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Helene Kile Larsen

Abstract

The crucian carp (*Carassius carassius*) manage to live without oxygen for months at low temperatures, and the only way to produce ATP is through the glycolytic pathway, which yields less than 10% of the ATP formed through aerobic metabolism. There are two options for compensating for the lowered ATP production efficiency; (1) increase the rate of ATP production and/or (2) reduce the rate of ATP consumption. An energy saving decrease of ion permeability ("channel arrest") is displayed by the red-eared slider turtle (*Trachemys scripta*) during anoxia. To examine if a similar strategy is used by the crucian carp, brain mRNA levels of α-subunits of voltage-gated Na^+ and Ca^{2+} (Na_v and Ca_v) channels were quantified by real-time RT-PCR after exposure to 1-7 days of anoxia and anoxia followed by reoxygenation (all at 11°C).

Heat shock proteins (Hsps) are known for protecting cells against detrimental effects of various stressors, including anoxia. Of the many functions proposed for Hsps, one is to refold proteins to their functional structure and another is to designate damaged proteins for degradation. In this thesis brain mRNA levels of Hsp90, Hsp70.1, Hsp70.2, Hsc70 and Hsp30 were quantified by real-time RT-PCR in crucian carp exposed to anoxia at two temperatures, 8°C and 13°C.

No changes were found in mRNA levels of the α-subunits of the voltage-gated ion channels. Thus, the results do not support the "channel-arrest" hypothesis in crucian carp brain. By contrast, for Hsps a significant increase was found in both Hsp70.1 and Hsp70.2 mRNA levels at 13°C, while the response of these two paralogs showed divergent changes at 8°C. During anoxia, a decrease was found in Hsc70 and Hsp90 mRNA at 8°C, while at 13°C a significant decrease was found in Hsc70 and Hsp30 mRNA. These findings support the possibility that the Hsps are involved in the anoxia response of crucian carp brain, and that temperature has an effect on the regulation of some Hsps.

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1 Introduction

Oxygen depletion (anoxia) is lethal to most vertebrates. However, there are exceptions and a few species like the crucian carp (*Carassius carassius*) and the red-eared slider turtle (*Trachemys scripta*) manage to cope with the detrimental effects of anoxia, surviving without oxygen for months at low temperatures (Ultsch, 1985; Piironen, 1986). During the anoxic state, such animals are completely dependent on anaerobic ATP production (glycolysis) to meet the energy needs. Glycolytic ATP production is about 10% less efficient than aerobic ATP production (Hochachka, 1984), indicating that a lowering of ATP use may be necessary to balance ATP expenditure during anoxia. In this regard, much attention has been directed towards brain tissue. Under normal conditions, the vertebrate brain has a high rate of ATP use, most of which is associated with ion pumping needed to maintain ion gradients across the cell membrane (Erecinska & Silver, 1989). Hence, by depressing these ion-fluxes the ATP usage of neurons could be lowered. Such a depression has been shown to occur in brain tissue of anoxic red-eared sliders where electroencephalogram (EEG) recordings indicate reduced neuronal activity in anoxic individuals (Fernandes *et al.*, 1997). Moreover, in anoxic red-eared slider turtles metabolic rate is reduced to 5-10% compared to normoxic turtles (Buck *et al.*, 1993), indicating a nearly comatose state (Feng *et al.*, 1988; Chih *et al.*, 1989). Similarly, in crucian carp sensory functions have been demonstrated to be depressed during anoxia (Johansson *et al.*, 1997), while heat production in brain tissue is reduced to 60% in response to anoxia (Johansson *et al.*, 1995). The depression of neuronal activity described in turtles and in crucian carp has for long been hypothesized to be mediated through a phenomenon termed “channel arrest”, the lowering of ion permeability of ion channels (Lutz *et al.*, 1985; Hochachka, 1988).

In nature, the anoxia tolerance of both crucian carp and slider turtles have evolved in response to over-wintering in anoxic conditions at near 0°C under the ice of ponds and streams. Crucian carp has been demonstrated to move to colder water during hypoxia (Rausch *et al.*, 2000), a strategy that will suppress the metabolic rate. Furthermore, studies have shown that anoxia tolerance is increased at low temperatures in both turtle and fish (Herbert & Jackson, 1985; Ultsch, 1985).

This thesis aims to investigate the validity of the channel arrest hypothesis in anoxic crucian carp tissue by looking at the relative mRNA expression of voltage-gated ion channels using real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR). Moreover, being exposed to anoxia is a stressor for most organisms, and heat shock proteins (Hsps) are well-known for having a protective role under cellular stress (Lindquist & Craig, 1988). Therefore, this thesis also investigates the relative mRNA expression of Hsps in anoxic brain tissue and in addition, at two different temperatures to study possible effects of temperature as well.

1.1 Anoxia

1.1.1 The anoxic disaster

In most vertebrates oxygen is needed to make sufficient amounts of ATP to fuel cellular pathways. Sufficient ATP is especially important for proper function of the Na^+/K^+-ATPase, which is responsible for keeping the ion distribution across the cell membrane and the membrane potential at a steady state. In brain the Na^+/K^+-ATPase may spend 50 % of the ATP produced, and the membrane potential is rapidly disturbed when ATP levels drop (Erecinska & Silver, 1989; Hochachka *et al.*, 1996). This implies that when oxygen supply is cut off, Na^+/K^+-ATPase activity will diminish, leading to collapse of ion gradients and a coinciding depolarization of neurons. A subsequent massive release of excitatory neurotransmitters results in additional depolarization, leading to an uncontrolled rise in the intracellular Ca^{2+} level. Consequently, cellular functions are disturbed to such a degree that the cell will disintegrate or go into apoptosis (Lutz, 2003) Thus, lack of oxygen in anoxia-sensitive brains will rapidly lead to cell death (Hansen, 1985; Silver & Erecinska, 1990).

1.1.2 Surviving anoxia

To survive prolonged anoxia, ATP production must balance cellular ATP demands. Thus, the cells of anoxia-tolerant vertebrates have two options for

compensating the lowered ATP production efficiency; (1) to increase the rate of ATP production and/or (2) to reduce the rate of ATP consumption.

The only ATP producing machinery in the absence of oxygen is the glycolytic pathway, and glycogen must therefore be available throughout the anoxic period. Glycogen makes up 15 % of the crucian carp liver, and this is the largest glycogen store of any known vertebrate (Hyvarinen *et al.*, 1985). Also, the glycogen stores in the crucian carp brain are larger than for other vertebrates study (Schmidt, 1988). Thus, the crucian carp is capable of keeping ATP production at a respectable level as long as glycogen is available throughout the anoxic period.

Both the red-eared slider turtle and the crucian carp respond to anoxia through approximately a doubling of brain blood flow within the first minutes. While this increase in brain blood flow is sustained in the anoxic crucian carp (Nilsson *et al.*, 1994), it falls back to almost pre-anoxic levels in the red-eared slider after about one hour of anoxia (Hylland *et al.*, 1994; Stecyk *et al.*, 2004), which probably correspond to the onset of deep hypometabolism in the turtle. Another outcome of the anoxic response is increased extracellular levels of adenosine, which probably is a result of a minor ATP breakdown (Nilsson, 1992). Adenosine seems to help prevent toxic levels of intracellular Ca^{2+} and cellular death in anoxic goldfish (*Carassius auratus*), another anoxia-tolerant animal that is a close relative to the crucian carp (Krumschnabel *et al.*, 2000; Lutz & Prentice, 2002; Lutz & Nilsson, 2004).

To reduce ATP consumption, the metabolic rate of the crucian carp is reduced to some 60-70 % during anoxia, as indicated by microcalorimetric measurements of heat production in crucian carp brain slices (Johansson *et al.*, 1995). Moreover, swimming activity is reduced by 50 % at 9 °C under anoxic conditions (Nilsson, 1993), and sensory functions like hearing (Suzue *et al.*, 1987) and vision (Johansson *et al.*, 1997) are also depressed during anoxia.

Glutamate is the main excitatory neurotransmitter in the central nervous system (CNS) and hence, the glutamate-receptors play a central role in mediating excitatory synaptic transmission. Recent data suggest that the mRNA levels of glutamate-receptors, such as the N-methyl-D-aspartate receptor (NMDAR) and the α-amino-5-hydroxy-3-methyl-4-isoxasole propionic acid receptor (AMPAR) are relatively unaffected by anoxia

in crucian carp, although a tendency towards a decrease was seen in some of the receptor-subunits (Ellefsen, 2006). In the case of the freshwater turtle, it has been shown that the NMDAR number falls in anoxia (Bickler & Buck, 1998).

Levels of gamma-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the CNS, show an 80-fold increase of normoxic levels in the red-eared slider turtle (Nilsson & Lutz, 1991), and a 2 fold increase is seen in the crucian carp after 5 h of anoxia at 10 °C (Hylland & Nilsson, 1999a). Higher levels of GABA may contribute to a neuronal depression that will reduce ATP use.

Neuronal depression may also be influenced by decreased ion channel permeability across cell membranes, referred to as "channel arrest" (Hochachka, 1986; Bickler & Buck, 1998). The "channel arrest" hypothesis proposes that key ion channels, such as voltage-gated ion channels, may alter gating properties via e.g. conformational changes or gene expression, to save energy during anoxia. Such an arrest of ion channels could also function to reduce a toxic increase of intracellular of Ca^{2+} levels (Bickler & Buck, 1998). However, investigations of a possible "channel arrest" where performed on brain tissue of anoxic crucian carp, due to ion permeability of Ca^{2+} and K^{+}, but no detectable changes in the ion permeability were found (Johansson & Nilsson, 1995; Thorén, 2000). Yet, reduced ion permeability of voltage-gated Na^{+} and Ca^{2+} channels might still be a possibility to reduce neuronal activity and decrease the ATP use. In this study, changes in mRNA levels of the pore-forming subunit of voltage-gated Na^{+} and Ca^{2+} channels were measured to examine the possibility of a transcriptional depression of these key ion channels during anoxia.

1.2 Voltage-gated ion channels

Voltage-gated ion channels consist of Na^{+}-, Ca^{2+}- and K^{+} channels, and are responsible for the formation and modulation of action potentials and transmitter release in neurons. The isoforms within each ion channel family are named according to differences in their α-subunit, the pore-forming unit. The number of subunits differs among the three ion channel families, but the α-subunit is similar. Often, expression of the α-subunit alone is sufficient for a functional ion channel (Goldin *et al.*, 1986;

Takahashi *et al.*, 1987), but auxiliary subunits may modify opening and closing mechanisms of the channels (Catterall, 2000a, 2000b).

In this thesis, mRNA levels of pore-forming α-subunits in voltage gated Na^+ and Ca^{2+} channels have been studied on anoxic crucian carp brain tissue. It should be noted that the following information is mostly based on studies of mammals, and may not necessarily apply to fish.

1.2.1 Voltage-gated Na^+ channels

Voltage-gated Na^+ (Na_v) channels make up a family of nine functionally expressed α-subunit isoforms. Out of these, four isoforms are present in the brain: Na_v 1.1, Na_v 1.2, Na_v 1.3 and Na_v 1.6 (Goldin, 2001). The Na_v channels in brain consist of one pore-forming α-subunit (260 kDa) and two auxiliary subunits β_1 (36 kDa) and β_2 (33 kDa) (Catterall, 1975; Messner & Catterall, 1985) (Figure 1.1). The β-subunits are important for kinetics of the Na_v channel and help regulate opening and closing of the channel (Isom *et al.*, 1992; Isom *et al.*, 1995).

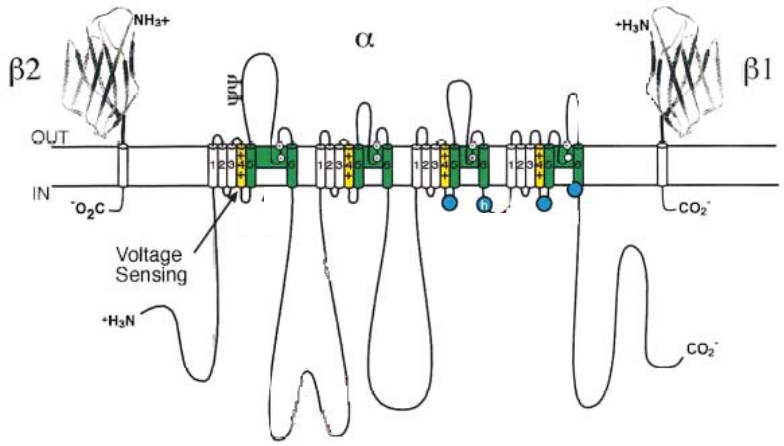

Figure 1.1. Structure of voltage-gated Na^+ channel. The three subunits are shown; α, β_1 and β_2. The pore-forming α subunit consists of four domains (I-IV) with six transmembrane segments (S1-S6) in each domain. From Catterall (2000a)

The functional role of Na_v channels is primarily to form and conduct action potentials in excitable cells, and they also influence subthreshold electric activity via persistent Na^+ currents (Ogata & Ohishi, 2002). Persistent Na^+ currents occur when Na_v channels remain open for longer periods (Taylor & Meldrum, 1995), and in rat neurons it has been shown that the amplitude of persistent Na^+ currents is increased in response to hypoxia (Ju *et al.*, 1996). By decreasing the conductivity through Na_v channels in mammalian CNS, neuronal injury induced by hypoxia may be reduced, and hence favor survival during oxygen depletion. In anoxic turtle brain Perez-Pinzon *et al.* (1992) reported a decrease of Na_v channels, and suggested this was due to the physiological events related to anoxia.

