reason for this could be that a higher metabolic rate leads to more severe metabolic stress, requiring a larger HSC70 response. Correspondingly, the HSC70 response in the heart was more pronounced than in the brain. This could be because the anoxic heart is required to maintain normoxic levels of contractile activity during anoxia (52), whereas the anoxic brain shows depressed activity (24, 54). Together, these data suggest a role for HSC70 in damage repair and/or damage prevention during initial sequences of metabolic stress in anoxia-exposed crucian carp. This is supported by prevailing views of HSC70 function (6), and similar inductions in HSC70 expression have been reported in short-term anoxic turtle brains (44, 45). Furthermore, in crucian carp heart, HSC70 showed increased expression in the reoxygenation group, resembling expression levels observed after 24 h of anoxia. The increased HSC70 expression could be due to increased requirement for antioxidant enzymes (32), which is, in part, controlled by HSPs (40). However, after 7 days of anoxia, the expression of HSC70 was reduced in brains, but not in hearts. This contradicts a general role for HSC70 in neuronal protection, as was suggested by Brown (6). Like most heat-shock proteins, HSC70 is known to exert a wide range of functions that are not necessarily restricted to stress protection (39). Indeed, HSC70 is likely to be important for organizing the postsynaptic density (57), and the decrease in anoxic brain HSC70 expression could be connected to the neuronal depression shown by this tissue in anoxia (24, 54).

In accordance with a previous study (47), cold-acclimated crucian carp (8°C) showed higher expression of HSC70 and HSP90 than warm-acclimated crucian carp (13° C). This resembles the response shown by HSP70 and suggests a role for these genes in hypothermic survival. However, unlike what was seen for HSP70, these elevations did not result in an altered response to anoxia, suggesting that their roles are confined to handling hypothermia.

HSP90

The observed decrease in HSP90 expression in brain of crucian carp during anoxia is likely to result in lowered HSP90 activity. In mammalian cells, HSP90 has been shown to associate with and to stabilize HIF1α (30). HIF1α is an important regulator of the cellular response to oxygen deprivation (48) and may be important also in the crucian carp (47, 51). Inhibition of HSP90 function has been shown to result in oxygen-independent destabilization of HIF1α during hypoxia (33). Thus, the decreased HSP90 expression seen in the anoxic crucian carp brain is likely to result in decreased HIF1α activity. This contradicts the existing paradigm for oxygen-deprivation survival (48). However, it has been suggested that HIF1α has to be suppressed during anoxia in anoxia-tolerant animals, where the whole body is anoxic, and a global induction of many HIF1α-mediated processes would be too costly and thereby maladaptive (35). Moreover, in mammalian cells, HSP90 has been found to be required for cellular proliferation, and inhibition of HSP90 function is considered a promising approach for cancer treatment (22, 49). Consequently, the reduced HSP90 expression seen in the anoxic crucian carp brain may result in lowered mitotic activity, reducing ATP consumption. Interestingly, we recently reported low AKT phosphorylation and high AMPK phosphorylation in anoxic crucian carp heart and brain (53). This may reflect a lowered mitotic activity, as AKT is believed to increase protein translation, while AMPK has an inhibitory effect (21).

HSP30

In contrast to the other heat-shock proteins assessed in this study, HSP30 expression showed similar changes in response to anoxia at 8°C and 13°C in both brain and heart. Interestingly, the expression decreased in anoxia, which is in contrast to studies of small HSPs in mammalian systems, where HSP27, particularly, has been ascribed a vital role during oxygen deprivation and excitatory seizures. Indeed, HSP27 has been shown to be inducible and protective (3, 29, 61), and it has been suggested to convey ischemic protection even more potently than HSP70 (29). The protective effect may be mediated by its ability to prevent protein aggregates. The decreased HSP30 expression in crucian carp could indicate that anoxia is only a moderate stress for this species. Interestingly, the expression was slightly reduced in the cold, supporting a minor role in anoxia. However, crucian carp HSP30 shows only 39% similarity with human HSP27 and 74% with zebrafish HSP30. This suggests divergent properties and function. Speculatively, the crucian carp HSP30 gene may have evolved to ensure proper normoxic function of proteins necessary for anoxic survival. In such a scenario, oxygen would represent the stressor. However, it is an acknowledged fact that while small heat shock proteins show little sequence conservation between species, they do show conservation of structural organization (18).