Several modulating factors can influence the activity of Na_v channels. Biochemical studies of Na_v channels in brain synaptosomes show that Na_v channels are rapidly phosphorylated by cAMP-dependent protein kinase (Costa & Catterall, 1984a), and studies have reported a reduction of peak Na^+ currents in brain neurons in response to these modulations (Li *et al.*, 1992). Also dopamine and protein kinase C (PKC) may modulate Na_v channels in such a way that Na^+ currents and generation of action potential are reduced (Costa & Catterall, 1984b; Calabresi *et al.*, 1987). Thus, modulations of the pore-forming subunit of Na_v channels may regulate the generation of action potentials in neuronal cells, and hence affect neuronal transmission and information processing. Under extreme conditions such as anoxia, modulating mechanisms may play a part in regulating Na_v channels to cope with the anoxic challenge.

1.2.2 Voltage-gated Ca^{2+} channels

The voltage-gated calcium (Ca_v) channels are divided into three families according to the characteristics of their α-subunits and type of Ca^{2+} currents that is mediated; Ca_v 1 (L-type), Ca_v 2 (P/Q-, N-, and R-type) and Ca_v 3 (T-type). Ca_v channels play roles in muscle contraction, hormone secretion, synaptic transmission, signal transduction and gene expression. In this thesis, only Ca_v 2 and Ca_v 3 channels have been

investigated, because the Ca_v 1 channels are not highly expressed in brain tissue (Catterall, 2000b).

Compared to functional Na_v channels which are comprised of three subunits, functional Ca_v channels consist of five subunits; one α_1-subunit (200-250 kDa), one α_2-subunit which forms a complex with one δ-subunit (170 kDa), one modulating β-subunit of 55 kDa and one transmembrane γ-subunit of 33 kDa (Curtis & Catterall, 1984; Takahashi *et al.*, 1987; Gurnett *et al.*, 1996).

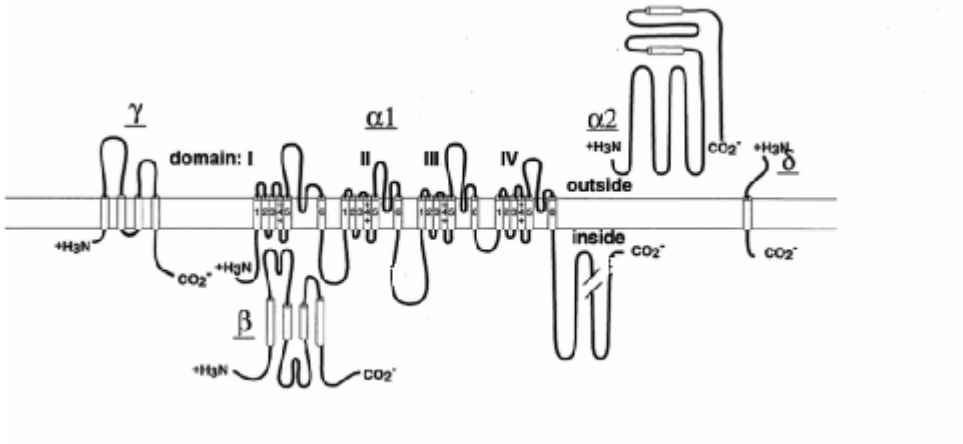

Figure 1.2. Structure of voltage-gated Ca^{2+} channel from skeletal muscle. The five subunits are shown; α_1, α_2, β, δ and γ. The pore-forming α_1 subunit consists of four domains (I-IV) with six transmembrane segments (S1-S6) in each domain. From Catterall (2000b).

The Ca_v 2 family includes three channels; Ca_v 2.1, Ca_v 2.2 and Ca_v 2.3. They are mostly found in synapses, and are the main mediators of Ca^{2+} fluxes associated with release of neurotransmitters. A regulation of these could as a result directly affect neuronal activity in the anoxic crucian carp. The members of the Ca_v 2 family need strong depolarization to be activated, and are therefore called high-voltage activated (HVA) Ca^{2+} channels (Catterall, 2000b).

Among many modulating factors that may influence the function of the Ca_v 2 channels, neurotransmitters and multiple G-protein coupled pathways are the most studied (Bean, 1989; Hille, 1994; Dolphin, 1995; Ikeda, 1996). Studies have shown that

G-protein coupled pathways inhibit Ca^{2+} currents in a manner that can be transiently reversed by strong depolarizations (Elmslie *et al.*, 1990; Ikeda, 1991; Hille, 1994). Also, reversion of G-protein coupled pathways can be obtained by phosphorylating intracellular sites of the Ca_v channels by neurotransmitters acting through protein kinase C (Swartz *et al.*, 1993).

The Ca_v 3 family also includes three channels; Ca_v 3.1, Ca_v 3.2, Ca_v 3.3, where each channel conducts transient Ca^{2+} currents (T-type Ca^{2+} currents). The Ca_v 3 channels are located in the soma and dendrites of neurons in contrast to the Ca_v 2 channels, which are mostly found in presynaptic areas. Because only weak depolarizations are needed to activate Ca_v 3 channels, they are also known as low-voltage-activated Ca^{2+} channels (LVA) (Catterall, 2000b). This implies that T-type Ca^{2+} currents are not likely to induce neurotransmitter release, and may function primarily as a modulator of synaptic efficacy, helping to shape and strengthen changes in local synaptic potentials, thereby controlling action potentials (McCobb & Beam, 1991; Huguenard, 1996; Lambert *et al.*, 1998; Yunker & McEnery, 2003). Furthermore, different splice variants exist for each of the three α_1 subunits in the Ca_v 3 family, a source of variation that may be important in membrane targeting, channel stability, and/or channel modulation (Yunker & McEnery, 2003).

Ca_v 3 channels are commonly observed in early development of cells and it has been shown that the density, amplitude and property of neuronal Ca_v 3 channels change over time (Gottmann *et al.*, 1988; Desmadryl *et al.*, 1998). Modulation of Ca_v 3 channels are not much studied, but Ca^{2+}/calmodulin-dependent protein kinase II has been found to affect gating of Ca_v 3 channels (Lu *et al.*, 1994). Moreover, it has been reported that angiotensin II acting via G-protein pathways, inhibits Ca^{2+} fluxes in neuroblastoma cells (Buisson *et al.*, 1992; Lu *et al.*, 1996).

Calcium entry is one of the central causes of cellular damage under anoxia, and a putative strategy for surviving anoxia would be to reduce the permeability of voltage-gated Ca^{2+} channels.

1.3 Heat shock proteins

Heat shock proteins (Hsps) constitute a large family of proteins classified into several groups according to molecular size and amino acid sequence. The Hsps are normally grouped into five subfamilies: the high molecular weight (100-110 kDa) family; the 83-90 kDa family; the 70 kDa family (66-78 kDa), the 60 kDa family, and the small Hsps (15-30 kDa) (Franklin *et al.*, 2005). In this thesis Hsp90, three members of the 70 kDa family and Hsp30 have been studied in crucian carp.

Hsps are known to protect vertebrate cells against a wide spectrum of stressors, like toxins, heavy metals, oxygen depletion, and variation in temperature (Feige & van Eden, 1996; Airaksinen *et al.*, 1998; Lee *et al.*, 2001; Ramaglia & Buck, 2004). In this respect, two main functions have been proposed for Hsps; a molecular chaperone function, and a protein degradation function (Burel *et al.*, 1992; Parsell & Lindquist, 1993). Acting as molecular chaperones, Hsps recognize unstable proteins and facilitate proper protein folding, and facilitate refolding of denatured proteins in both normal and stressful conditions. In protein degradation, Hsps are involved in presenting damaged substrates for proteolysis, thus repairing damage after periods of stress (Ohtsuka & Hata, 2000).

Hsps protect cells from stress in a complex and poorly understood manner, in which different Hsps serve different roles at different locations in the cells, some needing co-proteins for proper function (Freeman *et al.*, 1995; Demand *et al.*, 1998; Mayer & Bukau, 1998). Another aspect of this complexity is reflected through a cross-species view of Hsps, where different species respond to similar types of stress using different Hsps (Parsell & Lindquist, 1993).

When an organism is exposed to a stressor, cellular pathways are rapidly affected and it is a clear advantage to be able to minimize negative effects through a strong defense system. Findings show that most Hsps are expressed in both normal and stressed cells, with their synthesis being enhanced by exposure to stressors (Becker & Craig, 1994; Hartl *et al.*, 1994). This has led to the idea that Hsp function is required in normal cells, but that it is needed to an even greater extent in stressed cells (Parsell & Lindquist, 1993; Parsell *et al.*, 1993; Latchman, 2005). It could be reasonable to expect that stress-

tolerant animals could be adapted by having a raised defense against the stressor. As an example, high levels of Hsp70 found in normoxic turtle brain contrast with low Hsp70 levels in the anoxia-sensitive mammal brain, indicating that the anoxia-tolerant turtle is prepared for stressful situations (Prentice *et al.*, 2004).

1.3.1 Hsp70

The Hsp70 family, and more specifically Hsp70, is the best characterized of the heat shock proteins. Amino acid sequences of Hsp70 are ~50% identical between prokaryotes and eukaryotes (Gupta & Singh, 1992; Mayer & Bukau, 1998), indicating that Hsp70 function has been extremely well conserved throughout evolution (Parsell & Lindquist, 1993). Several functions have been denoted Hsp70, such as regulation of apoptosis, molecular chaperoning, antigene presentation, uncoating clathrin-coated vesicles, nuclear transport, synaptic vesicle trafficking and synaptic plasticity (Pelham, 1986; Rothman & Schmid, 1986; DeLuca-Flaherty *et al.*, 1990; Rordorf *et al.*, 1991; Ohtsuka & Suzuki, 2000). Hsp70 is expressed at low levels in normal cells, but the Hsp70 expression is highly induced by stressors, including ischemia and hypoxia (Murphy *et al.*, 1999; Snoeckx *et al.*, 2001).

Heat shock cognate 70 (Hsc70), a constitutively expressed member of Hsp70 family (Craig *et al.*, 1983), is highly expressed in non-stressed tissues and is only slightly inducible by stress (Snoeckx *et al.*, 2001). This indicates different functions between Hsp70 and Hsc70. Loones *et al.* (2000) suggested that Hsc70 is localized in neurons and that it is involved in axonal transport, possibly ensuring transport of properly folded proteins along the axon (Black *et al.*, 1991; Bechtold *et al.*, 2000).

Due to one additional gene duplication in fish compared to tetrapods, several paralogs of genes is often found. In this thesis two paralogs of Hsp70 (Hsp70.1 and Hsp70.2) and one paralog of Hsc70 were characterized and investigated in crucian carp, to assess potential differences in gene expression patterns in response to anoxia.

1.3.2 Hsp90

Hsp90 is abundantly expressed in unstressed cells, but its expression is normally enhanced by stress (Lindquist & Craig, 1988). For example, Hsp90 is essential for cellular function at all temperatures in yeast, indicating that Hsp90 might have vital basic functions in eukaryotic cells (Borkovich *et al.*, 1989).

Hsp90, and other members of the 83-90 kDa family of Hsps, are primarily located in cytosolic and nuclear compartments of eukaryotic cells (Parsell & Lindquist, 1993). These Hsps interact with several proteins, such as steroid-hormone receptors (Catelli *et al.*, 1985; Sanchez *et al.*, 1985), actin filaments (Koyasu *et al.*, 1986), different kinases (Miyata & Yahara, 1992, 1995), and calmodulin (Minami *et al.*, 1993). The binding of Hsp90 to steroid-hormone receptors has been shown to be crucial for regulating the receptor's ability to bind to promoter regions of genomic DNA, giving Hsp90 a role as a regulator of hormone receptor activity (Rose *et al.*, 1989). Like Hsc70, Hsp90 bind actin and may also play a role in cytosolic transport (Nishida *et al.*, 1986).

1.3.3 Hsp30

In accordance with the other families of Hsps, small Hsps have been suggested to play a role in stress responses, but they have also been assigned a role in growth and development (Lindquist & Craig, 1988). Small Hsps may exert their function through influencing signal transduction pathways, and through protection of microfilament networks during stress exposures (Landry & Huot, 1995). Compared to other families of Hsps, amino acid sequences of small Hsps have been less conserved throughout evolution (Parsell & Lindquist, 1993).

Growth and development is probably a low priority under anoxia, because energy use needs to be minimized. However, protection of the cytoskeleton and microfilaments may be given high priority under anoxic conditions, to preserve the integrity of the existing cells in due to a likely reduction in the formation of new cells.

1.4 Real-time RT-PCR

In this study real-time reverse transcription polymerase chain reaction (real-time RT-PCR) was used to estimate levels of mRNA of the genes of interest. The simplicity, specificity and sensitivity, together with the potential for high throughput has made real-time RT-PCR an attractive technology for detection and/or comparison of RNA levels (Schwaller *et al.*, 1997; Wang *et al.*, 1999) This method involves two steps: (1) synthesis of complementary DNA (cDNA), and (2) amplification of target genes by real-time RT-PCR. A fluorescent reporter molecule, SYBR green, intercalates with double-stranded DNA formed in the PCR reaction, and thereby makes it possible to monitor the accumulation of PCR products as the PCR reaction proceeds. Generally, two quantification strategies can be performed; absolute quantification and relative quantification (Freeman *et al.*, 1999). Absolute quantification attempts to determine the number of mRNA copy per cell by comparison with calibration curves (Pfaffl & Hageleit, 2001). This approach makes it possible to compare results between laboratories because the calibration curve is supposed to be reliable and stable, although the precise quantification of RNA for the construction of calibration curve can be problematic. In relative quantification, which is used in this thesis, mRNA levels of the genes of interest are normalized to the levels of an internal RNA reference gene, preferably a gene which show stable mRNA levels across the experimental groups of the study. Relative quantification is the most adequate approach to investigate physiological changes in gene expression levels and is the most common approach (Freeman *et al.*, 1999).

Traditional internal RNA controls include β-actin, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and peptidylpropyl isomerase A (PPIA). However, it has been found that mRNA levels of these genes change under some conditions (Schmittgen & Zakrajsek, 2000; Suzuki *et al.*, 2000; Radonic *et al.*, 2004), and a thorough evaluation of these genes is vital to avoid misinterpretations of gene expression data. This is particularly important in extreme experimental insults, such as anoxia. In such experiments, an external RNA reference can be used to monitor levels of the internal RNA references (Baker *et al.*, 2005). Additional approaches have been developed to improve normalization of relative gene expression data, such as the use of total RNA

(Bustin, 2002). The disadvantage of this approach is that total RNA includes large amounts of ribosomal RNA (rRNA) and transfer RNA (tRNA) which can vary relatively to the amount of mRNA, and that the amount of total RNA of a tissue has been found to change in response to various treatments (Smith *et al.*, 1999).

1.5 Aims of the study

Because a reduced or altered ion channel function has the potential to significantly lower the energy use of the brain, I found it relevant to look for changes in mRNA levels of selected voltage-gated ion channel subunits in brain tissue. Anoxia is an extreme stressor for most animals, and it is possible that Hsps could be involved in counteracting the effects of this stressor. Moreover, anoxia is likely to be a more severe challenge at high temperature since the rate of ATP use increase with temperature. Therefore, I chose to study the expression of heat shock proteins at two different temperatures under anoxic conditions.

Thus, the aim of this study was to investigate the effects of anoxia on the mRNA levels of the pore-forming subunits of voltage-gated Na^+ and Ca^{2+} channels, as well as to quantify the effects of anoxia at two temperatures on the mRNA levels of Hsps in crucian carp brain.

2 Materials and methods

This thesis consists of three experiments; (1) quantification of relative mRNA levels of voltage-gated ion channels in fish exposed to anoxia at 11°C (2), quantification of relative mRNA levels of Hsps in fish exposed to anoxia at 8°C and (3) quantification of relative mRNA levels of Hsps of fish exposed to anoxia at 13°C.

2.1 Animal handling

2.1.1 Animals

Crucian carp were captured in Tjernsrud pond, Oslo community. Experiments were performed at three different temperatures, using two different batches of fish. The first batch was captured in June 2004, and was used in the ion channel experiments at 11°C, while the second batch was captured in June 2005 and was used in Hsps experiments at 8°C and 13°C. Both batches were kept in 750-litre tanks at the aquarium facility of the Department of Molecular Biosciences, University of Oslo. These tanks were continuously supplied with aerated and dechlorinated water from Maridalsvannet, Oslo. The photoperiod was held at 12h light/12h darkness, and the fish were fed daily with commercial fish food (Tetrapond, Tetra), except during experiments. Experimental animals weighed 40±13g.

2.1.2 Anoxia exposure and tissue sampling

Anoxia exposures were performed at three temperatures, 8°C, 11°C and 13°C, where each temperature included four exposure groups; 7 days normoxia (N7), 1 day anoxia (A1), 7 days anoxia (A7), or 7 days anoxia followed by 3 or 7 days of normoxia (A7N3 or A7N7), see table 2.1 for details. Exposures were carried out in circular 25-litre tanks, continuously supplied with dechlorinated, aerated/de-oxygenated water (0.5-1 ml/second). De-oxygenation was achieved by N_2-bubbling (Nitrogen 99.99%), and oxygen levels were monitored using a galvanometric oxygen electrode (WTW 340i from Wissenschaftlich Technische werkstätten, Weilhem, Germany) connected to a printer

(Figure 2.1). Fish were left to acclimate in the 25-litre tanks for 12 hours prior to exposure, followed by removal of excrements and closure of tanks with tight lids.

Table 2.1. Overview of the experiments.

Crucian carp (time of capture)	Experimental period	Temp	Exposure groups
June 2004	November 2004	11 °C	N7, A1, A7, A7N7
June 2005	November 2005	13 °C	N7, A1, A7, A7N7
June 2005	January-February 2006	8 °C	N7, A1, A7, A7N3

N7= normoxia 7 days, A1=anoxia 1 day, A7=anoxia 7 days, A7N3/N7=anoxia 7 days followed by normoxia 3 or 7 days.

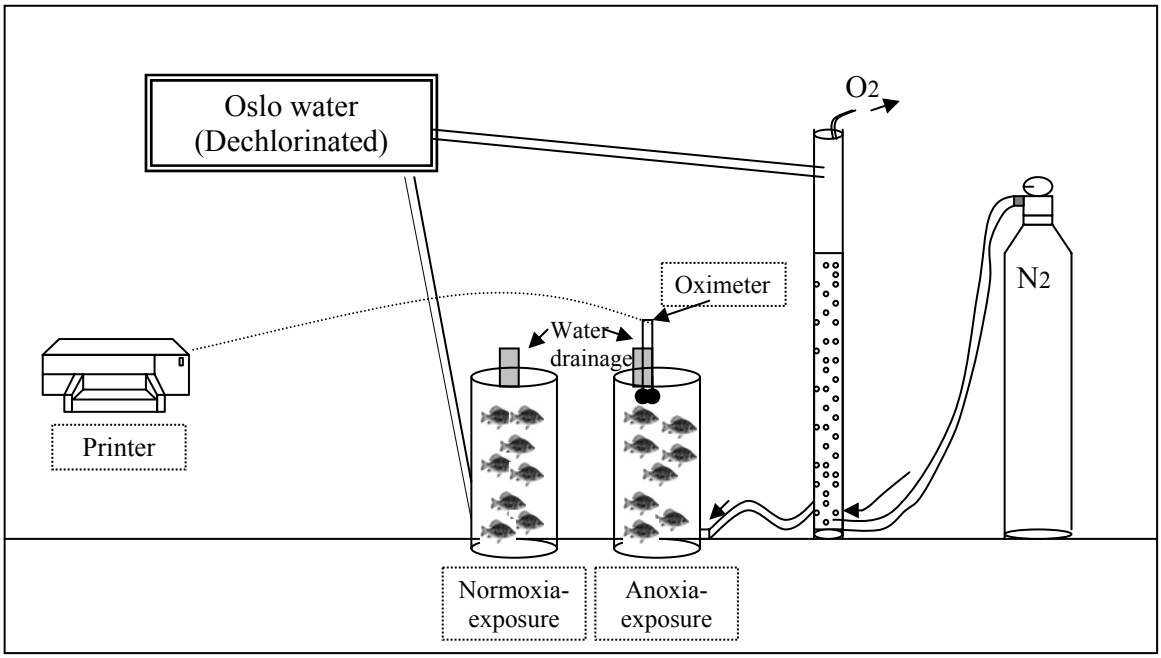

Figure 2.1. Set-up for anoxia exposure. Left circular tank represents normoxia experiment, while right circular tank represent anoxia experiment. The oxygen level and temperature were registered by an oxygen electrode, and recorded by a printer.

Immediately after the respective exposures, crucian carp was carefully captured and killed by cutting the spinal column, close to the head. Brain tissue was dissected excluding the olfactory tracts and spinal cord and snap-frozen in liquid nitrogen within 3

minutes of capture. The brain tissue was subsequently stored at -80°C until further analysis. The number of fish included in each exposure group is listed in table 2.2.

Table 2.2. Number of fish in each exposure group at the different temperatures

Exposure	8 °C	11 °C	13 °C
N7	6	5	6
A1	5	5	6
A7	7	6	6
A7N3 / A7N7	4	5	6

2.2 Isolation of total RNA

Brains were weighed in a frozen condition (8°C; 107.5±28 mg, 11°C; 129.7±19 mg, 13°C; 137.8±15 mg), and quickly transferred to a homogenizer placed on ice, containing 500 µl TRIzol (Invitrogen). Prior to homogenization, an external RNA reference (denoted; 2A-4, from cyanobacterium, Unpublished) was added on a pg-per-mg-basis in addition to a total volume of 15 µl TRIzol pr mg tissue. Total RNA from brain tissue was isolated according to the manufacturer's protocol (Invitrogen). Duall glass homogenizers (Kontes) were used to homogenize the brain tissue in the 11°C experiments, and an electrical homogenizer (Ultra-Turrax T 8, IKA) was used to homogenize the brain tissue in the 8°C and 13°C experiments.

2.2.1 Quality check of total RNA

To check the quality of the extracted total RNA, one microliter of 1:10 dilutions of total RNA solution was analyzed on a 2100 Bioanalyzer (Agilent) using the RNA 6000 Nano Lab Chip ® Kit (Agilent) according to the manufacturer's protocol. Ratios of 28S to 18S ribosomal RNA were estimated, and were found to be approximately 1.5, a value indicating high-integrity RNA.

To measure the concentration of RNA, optical density (OD) measurements were performed using a NanoDrop spectrophotometer (NanoDrop technologies) on 1:10

dilutions of total RNA. Technical triplicates were performed on each sample, and the concentrations were found to be in the order of 1500-2500 ng/µl undiluted total RNA solution. OD 260/280 ratio showed values between 1.8 and 2.1 and the 260/230 ratio showed values above 1.8. Lower values of OD 260/280 and OD 260/230 indicate polluted RNA.

2.3 cDNA synthesis

Two µg total RNA was treated with 2 units DNase I (amplification grade, Sigma), prior to cDNA synthesis to remove all traces of genomic DNA. However, during this master thesis the lab encountered problems with the DNase I treatment, and hence, total RNA from crucian carp exposed to different oxygen regimes at 8°C were not DNase I treated. Reverse transcription was performed using Superscript™ III Reverse Transcriptase (Invitrogen) and 500 ng oligo$(dT)_{18}$. Both DNase I treatment and cDNA synthesis were performed according to the manufacturer's protocol. For each RNA sample two cDNA syntheses were carried out, and the cDNA was diluted 1:25 with DEPC (diethylpyrocarbonate)-milliQ water. Note; all non-DNase I treated RNA samples were controlled for contamination of genomic DNA by performing real-time RT-PCR reactions on 1:750 dilutions of RNA.

2.4 Partial cloning and sequencing

Cloning and sequencing of the following genes were done from crucian carp brain to be able to design functional primers for the real-time RT-PCR assay; Na_v 1.1, Na_v 1.6, Ca_v 2.1, Ca_v 2.3, Ca_v 3.1, Ca_v 3.2, Hsp70.2 and Hsp90. For the genes Na_v 1.3, Hsp30, Hsp70.1 and Hsc70, sequences were available for the closely related goldfish[i] and thus, suitable sequences for synthesizing real-time RT-PCR primers for crucian carp (see table

[i] To point out the close relationship; 0.4 mutations pr 100bp were detected in Hsp30 sequences in between the two crucian carp and goldfish (acc. AB177389), and 3 mutations pr 100bp were detected in the sequences of Hsc70 (goldfish; acc. AB092840), after cloning of the real-time RT-PCR products from crucian carp.

2.3 for accession no.). Cloning was performed on cDNA from normoxic crucian carp brain tissue.

Primers for cloning were obtained by aligning nucleotide sequences of genes of interest from a number of species (retrieved from GenBank) using Clustal X (1.83) and Genedoc (version 2.6.002). Primers were designed in conserved regions of the genes using the web-based Primer3 resource (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), and synthesized by Invitrogen (see table 2.3). All primers had a melting temperature of approximately 60°C.

Table 2.3. Primers used for cloning and the accession number for the goldfish sequences.

Gene	Acc. nr	Forward primer 5'→3'	Reverse primer 5'→3'
NaV1.1		TGTAACAGAGTTTGTGGATTTGG	ACAGCCAGGATGAGGTTCAC
NaV1.3	AF372583		
NaV1.6		TGTAACAGAGTTTGTGGATTTGG	ACAGCCAGGATGAGGTTCAC
CaV2.1		ACTTCATCCCGCTCATCATC	TTCCTCAGAGACGCCCAATA
CaV2.3		TCCTCATCGTCTACAAGCTGTTT	CCCTGACGCAGGAGTTTAAT
CaV3.1		GCCCGTCATGAATTACAACC	CAAGACCTTCACAGGGGTGT
CaV3.2		CATCACGCTTGAGGAAATTG	ATCCATCTCCGCCTCCTC
Hsp70.1	AB092839		
Hsp70.2		ATCCTGACGATTGAGGATGG	CGGCTGGTTATCGGAATATG
Hsc70	AB092840		
Hsp90		CGTAATAGGGTAGCCAATGAACT	CGTAATAGGGTAGCCAATGAACT
Hsp30	AB177389		

Cloning of the genes of interest was achieved by using FastStart Taq DNA polymerase (Roche Diagnostics) and 39 cycles of amplification (PCR program; Taq activation at 94°C (10 min), 39 cycles of 94°C (10 sec), 50°C/55°C (1 min), 72°C (1 min), and extension at 72°C (10min)). PCR-products were ligated into pGEM®-T Easy Vector (Promega) and transformed into $CaCl_2$-competent cells, and subsequently applied on IPTG/X-Gal agar plates. A number of colonies were picked, and amplified by PCR

with primer annealing temperatures of 55°C, using M13 forward and reverse primers (Invitrogen). The colony PCR-products were sequenced by ABI-lab at CEES, Departments of Biology and Molecular Sciences, University of Oslo.

All obtained sequences can be found in appendix IV. It should be noted that cloning of three of the genes of interest; Na_v 1.2, Ca_v 2.2 and Ca_v 3.3, failed.

2.5 Quantification of mRNA expression with real-time RT-PCR

2.5.1 Primer design

Real-time RT-PCR primers were designed based on cloned crucian carp sequences and available goldfish sequences (see appendix IV for acc.), using the LightCycler Probe Design Software (version 1.0 Roche). The primers were synthesized by Invitrogen, and their specificity was verified by cloning and sequencing the real-time RT-PCR products. See table 2.4 for information on real-time primers.

Table 2.4. Primers used for real-time RT-PCR.