Perspectives and Significance

Anoxia exposure significantly altered the expression of all measured heat shock proteins in crucian carp. Interestingly, the results indicated a particular level of HSP70 expression for anoxia-tolerance in crucian carp. Moreover, it suggests that induction of HSC70 and HSP90 expression may be important for the early defense against anoxia, but not for long-term survival. In cold-acclimated crucian carp, this may be obtained through temperature-cued preconditioning. This could be important when studying long-term anoxic survival, because different mechanisms may be involved at different temperatures. A large-scale investigation of gene-expression profiles at different temperatures in anoxia is clearly warranted.

GRANTS

This study was supported by the Research Council of Norway (to G. E. Nilsson) and Ullevål University Hospital (to K. O. Stensløkken and J. Vaage).

DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES

1. **Airaksinen S, Jokilehto T, Rabergh CMI, Nikinmaa M.** Heat- and cold-inducible regulation of HSP70 expression in zebrafish ZF4 cells. *Comp Biochem Physiol B* 136: 275–282, 2003.
2. **Airaksinen S, Rabergh CMI, Sistonen L, Nikinmaa M.** Effects of heat shock and hypoxia on protein synthesis in rainbow trout (*Oncorhynchus mykiss*) cells. *J Exp Biol* 201: 2543–2551, 1998.
3. **Akbar MT, Lundberg AMC, Liu K, Vidyadaran S, Wells KE, Dolatshad H, Wynn S, Wells DJ, Latchman DS, de Belleroche J.** The neuroprotective effects of heat shock protein 27 overexpression in transgenic animals against kainate-induced seizures and hippocampal cell death. *J Biol Chem* 278: 19956–19965, 2003.