Gene	Forward primer 5'→3'	Reverse primer 5'→3'
NaV1.1	CGTAATCCTGACTATGGC	CAAAGAAGATCATATAGGGCT
NaV1.3	AGCATCATGGGAGTCAA	GTAATACTGCGTGCTGTT
NaV1.6	GGTCGCAATCCAAACTATG	GCTCTCAGGGTCAGCA
CaV2.1	GGTGATTTTGGCTGACG	CCTTATATCTCCATCTCTATCCT
CaV2.3	GTGCCAAACCTTTGACT	GTGTTTAAGGGCGATCAT
CaV3.1	AAATCGAGGTCAATGCTT	AAGAGGAGACCCAGATTC
CaV3.2	TTCCTCACTTTGTTCCGC	CCCAGCACGAATGTCA
Hsp70.1	ACAAGCGACTAAAGACG	GTACGCCAACAGCTTC
Hsp70.2	CATCCTGATGGGCGAC	GGTTATCGGAATATGTGGAGA
Hsc70	GCTATTGCTTACGGTCTG	CCGCGAACTTGAGACA
Hsp90	GGAATCTTCCGCTGGAG	CGAGTGCTTCTTGACGA
Hsp30	GACGCTGGACACTAAAG	ACTGCCGACTAAATGACC

2.5.2 Real-time RT-PCR

Quantification of mRNA levels of the genes of interest (see table 2.4) were performed on a Lightcycler®2.0 instrument, using LightCycler FastStart DNA Master^{PLUS} SYBR Green I kit (Roche Diagnostics). The expression of each gene was quantified by performing four real-time RT-PCR reactions on each fish; two reactions on each of the two cDNA syntheses (Figure 2.2).

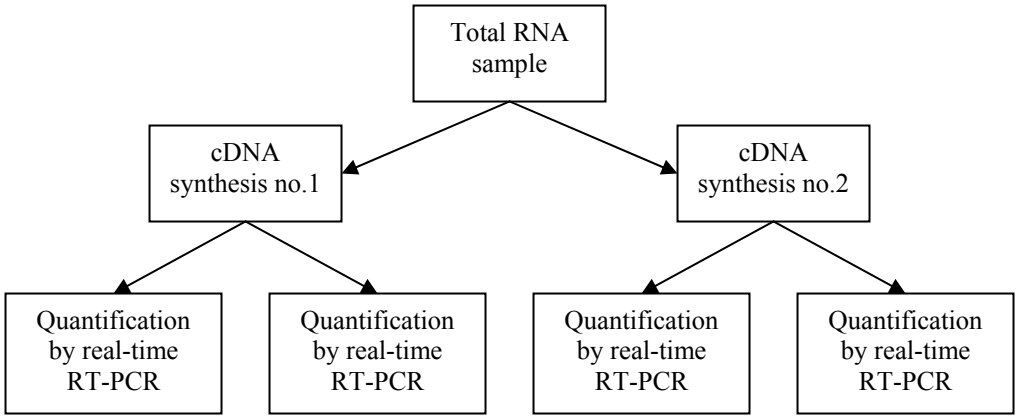

Figure 2.2. Overview of the quantification procedure. Two cDNA syntheses were performed on total RNA of each fish, whereby each cDNA was quantified twice on every primer pair using real-time RT-PCR.

β-actin and GAPDH was chosen as internal RNA reference genes on the basis of previous testing of the stability of three putative internal RNA reference genes; β-actin, GAPDH and PPIA during the anoxia exposures (Ellefsen. S, Unpublished) (Figure 2.3). The expression profiles of these three genes were related to the external RNA reference gene (2A-4) added to the brain tissue prior to RNA extraction on a pg-per-mg basis. The external RNA reference gene enables comparison of gene expression between the different exposure groups. PPIA mRNA levels showed a significant change at 13°C and therefore discarded as internal reference gene. β-actin was chosen as internal RNA reference gene at 11°C, because of a nearly significant increase in mRNA of GAPDH in the A7 group (P=0.0597, One-way ANOVA, Tukey-Kramer post test), indicating a possible response to anoxia. GAPDH was found to be the most stable RNA reference gene at 8°C and 13°C, while the mRNA levels of β-actin showed a relatively large variance at 13°C (P=0.1053, One-way ANOVA, Tukey-Kramer post test). Therefore,

real-time RT-PCR was performed using β-actin as the internal reference gene for the 11°C experiment, and GAPDH as the internal reference gene for the 8°C and 13°C experiments. As reference genes, the internal reference genes were preferred to the external reference gene (2A-4), since there was a considerably higher sample-to-sample variation in 2A-4 compared to the external reference genes. There was also an aliquot-to-aliquot variation in 2A-4 mRNA levels between the three temperatures, but this will not influence the results obtained within each of the three experiments (8°C, 11°C, and 13°C) as the RNA extraction of each experiment was performed using the same aliquot.

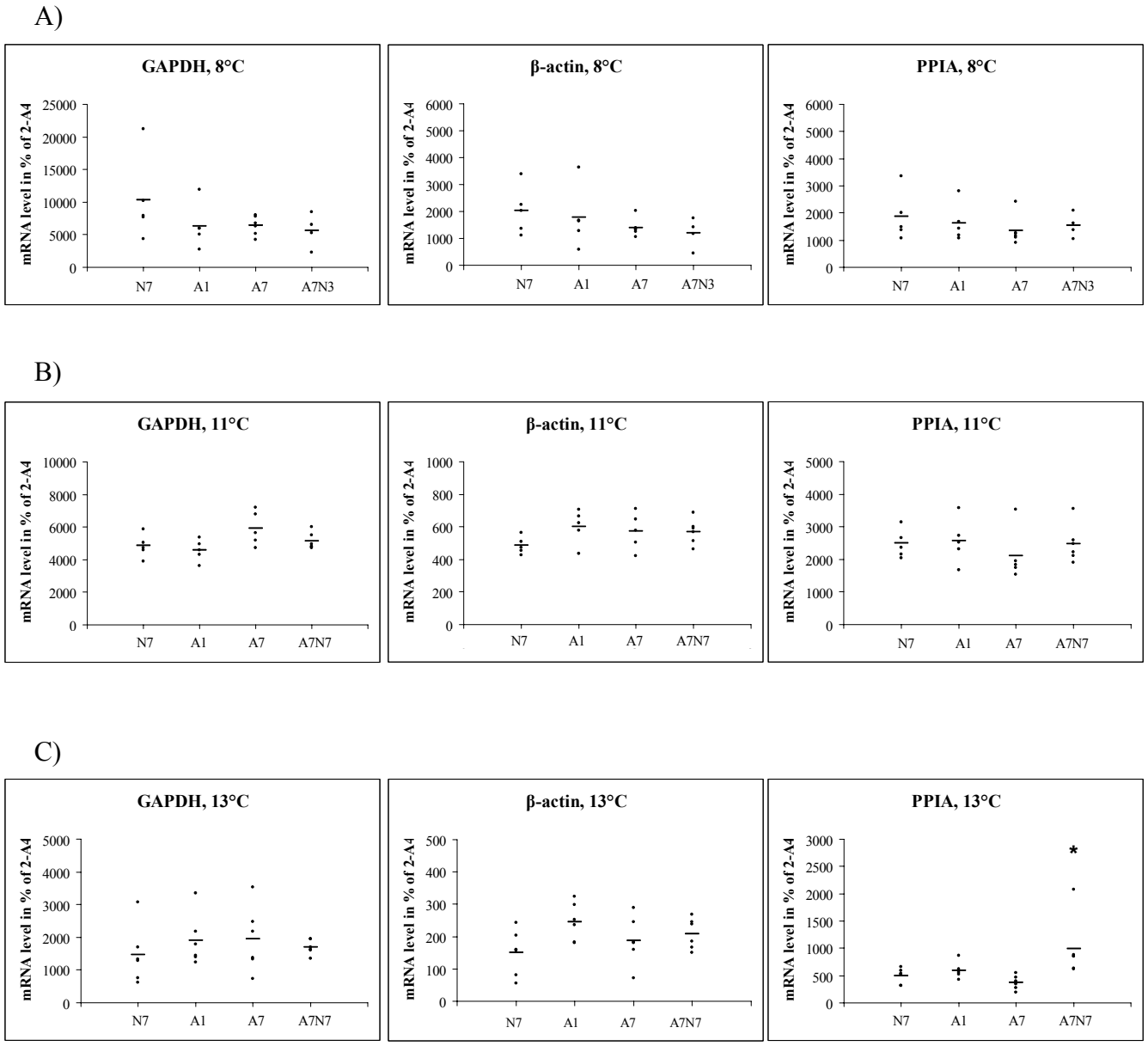

Figure 2.3. Stability of internal reference genes during anoxia exposures at different temperatures. mRNA levels of internal reference genes at 8°C (A), 11°C (B) and 13°C (C), normalized to the external reference gene. GAPDH was chosen as internal reference gene for experiments at 8°C and 13°C, while β-actin was chosen as internal reference gene for experiments at 11°C. P-values for the 8°C experiments (all three by Kruskal-Wallis, Dunn's post test); GAPDH P=0.4678, β-actin P=0.5249, and PPIA P=0.5783. P-values for the 11°C experiments; GAPDH P=0.0597 (One-way ANOVA, Tukey-Karmer post test), β-actin P=0.2370 (One-way ANOVA, Tukey-Kramer post test) and PPIA P=0.3344 (Kruskal-Wallis, Dunn's post test). P-values for the 13°C experiments; GAPDH P=0.5385 (Kruskal-Wallis, Dunn's post test), β-actin P=0.1053 (One-way ANOVA, Tukey-Kramer post test) and PPIA P=0.0039 (Kruskal-Wallis test, Dunn's post test).

Real-time RT-PCR was performed according to the manufacturer's protocol, with the adjustment of halving each reaction from 20 µl to 10 µl. Five µl of 1/25 dilutions of cDNA was used as template. The real-time RT-PCR program was set to 40 cycles of 10 sec at 95°C, 12 sec at 60°C and 8 sec at 72°C after 10 min (95°C) of Taq activation. A melting curve analysis was generated according to the manufacturer's protocol.

To correlate real-time RT-PCR reaction curves to amounts of initial starting template, a reference point in the curve is needed. This point is called crossing point (Cp) and is defined as the fractional cycle number at which the fluorescence has reach a threshold value. The following information was calculated from each real-time RT-PCR reaction; (1) Cp was calculated using the second derivative maximum method (LightCycler Software version 3.5, Roche Diagnostics) and (2) efficiency (E) was estimated for each PCR reaction using LinRegPCR (version 7.5), (Ramakers *et al.*, 2003) wherein each efficiency calculation was visually inspected. Efficiencies lying in suboptimal regions of the graphs were manually corrected. These individual efficiency estimations were subsequently used to calculate the mean efficiency of every amplicon. This mean efficiency was used to calculate the level of relative mRNA abundance. Moreover, quantification of GAPDH and Hsp mRNA levels was performed on both 8°C and 13°C brains. For these genes a mutual mean efficiency was calculated based on data from both temperatures. It should be noted that Cp values within the acceptable range (<36 cycles) could not be retrieved for the gene Hsp70.2 in the 13°C group of fish. The FitPoint method (LightCycler Software version 3.5, Roche Diagnostics) was therefore used to define the level of expression (Rasmussen, 2001).

$$\textbf{Relative mRNA level} = \frac{{}_{\text{Ref}}E^{Cp_{exp}}}{{}_{\text{Tar}}E^{Cp_{exp}}}$$

Formula for calculating relative mRNA levels of each sample. The mean efficiency for each gene of interest was calculated using LinRegPCR (version 7.5). E=efficiency, Cp=crossing point, Ref= (internal) reference gene, Tar=target gene, exp=exposure group (N7, A1, A7 or A7N3/N7).

2.6 Statistical analyzes

The data were normality tested using the method of Kolmogorov and Smirnov. Bartlett's test was used to test for significant differences in SD. One-way ANOVA with Dunnett multiple comparison tests was used to test for significant changes in gene expression compared to control (N7) in data sets passing the normality test. Kruskal-Wallis test with Dunn's post test was used when data sets showed significant variation in SD or did not pass the normality test. $P \leq 0.05$ was considered significant. The statistical calculations were done using GraphPad InStat (GraphPad® version 3.06).

3 Results

Normoxia/anoxia exposures were carried out at three temperatures; 8°C, 11°C and 13°C. Fish exposed to 7 days anoxia at 8°C and 11°C were still active, while fish exposed to 7 days anoxia at 13°C showed a highly reduced activity.

The exposure groups are here abbreviated as N7 (normoxia 7 days), A1 (anoxia 1 day), A7 (anoxia 7 days), and A7N3 or A7N7 (anoxia 7 days followed by normoxia for 3 or 7 days, respectively). Statistical information for all results is listed in appendix III.

3.1 Real-time RT-PCR efficiency calculations

Mean efficiencies for the genes that were studied are presented in table 3.1. Quantification of GAPDH and heat shock protein mRNA levels was performed on both 8°C and 13°C brains, and a common mean efficiency was calculated based on data from both temperatures.

Table 3.1. Mean efficiencies calculated from LightCycler real-time RT-PCR reactions.

Gene of interest	Mean efficiency, 11°C	SD
β-actin	1.833	0.020
Ca_v 2.1	1.848	0.012
Ca_v 2.3	1.903	0.016
Ca_v 3.1	1.874	0.012
Ca_v 3.2	1.892	0.030
Na_v 1.1	1.892	0.015
Na_v 1.3	1.888	0.013
Na_v 1.6	1.889	0.021

Gene of interest	Mean efficiency, 8°C and 13°C	SD
GAPDH	1,890	0,038
Hsp30	1,886	0,024
Hsp70.1	1,866	0,033
Hsp70.2	1,835	0,025
Hsc70	1,879	0,031
Hsp90	1,894	0,038

Individual efficiencies for each PCR reaction were initially estimated using LinReg software. n=84 for voltage-gated ion-channels and β-actin; n=184 for heat shock proteins and GAPDH (8°C; n=88 and 13°C; n=96).

3.2 Relative mRNA levels of voltage-gated ion channels

Quantifications of mRNA levels of the α-subunit of voltage-gated ion channels were performed on total RNA from brains of crucian carp exposed to different oxygen regimes at 11°C. Gene expression data of each gene was normalized using β-actin as internal reference gene.

With regard to Na_v channels only mRNA levels the Na_v 1.3 channel α-subunit in the A1 group showed a significant decrease compared to N7 (Kruskal-Wallis, Dunn's post test, $P<0.05$) (Figure 3.1). No significant changes were detected in the expression of the α-subunits of Na_v 1.1 compared to the N7 group, although the one-way ANOVA indicated differences between the exposure groups ($P=0.0289$). With regard to the Na_v 1.6 α-subunit, no significant changes were detected compared to the N7 group.

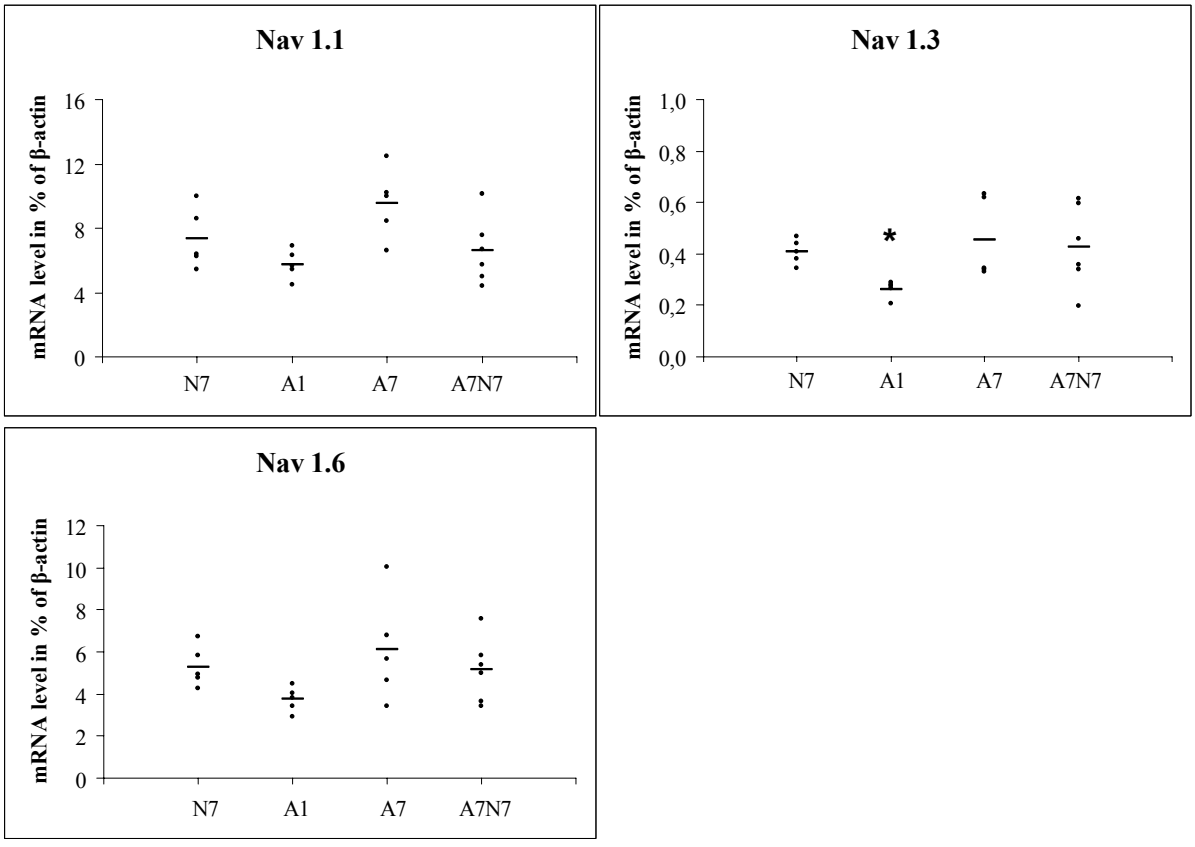

Figure 3.1. Relative mRNA levels of members of the Na_v channels in the brain of crucian carp, exposed to different oxygen regimes at 11°C. The data are normalized using β-actin as internal reference gene and are presented as individual values with lines representing mean values. * =$P<0.05$, compared to N7. For number of fish in each exposure group, see table 2.2. For statistical details, see appendix III.

No significant changes were found in mRNA levels of the α-subunits in the Ca_v 2 family (Ca_v 2.1 and Ca_v 2.3) in response to anoxia in crucian carp brain (Figure 3.2). Noticeable, the relative amount of mRNA were similar for both Ca_v 2 channels.

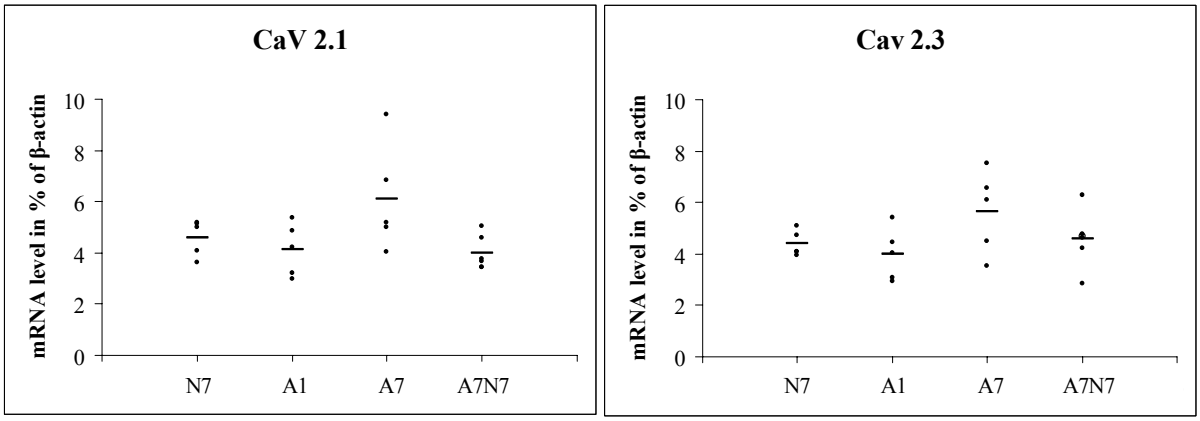

Figure 3.2. Relative mRNA levels of members of the Ca_v 2 channel subunits in the brain of crucian carp, exposed to different oxygen regimes at 11°C. The data are normalized using β-actin as internal reference gene and are presented as individual values with lines representing mean values. For number of fish in each exposure group, see table 2.2. For statistical details, see appendix III.

Similarly, the Dunnett post-test did not detect any changes in the mRNA levels of the α-subunits of the Ca_v 3 family compared to the N7 group (Figure 3.3). However, for the Ca_v 3.1 α-subunit, a P value of 0.0087 was obtained in the one-way ANOVA, indicating differences between the exposure groups.

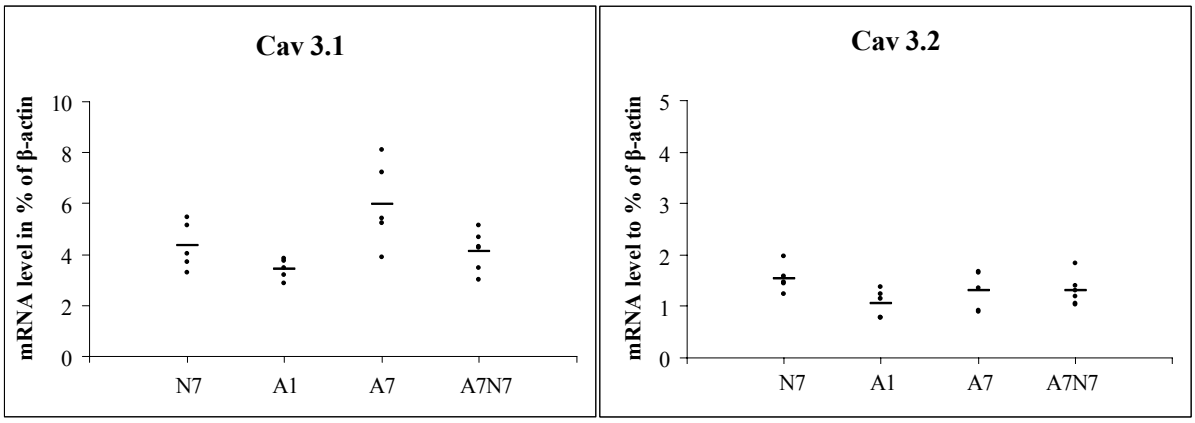

Figure 3.3. Relative mRNA levels of members of the Ca_v 3 channel subunits in the brain of crucian carp, exposed to different oxygen regimes at 11°C. The data are normalized using β-actin as internal reference gene and are presented as individual values with lines representing mean values. For number of fish in each exposure group, see table 2.2. For statistical details, see appendix III.

3.3 Relative mRNA levels of heat shock proteins

Relative quantifications of mRNA levels of heat shock proteins were performed on total RNA from brains of crucian carp exposed to different oxygen regimes at 8°C or 13°C. Gene expression data of each gene was normalized using GAPDH as internal RNA reference gene.

Two paralogs of Hsp70 were found in the crucian carp transcriptome, and these are here denoted Hsp70.1 and Hsp70.2.

At 8°C, Hsp70.1 mRNA levels were found to be significantly higher in the A7N3 group compared to N7 ($P<0.05$, Kruskal-Wallis with Dunn's post test) (Figure 3.4). At 13°C the mRNA expression of Hsp70.1 showed a quite different response with a 9 fold higher mRNA level in the A7 group compared to the N7 group ($P<0.001$, Kruskal-Wallis with Dunn's post test) followed by a decrease of mRNA in the A7N7 group. Interestingly, relative mRNA levels at N7 were found to be 7.5 times higher at 8°C than they were at 13°C.

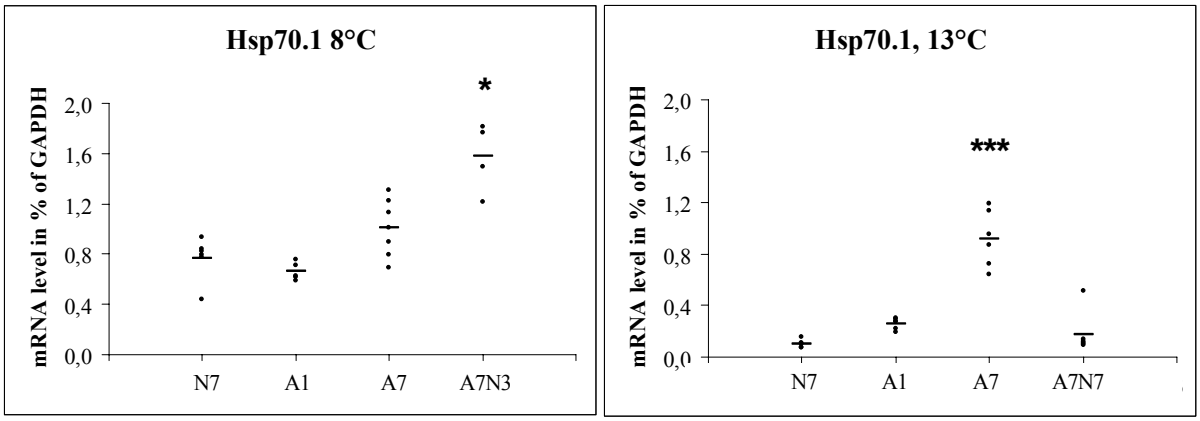

Figure 3.4. Relative mRNA levels of Hsp70.1 in the brain of crucian carp, exposed to different oxygen regimes at 8°C or 13°C. The data are normalized using GAPDH as internal reference gene and are presented as individual values with lines representing mean values. * =P<0.05, compared to N7, *** =P<0.001, compared to N7. For number of fish in each exposure group, see table 2.2. For statistical details, see appendix III.

At 8°C the relative mRNA levels of Hsp70.2 showed significantly higher values in both A1 and A7 compared to N7 (P<0.05, Kruskal-Wallis with Dunn's post test) (Figure 3.5). Significantly higher levels of mRNA in the A7 group were also found at 13°C (P<0.05, Kruskal-Wallis with Dunn's post test). The mRNA levels of Hsp70.2 were found to be expressed at lower levels at both temperatures in the N7 group compared to its paralog, Hsp70.1.

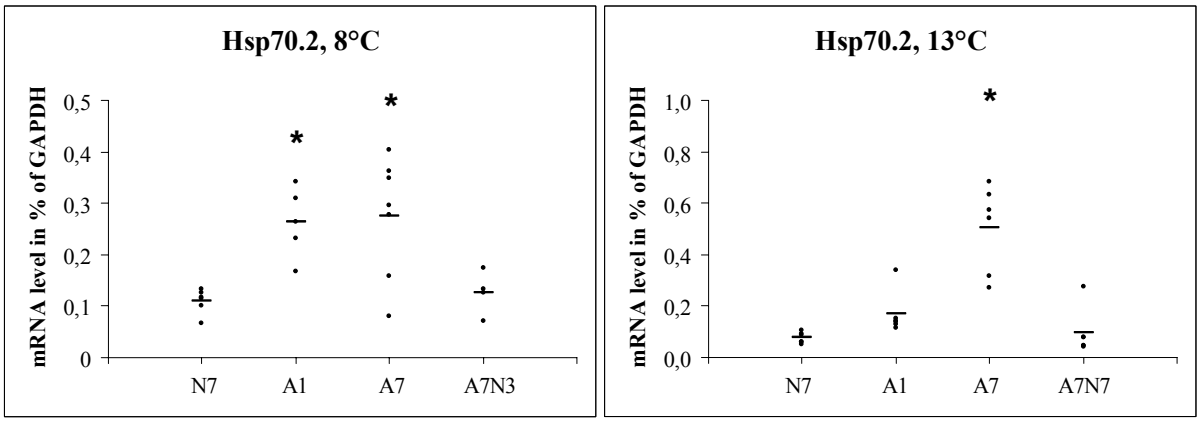

Figure 3.5. Relative mRNA levels of Hsp70.2 in the brain of crucian carp, exposed to different oxygen regimes at 8°C or 13°C. The data are normalized using GAPDH as internal reference gene and are presented as individual values with lines representing mean values. * =P<0.05, compared to N7. For number of fish in each exposure group, see table 2.2. For statistical details, see appendix III.