4. **Basu N, Todgham AE, Ackerman PA, Bibeau MR, Nakano K, Schulte PM, Iwama GK.** Heat shock protein genes and their functional significance in fish. *Gene* 295: 173–183, 2002.
5. **Beere HM.** Death versus survival: functional interaction between the apoptotic and stress-inducible heat shock protein pathways. *J Clin Invest* 115: 2633–2639, 2005.
6. **Brown IR.** Heat shock proteins and protection of the nervous system. In: *2nd World Conference on Stress*, edited by Csermely P, Korcsmaros T, and Sulyok K. Boston, MA: Blackwell 2007, p. 147–158.
7. **Currie S, Moyes CD, Tufts BL.** The effects of heat shock and acclimation temperature on hsp70 and hsp30 mRNA expression in rainbow trout: in vivo and in vitro comparisons. *J Fish Biol* 56: 398–408, 2000.
8. **Daugaard M, Rohde M, Jaattela M.** The heat shock protein 70 family: Highly homologous proteins with overlapping and distinct functions. *FEBS Lett* 581: 3702–3710, 2007.
9. **Deane EE, Woo NYS.** Cloning and characterization of the hsp70 multigene family from silver sea bream: Modulated gene expression between warm and cold temperature acclimation. *Biochem Biophys Res Commun* 330: 776–783, 2005.
10. **Delaney MA, Klesius PH.** Hypoxic conditions induce Hsp70 production in blood, brain and head kidney of juvenile Nile tilapia *Oreochromis niloticus* (L.). *Aquaculture* 236: 633–644, 2004.
11. **Ehrnsperger M, Graber S, Gaestel M, Buchner J.** Binding of non-native protein to Hsp25 during heat shock creates a reservoir of folding intermediates for reactivation. *EMBO J* 16: 221–229, 1997.
12. **Ellefsen S, Sandvik GK, Larsen HK, Stensløkken KO, Hov DA, Kristensen TA, Nilsson GE.** Expression of genes involved in excitatory neurotransmission in anoxic crucian carp (*Carassius carassius*) brain. *Physiol Genomics* 35: 5–17, 2008.
13. **Ellefsen S, Stensløkken KO, Fagernes CE, Kristensen TA, Nilsson GE.** Expression of genes involved in GABAergic neurotransmission in anoxic crucian carp brain (*Carassius carassius*). *Physiol Genomics* 36: 61–68, 2009.
14. **Ellefsen S, Stensløkken KO, Sandvik GK, Kristensen TA, Nilsson GE.** Improved normalization of real-time reverse transcriptase polymerase chain reaction data using an external RNA control. *Anal Biochem* 376: 83–93, 2008.
15. **Gamperl AK, Vijayan MM, Pereira C, Farrell AP.** β-receptors and stress protein 70 expression in hypoxic myocardium of rainbow trout and chinook salmon. *Am J Physiol Regul Integr Comp Physiol* 274: R428–R436, 1998.
16. **Gracey AY, Fraser EJ, Li WZ, Fang YX, Taylor RR, Rogers J, Brass A, Cossins AR.** Coping with cold: An integrative, multitissue analysis of the transcriptome of a poikilothermic vertebrate. *Proc Natl Acad Sci USA* 101: 16970–16975, 2004.
17. **Hansen LK, Houchins JP, Oleary JJ.** Differential regulation of Hsc70, Hsp70, Hsp90-α, and Hsp90-β messenger-RNA expression by mitogen activation and heat-shock in human-lymphocytes. *Exp Cell Res* 192: 587–596, 1991.
18. **Haslbeck M, Franzmann T, Weinfurtner D, Buchner J.** Some like it hot: the structure and function of small heat-shock proteins. *Nat Struct Mol Biol* 12: 842–846, 2005.
19. **Hochachka PW.** Defense strategies against hypoxia and hypothermia. *Science* 231: 234–241, 1986.
20. **Hutter MM, Sievers RE, Barbosa V, Wolfe CL.** Heat-shock protein induction in rat hearts—a direct correlation between the amount of heat-shock protein-induced and the degree of myocardial protection. *Circulation* 89: 355–360, 1994.
21. **Inoki K, Zhu TQ, Guan KL.** TSC2 mediates cellular energy response to control cell growth and survival. *Cell* 115: 577–590, 2003.
22. **Isaacs JS, Xu WP, Neckers L.** Heat shock protein 90 as a molecular target for cancer therapeutics. *Cancer Cell* 3: 213–217, 2003.
23. **Jaillon O, Aury JM, Brunet F, Petit JL, Stange-Thomann N, Mauceli E, Bouneau L, Fischer C, Ozouf-Costaz C, Bernot A, Nicaud S, Jaffe D, Fisher S, Lutfalla G, Dossat C, Segurens B, Dasilva C, Salanoubat M, Levy M, Boudet N, Castellano S, Anthouard R, Jubin C, Castelli V, Katinka M, Vacherie B, Biemont C, Skalli Z, Cattolico L, Poulain J, de Berardinis V, Cruaud C, Duprat S, Brottier P, Coutanceau JP, Gouzy J, Parra G, Lardier G, Chapple C, McKernan KJ, McEwan P, Bosak S, Kellis M, Volff JN, Guigo R, Zody MC, Mesirov J, Lindblad-Toh K, Birren B, Nusbaum C, Kahn D, Robinson-Rechavi M, Laudet V, Schachter V, Quetier F, Saurin W, Scarpelli C, Wincker P, Lander ES, Weissenbach J, Crollius HR.** Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype. *Nature* 431: 946–957, 2004.