The relative mRNA levels of Hsc70 at 8°C were significantly lower in the A7 group compared to the N7 group (P<0.05, Kruskal-Wallis with Dunn's post test) (Figure 3.6). A similar response was detected at 13°C, although with a more substantial decrease of Hsc70 mRNA levels in the A7 group (P< 0.01, One-way ANOVA with Dunnett post test).

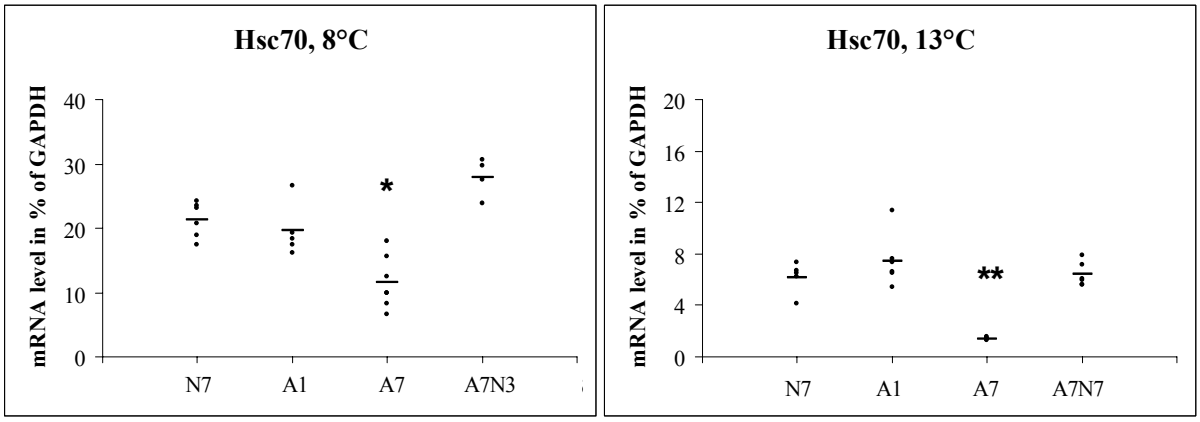

Figure 3.6. Relative mRNA levels of Hsc70 in the brain of crucian carp, exposed to different oxygen regimes at 8°C or 13°C. The data are normalized using GAPDH as internal reference gene and are presented as individual values with lines representing mean values. * =P<0.05, compared to N7, ** =P<0.01, compared to N7. For number of fish in each exposure group, see table 2.2. For statistical details, see appendix III.

At 8°C Hsp90 mRNA levels were found to be significantly lower in the A7 group compared to the N7 (Kruskal-Wallis Test, P=0.0013) (Figure 3.7). The tendency was similar at 13°C although no significant change of mRNA was detected at this temperature.

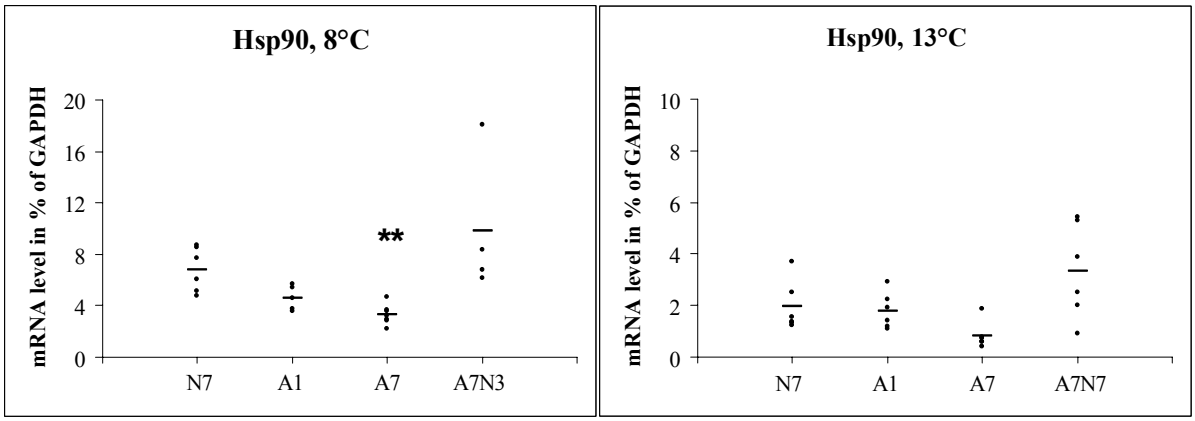

Figure 3.7. Relative mRNA levels of Hsp90 in the brain of crucian carp, exposed to different oxygen regimes at 8°C or 13°C. The data are normalized using GAPDH as internal reference gene and are presented as individual values with lines representing mean values. ** =P<0.01, compared to N7. For number of fish in each exposure group, see table 2.2. For statistical details, see appendix III.

No significant changes of Hsp30 mRNA levels were detected at 8°C (Figure 3.8). However, at 13°C significantly lower mRNA levels of Hsp30 were seen in the A1 (P<0.05, One-way ANOVA with Dunnett post test) and A7 groups (P<0.01, One-way ANOVA with Dunnett post test), compared to N7.

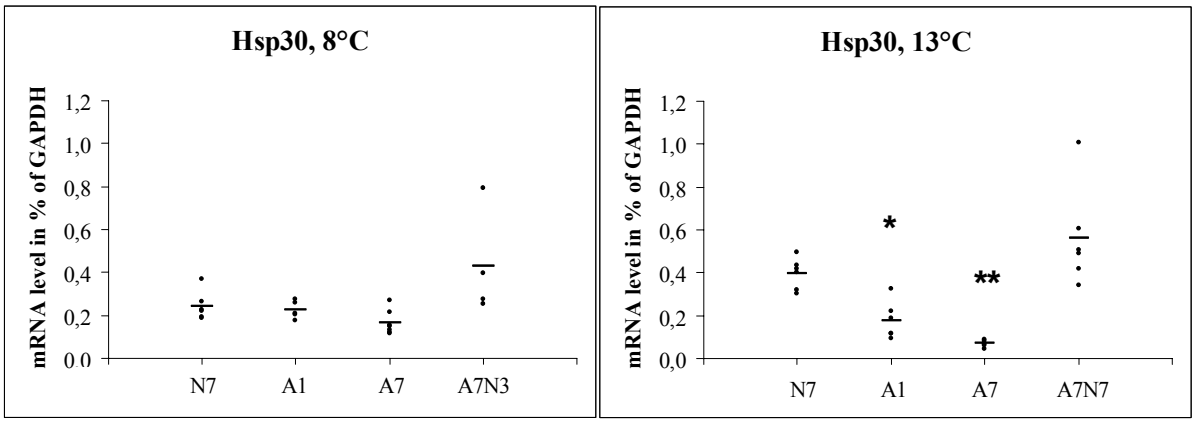

Figure 3.8. Relative mRNA levels of Hsp30 in the brain of crucian carp, exposed to different oxygen regimes at 8°C or 13°C. The data are normalized using GAPDH as internal reference gene and are presented as individual values with lines representing mean values. * =P<0.05, compared to N7, ** =P<0.01. For number of fish in each exposure group, see table 2.2. For statistical details, see appendix III.

4 Discussion

In the present study, mRNA levels of 12 different genes hypothesized to be related to anoxic survival strategies were quantified in brain tissue of crucian carp, as a first step to assess their role in anoxia tolerance. The studied genes included subunits of voltage-gated Na^+ (Na_v) and Ca^{2+} (Ca_v) channels and heat shock proteins (Hsps).

Relative mRNA levels of the α-subunits of seven voltage-gated ion channels were quantified to investigate the potential presence of "channel arrest" in brain tissue of crucian carp exposed to anoxia. Even though the crucian carp remains active during anoxia, its activity is reduced indicating that a reduction of neuronal ion permeability could form a part of its survival strategy.

Hsps are well-known to play protective roles in cells subjected to stress (Brown, 1990; Airaksinen *et al.*, 1998; Lee *et al.*, 2001). Therefore, the mRNA of five different Hsps was quantified in crucian carp brain to assess the possibility that these proteins are involved in protective mechanisms during anoxia. This was done in crucian carp kept at 8°C or 13°C to examine if temperature affects anoxic survival mechanisms.

4.1 Methodological considerations

As noted in section 2.4, cloning of three voltage-gated ion channel isoforms failed; Na_v 1.1, Ca_v 2.2 and Ca_v 3.3, and it may be several reasons for this. Firstly, homology in-between ion channel families could complicate the cloning. Moreover, the primers were designed primarily from zebrafish (*Danio rerio*) sequences, and although this species is a close relative to the crucian carp, these particular gene sequences may have evolved differently in crucian carp and zebrafish. Therefore, the non-crucian carp specific primers may have failed to align to the crucian carp sequences of these three ion channel isoforms. Other possibilities are that these isoforms are lacking in the crucian carp genome or are not expressed, or only expressed at very low levels.

Most current knowledge on the functions of voltage-gated ion channels comes from mammalian studies, and the properties of these proteins may not necessarily be the same in fish. Moreover, one additional genome duplication is found in fish compared to

mammals. Thus, it is possible that fish show an even more complex expression of ion channel subunits. An ion channel diversity that appears higher than in mammals has been observed for other ion channels in crucian carp, such as AMPAR and NMDAR (Ellefsen, S., Unpublished).

In the crucian carp habitat, anoxia is most likely to occur during the winter, and hence at temperatures close to 0°C. The temperatures at which crucian carp were exposed to anoxia in this thesis are likely to be higher than those naturally experienced by anoxic crucian carp. Still, virtually all previous studies on physiological and molecular responses to anoxia of anoxia tolerant vertebrates have been done at temperatures considerably higher than 0°C. With regard to goldfish and turtles, most studies have been done at room temperature. Although it can be argued that such studies are better performed at low "natural" temperatures, running anoxia exposures at higher temperatures are generally more practical and may also speed up the anoxic responses and even exaggerate them to make them more clear cut. After all, these animals do tolerate anoxia at temperatures above 0°C. Indeed, the observed differences in mRNA expression between fish going through anoxia at 13°C compared to 8°C are likely to provide additional insight in to the survival mechanisms involved.

It should be acknowledged that studying heat shock proteins and voltage-gated ion channels at the level of mRNA, may not necessarily translate into changes in protein function, which is a general problem associated with surveying mRNA levels. Smith *et al.* (1999) found a general decrease in the rate of RNA synthesis in anoxic crucian carp brain, while an earlier study by the same authors failed to detect a general decrease in brain protein synthesis under the same conditions (Smith et al., 1996). Still, analyzing molecular responses on the mRNA level has proven to give valuable insights in numerous physiological and pathological processes and is the technical fundament of functional genomics.

4.2 Effect of anoxia on mRNA levels of voltage-gated ion channels.

As described in section 2.5.2, mRNA levels of the α-subunit of voltage-gated ion channels were normalized to β-actin as internal reference gene, since the β-actin

expression profile remained relatively unaffected by anoxia at the respective temperature (Figure 2.3 B).

4.2.1 Relative mRNA expression of voltage-gated Na^+ channels

Na_v channels are important for conducting action potentials along neurons and a decrease in their expression was hypothesized to occur in neurons of anoxic crucian carp to save energy.

No significant changes in mRNA levels of the α-subunits of Na_v 1.1 or in Na_v 1.6 were seen during anoxia (Figure 3.1). However, mRNA levels of the Na_v 1.3 α-subunit showed a significant decrease in the A1 group compared to N7, but the expression of this subunit appeared to recover after seven days of anoxia. This trend was seen in all Na_v channel subunits studied with the common feature of coinciding levels of mRNA in the N7 and A7N7 groups. The results may indicate that the crucian carp alters the expression of the Na_v channel genes slightly as an immediate response to anoxia, but overall the Na_v channel α-subunit expression remained strikingly stable in face of anoxia. These findings were not in accordance with our hypotheses where a decrease in mRNA levels in response to anoxia was expected. However, other factors could influence the opening properties of the Na_v channels such as phosphorylation by cAMP-dependent kinase or protein kinase C (PKC) (Catterall, 2000a), and a modulation of the auxiliary subunits can also occur (Isom *et al.*, 1992; Isom *et al.*, 1995). Bickler and Buck (1998) suggested that an effect from PKC may influence the activity of ion channel activity by labeling the ion channel for internalization and hence, put it out of action.

4.2.2 Relative mRNA expression of voltage-gated Ca^{2+} channels

Disruption of the Ca^{2+} gradient in cells under anoxia may contribute to uncontrolled intracellular signaling and eventually cell death (Bickler & Buck, 1998). Moreover, a reduced Ca^{2+} permeability is likely to suppress neurotransmission and thereby reduce neuronal energy use. It was therefore hypothesized that the expression of Ca_v channels would change in response to anoxia.

However, none of the Ca_v channel subunits investigated showed a significantly altered expression in response to the different exposures (Figure 3.2 and 3.3). It could be noted that all Ca_v channel subunits displayed a similar trend of mRNA expression as the Na_v channel subunits, tending to show a small immediate response to anoxia, but in general their expression was strikingly stable.

Like with Na_v channels, there are several ways of controlling the activity of Ca_v channels except altering their expression. These include modulation by G-protein-coupled pathways and phosphorylation of the ion channels by kinases such as cAMP-dependent kinase or protein kinase C (Brown & Birnbaumer, 1990; Hille, 1994; Ikeda, 1996; Catterall, 2000b). Alterations of the auxiliary subunits may also, affect the opening mechanisms of the ion channels (Gurnett *et al.*, 1996). Recent data suggest that the α_2-subunit control internalization via conformational effects exert by the pore-forming subunit (Bernstein & Jones, In press).

4.3 Effect of anoxia and temperature on mRNA levels of Hsps

Hsp mRNA levels were normalized to GAPDH as internal RNA reference gene and quantified at two different temperatures. GAPDH was chosen since it showed the most stable expression profile in these experimental groups (Figure 2.3 A and C).

Brain tissue of crucian carp exposed to anoxia showed increased mRNA levels of Hsp70.1 (Figure 3.4). A moderate but continuous increase of Hsp70.1 mRNA levels was seen at 8°C, in contrast to a stronger increase in response to 7 days of anoxia at 13°C, followed by a decrease after reoxygenation. Moreover, the relative Hsp70.1 mRNA level in normoxia at 8°C was more than 7 times higher compared to normoxia at 13°C. However, it can not be excluded that these apparent differences were, at least partly, related to a change in the expression of the reference gene (GAPDH) in response to temperature.

The mRNA levels of Hsp70.2 at 8°C showed an increase in response to anoxia (both in A1 and A7), but in contrast to Hsp70.1, it fell back to nearly pre-anoxic levels after reoxygenation (Figure 3.5). Moreover, at 8°C the mRNA level of Hsp70.2 in the N7 group was about 1/7 of those of Hsp70.1 in the same group. However, at 13°C, the

response in mRNA expression was quite similar for both paralogs, with a distinct increase of mRNA in the A7 group. Figure 4.1 summarizes the data on Hsp70.1 and Hsp70.2 expression in crucian carp brain tissue at 8°C and 13°C. Interestingly, the sharp rise in the Hsp70.1 mRNA level at 13°C made it coincide with that at 8°C in the A7 group, while the mRNA levels of Hsp70.2 coincide at both temperatures in the N7 and A7N3/N7 group. But then again, this could be an effect of possible changes in the expression of the internal reference gene, GAPDH, in response to temperature.

These findings indicate that mRNA expressions of both Hsp70.1 and Hsp70.2 are induced by anoxia, but that they may serve slightly different functions in brain tissue of crucian carp exposed to anoxia at 8°C.

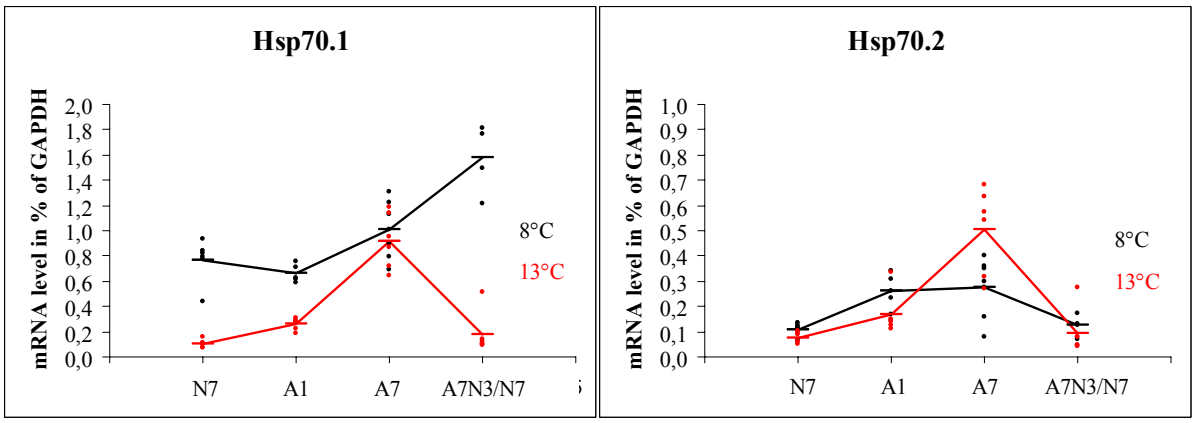

Figure 4.1. Relative mRNA level of Hsp70.1 in crucian carp brain at both 8°C (black) and 13°C (red). Data are normalized using GAPDH as internal reference gene, and are presented as individual values, with lines representing mean values.

Because anoxia is probably most likely to occur during the winter when ice covers the lakes, and the water temperature is close to 0°C, the changes in Hsp70.1 expression may reflect the expectation of an approaching anoxic winter. Previous experiments on an anoxia-tolerant tropical species, the epaulette shark, have shown significant increase of Hsp70 levels in response to anoxic preconditioning, while acute hypoxia exposure was without effect (Renshaw *et al.*, 2004). In the case of the epaulette shark, which live in a habitat where a few short hypoxic episodes are often followed by longer more severe hypoxia, hypoxic preconditioning may be important for hypoxic survival. For the crucian carp, low temperatures could serve as a similar function by

triggering physiological and molecular changes that prepares the fish for the anoxic winter. At this temperature a week-long anoxic bout may serve a similar preparatory function, as indicated by the continued rise in Hsp70.1 mRNA levels at 8°C. Thus, the present data suggest that both low temperature and low oxygen serve as stimuli to increase the expression of Hsp70.1 in crucian carp brain.

Relative mRNA levels of Hsc70 showed a significant decrease in the A7 group at both temperatures (Figure 3.7). However, mRNA levels of Hsc70 were found to be nearly 4 fold higher in the N7 group at 8°C compared to the N7 group at 13°C (assuming that GAPDH mRNA expression was unaltered by temperature). The decrease of mRNA levels in the A7 groups compare to the N7 was greater at 13°C than at 8°C. Furthermore, at 8°C normoxic Hsc70 mRNA levels were almost 30 times higher than Hsp70.1, and almost 200 times higher than Hsp70.2. Hsc70 is generally regarded to be highly expressed under normal conditions while Hsp70 is normally expressed at low levels and is induced by stressors. A suggested function for Hsc70 is axonal transport of properly folded proteins to synapses (Black *et al.*, 1991), and the depression of Hsc70 mRNA expression found in this study may reflect a reduced synaptic transmission and, thus, suppressed neuronal activity. Previous studies have shown a significant increase in the level of extracellular GABA in anoxic crucian carp brain, which is likely to cause a reduction in synaptic transmission (Hylland & Nilsson, 1999b).

Figure 4.2 illustrates mRNA expression of Hsc70 in crucian carp heart at 8°C and 13°C (Ellefsen, S., Stensløkken, K-O, & Nilsson, G., Unpublished), where no significant decrease is detected in response to anoxia. Instead, an increase of mRNA expression is found in heart in A7 and A7N3/N7 groups compared to normoxia. The difference in Hsc70 expression pattern in brain versus heart strengthens the hypotheses that brain Hsc70 plays a role in the synapses and that the need for synaptic transport is reduced in anoxia.

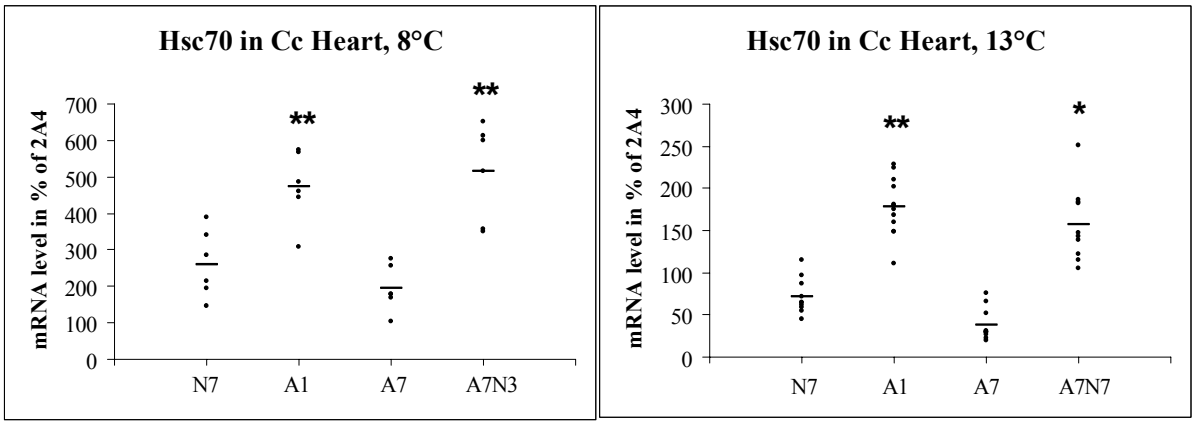

Figure 4.2. Relative mRNA level of Hsc70 in crucian carp heart at 8°C and 13°C. The data are normalized using external reference gene (2A-4) and are presented as individual values with lines representing mean values. * = P<0.05, **=P<0.01, One-way ANOVA, Dunnett post test at 8°C, and Kruskal-Wallis, Dunn's post test, at 13°C. (Unpublished data from Ellefsen, S., Stensløkken, K-O., and Nilsson, G.)

A significant decrease of Hsp90 mRNA was detected at 8°C after 7 days of anoxia while at 13°C no significant change was found (Figure 3.8). With regard to Hsp30, a significant decrease in the mRNA levels was found at 13°C in response to anoxia, but no change was seen at 8°C. Both proteins may have roles in maintaining the cytoskeleton and interact with microfilaments, and a fall in their mRNA levels might indicate that intracellular transport and formation of new cells are given a lower priority under anoxic conditions or are suppressed to save energy. Mitosis has been found to be depressed in anoxic crucian carp tissues (gills, liver and intestine) (Sollid *et al.*, 2005), although the brain remains to be examined in this respect.

4.4 Conclusions

Relative mRNA levels of voltage-gated ion channels did not change significantly in the crucian carp brain, except for the temporarily fall in the mRNA level of Na_v 1.3 α-subunit. This was not in accordance with the "channel-arrest" hypothesis. However, a reduced channel activity cannot be excluded since channel function can be modulated in many other ways than by changes in mRNA levels. Moreover, the results do support an earlier study on crucian carp brain showing a lack of change in membrane Ca^{2+} permeability (Thorén, 2000). Also, a study of K^+ permeability of crucian carp brain failed to detect a change in anoxia (Johansson & Nilsson, 1995). Thus, Nilsson (2001) suggested that "channel arrest" may be a too drastic strategy for anoxic metabolic depression in crucian carp, since this animal, in contrast to turtles, remain active in anoxia. For crucian carp, altered neurotransmitter release may represent a better and more subtle way of depressing energy use in anoxia.

An increased mRNA expression of Hsp70.1 at 8°C suggests a possible adaptation of the crucian carp for an approaching anoxic winter. Two forms of Hsp70 were found and the dissimilar expression of Hsp70.1 compared to Hsp70.2 at 8°C points to some difference in the functions of these two forms, although at 13°C both paralogs were induced by anoxia in a similar fashion.

A significant decrease in Hsc70 mRNA levels was found after 7 days of anoxia at both 8°C and 13°C. This could relate to the suggested function of Hsc70 in axonal transport which may play a part in anoxia-induced suppression of neuronal transmission.

A significant decrease was found in Hsp90 and Hsp30 compared to normoxia, at 8°C and 13°C, respectively. Both proteins have been suggested to participate in maintenance of the cytoskeleton and microfilaments, and the changes may reflect a reduced formation of new cells and lowered cytosolic trafficking during anoxia.

Appendices

Appendix I

Abbreviations

A1	anoxia exposure 1 day
A7	anoxia exposure 7 days
A7N3	anoxia exposure 7 days followed by normoxia 3 days
A7N7	anoxia exposure 7 days followed by normoxia 7 days
Acc.	accession number (from GenBank)
AMPA	α-amino-5-hydroxy-3-methyl-4-isoxasole propionic acid receptor
ANOVA	analysis of variance
ATP	adenosine triphosphate
cAMP	cyclic adenosine 5'-monophosphate-activated protein
Ca	Carassius auratus (goldfish)
Ca_v	voltage-gated calcium (channel)
Cc	Carassius carassius (crucian carp)
cDNA	complementary deoxyribo nucleic acid
CNS	central nervous system
EEG	electroencephalogram
EST	expressed sequence tag
GABA	gamma-aminobutyric acid
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
Hsc	cognate heat shock protein
Hsp	heat shock protein
IPTG	isopropyl-beta-D-thiogalactopyranoside
mRNA	messenger ribonucleic acid
N7	normoxia 7 days
Na_v	voltage-gated sodium (channel)
NMDA	N-methyl-D-aspartate receptor
OD	optical density
PCR	polymerase chain reaction
PKC	protein kinase C
PPIA	peptidylpropyl isomerase A
RT	reverse transcriptase
SD	standard deviation
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

Appendix II

Reagents, equipment and software

The following reagents, equipment and software were used in this thesis.