24. **Johansson D, Nilsson GE, Tornblom E.** Effects of anoxia on energy-metabolism in crucian carp brain-slices studied with microcalorimetry. *J Exp Biol* 198: 853–859, 1995.
25. **Kakela R, Mattila M, Hermansson M, Haimi P, Uphoff A, Paajanen V, Somerharju P, Vornanen M.** Seasonal acclimatization of brain lipidome in a eurythermal fish (*Carassius carassius*) is mainly determined by temperature. *Am J Physiol Regul Integr Comp Physiol* 294: R1716–R1728, 2008.
26. **Kondo H, Watabe S.** Temperature-dependent enhancement of cell proliferation and mRNA expression for type I collagen and HSP70 in primary cultured goldfish cells. *Comp Biochem Physiol A* 138: 221–228, 2004.
27. **Lanneau D, Brunet M, Frisan E, Solary E, Fontenay M, Garrido C.** Heat shock proteins: essential proteins for apoptosis regulation. *J Cell Mol Med* 2008.
28. **Latchman DS.** Heat shock proteins and cardiac protection. *Cardiovasc Res* 51: 637–646, 2001.
29. **Latchman DS.** HSP27 and cell survival in neurones. *Int J Hyperthermia* 21: 393–402, 2005.
30. **Liu YV, Semenza GL.** RACK1 vs. HSP90—competition for HIF-1 alpha degradation vs. stabilization. *Cell Cycle* 6: 656–659, 2007.
31. **Lund SG, Ruberte MR, Hofmann GE.** Turning up the heat: The effects of thermal acclimation on the kinetics of hsp70 gene expression in the eurythermal goby, *Gillichthys mirabilis*. *Comp Biochem Physiol A* 143: 435–446, 2006.
32. **Lushchak VI, Bagnyukova TV, Lushchak OV, Storey JM, Storey KB.** Hypoxia and recovery perturb free radical processes and antioxidant potential in common carp (*Cyprinus carpio*) tissues. *Int J Biochem Cell B* 37: 1319–1330, 2005.
33. **Minet E, Mottet D, Michel G, Roland I, Raes M, Remacle J, Michiels C.** Hypoxia-induced activation of HIF-1, role of HIF-1 α-Hsp90 interaction. *FEBS Lett* 460: 251–256, 1999.
34. **Murry CE, Jennings RB, Reimer KA.** Preconditioning with ischemia—a delay of lethal cell injury in ischemic myocardium. *Circulation* 74: 1124–1136, 1986.
35. **Nilsson G, Oijordsbakken M, Ellefsen S, Stensløkken K, Stecyk J.** Hypoxia inducible factor (HIF). A threat to hypoxic survival? *Comp Biochem Physiol A* 150: S118–S119, 2008.
36. **Nilsson GE, Lutz PL.** Anoxia-tolerant brains. *J Cereb Blood Flow Metab* 24: 475–486, 2004.
37. **Nilsson GE, Renshaw GMC.** Hypoxic survival strategies in two fishes: extreme anoxia tolerance in the North European crucian carp and natural hypoxic preconditioning in a coral-reef shark. *J Exp Biol* 207: 3131–3139, 2004.
38. **Nilsson GE, Rosen P, Johansson D.** Anoxic depression of spontaneous locomotor activity in crucian carp quantified by a computerized imaging technique. *J Exp Biol* 180: 153–162, 1993.
39. **Obrenovitch TP.** Molecular physiology of preconditioning-induced brain tolerance to ischemia. *Physiol Rev* 88: 211–247, 2008.
40. **Parcellier A, Schmitt E, Brunet M, Hammann A, Solary E, Garrido C.** Small heat shock proteins HSP27 and αB-crystallin: cytoprotective and oncogenic functions. *Antioxid Redox Signal* 7: 404–413, 2005.
41. **Parsell DA, Lindquist S.** The function of heat-shock proteins in stress tolerance—degradation and reactivation of damaged proteins. *Annu Rev Genet* 27: 437–496, 1993.
42. **Pearl LH, Prodromou C.** Structure and mechanism of the Hsp90 molecular chaperone machinery. *Annu Rev Biochem* 75: 271–294, 2006.
43. **Piironen J, Holopainen IJ.** A note on seasonality in anoxia tolerance of crucian carp (*Carassius-carassius* (L)) in the laboratory. *Ann Zool Fenn* 23: 335–338, 1986.
44. **Prentice HM, Milton SL, Scheurle D, Lutz PL.** The upregulation of cognate and inducible heat shock proteins in the anoxic turtle brain. *J Cereb Blood Flow Metab* 24: 826–828, 2004.
45. **Ramaglia V, Buck LT.** Time-dependent expression of heat shock proteins 70 and 90 in tissues of the anoxic western painted turtle. *J Exp Biol* 207: 3775–3784, 2004.
46. **Ramakers C, Ruijter JM, Deprez RHL, Moorman AFM.** Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett* 339: 62–66, 2003.
47. **Rissanen E, Tranberg HK, Sollid J, Nilsson GE, Nikinmaa M.** Temperature regulates hypoxia-inducible factor-1 (HIF-1) in a poikilothermic vertebrate, crucian carp (*Carassius carassius*). *J Exp Biol* 209: 994–1003, 2006.

48. **Semenza GL.** Hypoxia-inducible factor 1 (HIF-1) pathway [Online]. *Sci STKE* 2007: cm8, 2007.
49. **Sidera K, Patsavoudi E.** Extracellular HSP90: an emerging target for cancer therapy. *Curr Signal Transduct Therapy* 4: 51–58, 2009.
50. **Sollid J, Kjernsli A, De Angelis PM, Rohr AK, Nilsson GE.** Cell proliferation and gill morphology in anoxic crucian carp. *Am J Physiol Regul Integr Comp Physiol* 289: R1196–R1201, 2005.
51. **Sollid J, Rissanen E, Tranberg HK, Thorstensen T, Vuori KAM, Nikinmaa M, Nilsson GE.** HIF-1α and iNOS levels in crucian carp gills during hypoxia-induced transformation. *J Comp Physiol [B]* 176: 359–369, 2006.
52. **Stecyk JAW, Stensløkken KO, Farrell AP, Nilsson GE.** Maintained cardiac pumping in anoxic crucian carp. *Science* 306: 77–77, 2004.
53. **Stensløkken KO, Ellefsen S, Stecyk JA, Dahl MB, Nilsson GE, Vaage J.** Differential regulation of AMP-activated kinase and AKT kinase in response to oxygen availability in crucian carp (*Carassius carassius*). *Am J Physiol Regul Integr Comp Physiol* 295: R1803–R1814, 2008.
54. **Stensløkken KO, Milton SL, Lutz PL, Sundin L, Renshaw GM, Stecyk JA, Nilsson GE.** Effect of anoxia on the electroretinogram of three anoxia-tolerant vertebrates. *Comp Biochem Physiol A* 150: 395–403, 2008.
55. **Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG.** The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25: 4876–4882, 1997.
56. **Tiitu V, Vornanen M.** Cold adaptation suppresses the contractility of both atrial and ventricular muscle of the crucian carp heart. *J Fish Biol* 59: 141–156, 2001.
57. **Tobaben S, Thakur P, Fernandez-Chacon R, Sudhof TC, Rettig J, Stahl B.** A trimeric protein complex functions as a synaptic chaperone machine. *Neuron* 31: 987–999, 2001.
58. **Vornanen M.** Seasonal adaptation of Crucian Carp (*Carassius carassius* L) heart—glycogen stores and lactate-dehydrogenase activity. *Can J Zool* 72: 433–442, 1994.
59. **Vornanen M.** Seasonal and temperature-induced changes in myosin heavy-chain composition of crucian carp hearts. *Am J Physiol Regul Integr Comp Physiol* 267: R1567–R1573, 1994.
60. **Vornanen M, Paajanen V.** Seasonal changes in glycogen content and Na^+-K^+-ATPase activity in the brain of crucian carp. *Am J Physiol Regul Integr Comp Physiol* 291: R1482–R1489, 2006.
61. **Wagstaff MJD, CollacoMoraes Y, Aspey BS, Coffin RS, Harrison MJG, Latchman DS, deBelleroche JS.** Focal cerebral ischaemia increases the levels of several classes of heat shock proteins and their corresponding mRNAs. *Mol Brain Res* 42: 236–244, 1996.
62. **Yellon DM, Hausenloy DJ.** Myocardial reperfusion injury. *N Engl J Med* 357: 1121–1135, 2007.
63. **Yin C, Salloum FN, Kukreja RC.** A novel role of microRNA in late preconditioning: upregulation of endothelial nitric oxide synthase and heat shock protein 70. *Circ Res* 104: 572–575, 2009.