Reagents (product, catalogue number and manufacturer)

Agarose, SeaKem®, 50004, Cambrex
Ampicillin (D[-]-α-Aminobenzylpenicillin), A-9518, Sigma
Chloroform, C2432, Sigma
DEPC (dietyl pyrokarbonat), SD5758, Sigma
DNase I (Amplification grade), 18068-015, Invitrogen
dNTP-mix, 10297-018, Invitrogen
Etidium bromide, 443922U, BDH-Electron
FastStart Taq Polymerase, 03515885001, Roche Diagnostics
LightCycler® FastStart DNA Master^{PLUS} SYBR Green I, 03515885001, Roche Diagnostics
pGEM®-T East Vector Systems, A1360, Promega
RNA 6000 Nano Lab Chip ® Kit, 5065-4474, Agilent
RNA 6000 Nano Reagents PartI, 5067-1511, Agilent
SOC medium, 15544-034, Invitrogen
Superscript^{TM} III Reverse Transcriptase, 18080-(044), Invitrogen
T7 RNA Polymerase, 18033-019, Invitrogen
TRIzol® Reagent, 15596-018, Invitrogen
X-Gal, V3941, Promega

Equipment (product, catalogue number/model and manufacturer)

Agilent Bioanalyzer, G2938B, Agilent
BioDoc-It^{TM} System, UVP
Eppendorf centrifuge, 5417R, Eppendorf
Finnpipettes, U23386, T27033, T27388, T28301, Thermo Labsystems
Homogenizer, Duall® 22, Kontes glass
Homogenizer, Ultra-Turrax T 8, IKA
LightCycler Capillaries (20 µl), 11909339001, Roche Diagnostics
LightCycler Carousel Centrifuge, 12189682001, Roche Diagnostics
LightCycler 2.0 Instrument, 03531414201, Roche Diagnostics
NanoDrop®, Model ND-1000, NanoDrop Technologies
N_2-gas, AGA

Software

Bioanalyzer 2100 expert, version B.01.02 SII36, Agilent Technologies
GraphPad InStat 3.06, GraphPad Software Inc.
LightCycler3 Data Analysis, version 3.5.28, Idaho Technology Inc.
LightCycler3 Front, version 3.5.17, Idaho Technology Inc.

LightCycler Probe Design Software, version 1.0, Roche Diagnostics
LightCycler3 Run, version 5.32, Idaho Technology Inc./Roche Diagnostics
NanoDrop 3.0.1, Coleman Technologies Inc.

Freeware

BioEdit Sequence alignment editor, version (http://www.mbio.ncsu.edu/BioEdit/page2.html), © 1997-2005 by Tom Hall
ClustalX, version 1.81, June 2000 (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/)
GeneDoc (http://www.psc.edu/biomed/genedoc/), © 2000 by Karl Nicholas
GENSCAN (http://gens.mit.edu/GENSCAN.html) © 1997-2000 by Christopher Burge
LinReg, version 7.5 (Available on request; e-mail: bioinfo@amc.uva.nl; subject: LinReg PCR), Dr. J. M. Ruijter, Department of Anatomy and Embryology, Academic Medical Centre, Amsterdam, Netherlands
Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), © 2004 Whitehead Institute for Biomedical Research

Appendix III

Statistical details

The following information shows statistical details of each gene of interest, with the reference to where a graphical presentation is found in this thesis.

Gene of interest	Test	P-value	Post-test	n in each group	Reference
Nav 1.1	One-way ANOVA	0.0289	Dunnett	5 and 6(A7N7)	Figure 3.1
Nav 1.3	Kruskal-Wallis	0.0361	Dunn's	5 and 6(A7N7)	Figure 3.1
Nav 1.6	One-way ANOVA	0.1595	Dunnett	5 and 6(A7N7)	Figure 3.1
Cav 2.1	One-way ANOVA	0.0545	Dunnett	5 and 6(A7N7)	Figure 3.2
Cav 2.3	One-way ANOVA	0.1565	Dunnett	5 and 6(A7N7)	Figure 3.2
Cav 3.1	One-way ANOVA	0.0087	Dunnett	5 and 6(A7N7)	Figure 3.3
Cav 3.2	One-way ANOVA	0.1425	Dunnett	5 and 6(A7N7)	Figure 3.3
Hsp70.1, 8°C	Kruskal-Wallis	0.0026	Dunn's	6,5,7,4	Figure 3.4
Hsp70.2, 8°C	Kruskal-Wallis	0.0111	Dunn's	6,5,7,4	Figure 3.5
Hsc70, 8°C	Kruskal-Wallis	0.0012	Dunn's	6,5,7,4	Figure 3.7
Hsp90, 8°C	Kruskal-Wallis	0.0013	Dunn's	6,5,7,4	Figure 3.8
Hsp30, 8°C	Kruskal-Wallis	0.0190	Dunn's	6,5,7,4	Figure 3.9
Hsp70.1, 13°C	Kruskal-Wallis	0.0004	Dunn's	6	Figure 3.4
Hsp70.2, 13°C	Kruskal-Wallis	0.0008	Dunn's	6	Figure 3.5
Hsc70, 13°C	One-way ANOVA	< 0.0001	Dunnett	6	Figure 3.6
Hsp90, 13°C	Kruskal-Wallis	0.0100	Dunn's	6	Figure 3.7
Hsp30, 13°C	One-way ANOVA	< 0.0001	Dunnett	6	Figure 3.8

Appendix IV

Sequences and real-time RT-PCR products

The following sequences of crucian carp (Cc) were obtained by cloning and sequencing of cDNA. Sequences of goldfish (Ca) were retrieved from GenBank and are listed with the respective accession number. The real-time RT-PCR products from each gene are highlighted in blue.

Na_v 1.1_Cc_637bp **real-time RT-PCR-Product: 439-585**

CGTAACAGATTTTGTGGATTTGGGCAATGTCTCAGCACTGAGAACCTTCAGGGTTTTACGAGC
ACTCAAAACTATATCAGTGATTCCAGGTCTGAAGACTATCGTTGGCGCTCTAATCCAGTCGGT
GAAGAAGCTGTCAGATGTGATGATCCTGACTGTGTTCTGTCTGAGTGTGTTTGCTCTGATTGGA
CTGCAGCTCTTCATGGGAAACCTGCGGCAGAAGTGTGTGAAGATTCCAKAGGTCAATGTCACT
GAAAACCTGACCGCCACGCTGAACTACAGCACCGGTTTAGAGCTGCACAGCACCTTTAACTGG
ACAGAGTACATTAGTGATGCGAGCAATTACTATTTCCTCCCAGACCGCAGAGATCCTTTGCTA
TGTGGAAATTCRAGTGATGCAGGGCAGTGTCCAGAGGGCTTCATGTGTATAAAAGCAGGTCGT
AATCCTGACTATGGCTACACAAGCTTTGACACATTCAGCTGGGCCTTCCTCTCACTCTTCCGAC
TCATGACACAAGACTTCTGGGAAAACCTCTACCAACAGAACCCTGCGTGCAGCTGGAAAGCC
CTATATGATCTTCTTTGTTCTTGTGATTTTCCTGGGCTCCTTCTATCTGGTGAACCTCATCCTGG
CTGT

Na_v 1.3_Ca_589bp (Acc. AF372583) **real-time RT-PCR-Product: 385-514**

AGCGCTGGCATTTGAAGACATTTACATTGAGCAAAGGAAAGTGGTCAAAGTGGTGCTTGAAT
ACGCAGACAAGATCTTCACCTACATCTTCATCTTGGAAATGGGTCTGAAGTGGATCGCCTACG
GTTTCAGGAAGTACTTCACCAACTACTGGTGCTGGCTTGACTTCCTCATTGTGGATGTGTCTCC
CGTAAGCCTTGTAGCAAACACGCTGGGCTACTCGGACTTTTCTGCCATCAAATCTCTCCGAAC
GCTTAGAGCCCTCAGACCGCTGAGAGCACTGTCCCGGTTTGAAGGCATGAGGGTGGTGGTGA
ACGCTCTGATCGGGGCGATCCCGTCCATCATGAACGTGCTGCTGGTGTGTCTCATCTTCTGGCT
CATCTTCAGCATCATGGGAGTCAATCCGTTCGCCGGGAAGTTCGGCCGCTGTGTCAACCGCAC
CGGCTTCATCTTCAACGCCTCCTTCATCAACAACAAGAGCGAGTGTCTGGAGATGAACAGCAC
GCAGTATTACTGGAGCAAAGTCAAGGTTAACTTCGATAACGTGGGCGCTGGGTACCTCGCTCT
GCTGCAGGTGGCCACGTTCAAG

Na_v 1.6_Cc_609bp **real-time RT-PCR-Product: 409-528**

TGTAACAGAGTTTGTGGATTTGGGGAATGTATCTGCGCTGAGAACATTCAGAGTTCTCCGAGC
ATTGAAAACTATCTCTGTCATTCCAGGTCTGAAGACCATTGTTGGCGCTTTGATCCAGTCGGTG
AAGAAGCTGTCAGATGTGATGATCCTCACCGTTTTCTGCCTCAGTGTCTTTGCCCTCATTGGTC
TGCAGCTCTTCATGGGAAATCTGCGGCACAAGTGTGTCATCTGGCCCATCAACATGACAGAAA
AATACCAGGCCAATGGCACCCATGCATTCAACTGGGATGAGTACATCATGAATGATACCAATT
TCTACTTCCTGCCGGACCAGCTTGATGCTCTTCTATGTGGAAACAGCTCTGATTCAGGTCGCTG
TCCTGAAGGCTACACATGCATGAAGGCTGGTCGCAATCCAAACTATGGGTACACCAGCTTTGA
CAGTTTTGGCTGGGCTTTCCTGGCTCTATTCCGCCTCATGACGCAGGACTTTTGGGAGAACTTG
TACATGCTGACCCTGAGAGCAGCGGGGAAAACATACATGATTTTCTTTGTGCTGGTTATCTTT
GTTGGCTCATTCTACCTTGTGAACCTCATCCTGGCTGT

Ca_v 2.1_Cc_411bp **real-time RT-PCR-Product: 278-408**

CCTTCATCCCTATCATCATCTGGAGGGCTGGACTGACCTGCTTATACTATAGCAATGATGCCTC
AGGGAGTGCATGGAACTGGATGTACTATCATCCCCCTTCATCATCATCGGCTCCTTTCTTCATG
CTCAATCTTGTGCTGGGTGTGCTGTCAGGGTGAGTTTGCCAAAGAAAGGGAACGTGTGGAGA
ACAGGAGAGAGTTTCTTAAGCTGAAGCGTCAGCAACAGATAGAGAGAGAGCTGAACGGATAT
CTGGAGTGGATCTGCAAAGCAGAAGAGGTGATTTTGGCTGACGAGGACAATGACCCAGATGA
CCGGATACCCTTTTGACTTTCGCGGAGGAGGCCTACCATCAAGAAGAATAAAACAGACCTGC
AAGATGCAGAGGATAGAGATGGAGATATAAGGTTC

Ca_v 2.3_Cc_634bp **real-time RT-PCR-Product: 476-598**

TCCTCATCGTCTACAAGCTGTTTATGTTCATATTTGCAGTCATTGCAGTGCAGCTTTTCAAAGG
GAAATTTTACTACTGTACCGACAGTTCCAAGGACACAGAAAACGAGTGCAAGGGTTACTACA
TTGACTATGACAAAGACAAGAAAAAGGAGAAACGAGAGTGGAAGAGACGTGATTTTCATTAT
GATAACATCATCTGGGCTCTGTTGACTCTCTTCACTGTATCCACTGGGGAAGGGTGGCCACAA
GTTTTGCAGCACTCTGTGGATGTGACCGAGGAGGACCGGGGTCCCAGTCAGGGAAACAGAAT
GGAGATGTCCATCTTTTACGTCATTTATTTTGTGGTGTTTCCCTTCTTCTTCGTCAACATATTGT
GGCCCTCATCATCATCACCTTCCAAGAGCAGGGTGACAAAATGATGGAGGAGTGCAGTCTGG
AGAAAAACGAGAGGGCTTGTATCGACTTTGCCATCAGTGCCAAACCTTTGACTCGCTACATGC
CCCAGAACAGACAGACCTTACAGTACCGGCTGTGGCACTTTGTGGTGTCTCCTGTTTATGAAT
ACACCATACTGACGATGATCGCCCTTAAACACCATCGTTCTCATGATGAAGCATCATGAACCT
ATTGA

Ca_v 3.1_Cc_687bp **real-time RT-PCR-product: 516-685**

GCCCGTCATTTATTACAACCCATGGATGCTGCTCTATTTCATCTCCTTCTTGCTGATTGTGGCTT
TCTTCGTGCTCAACATGTTCGTTGGCGTGGTGGTCGAGAACTTTCACAAGTGCCGGCGGCACC
AGGAAGCCGAAGAAGCCAAACGGCGTGAGGAAAAGCGCCTTAAACGCATGGAGAAAAAAAA
GAAGGAAAGCCCAGAGTAAACCGTATTACTCCGATTACTCACCTACACGGCTTCTCATCCACA
AGATATGCACCAGCCACTATCTGGACCTGTTCATCACAATTGTCATCGGGCTCAATGTTATCA
CCATGTCCATGGAGCACTACCATCAACCTAAGGTTCTGGATGAGGCTTTGAAGATCTGTAACT
ACATTTTTACAATCATATTTGTCCTAGAATCCGTCTTCAAACTTGTTGCATTTGGATTTCGCCG
CTTCTTCAAGGACAGGTGGAACCAGTTGGATCTTGCCATTGTTCTGTTGTCTATCATGGGCATA
ACACTGGAAGAAATCGAGGTCAATGCTTCCCTTCCCATCAACCCCACCATAATTCGCATCATG
AGGGTGCTGCGGATTGCTCGTGTGCTGAAGCTGCTCAAGATGGCAGTGGGAATGCGGGCTCTG
TTAGACACTGTGATCCAGGCCCTGCCTCAGGTGGGGAATCTGGGTCTCCTCTTCA

Ca_v 3.2_Cc_452bp **real-time RT-PCR-product: 307-449**

CACCACGCTTTAGGAAATTGACCTGAATGCCTCACTACCCATCAACCCAACCATTATTCGCAT
AATGAGGGTGCTGCGCATCGCCAGAGTGCTGAAGCTGTTAAAGATGGCCACAGGAATGAGAT
CTCTTCTGGATACAGTAGTTCAAGCTTTACCGCAGGTTGGTAATCTGGGTCTGCTATTTATGCT
GCTGTTCTTCATCTATGCAGCGCTGGGAGTTGAACTTTTTGGCAAACTTGAATGTGCAGAGGA
GAATCCATGTGAGGGATTGAGCAGACATGCTACATTTCAGAATTTCGGCATGGCATTCCTCAC
TTTGTTCCGCATTTCCACCGGAGATAACTGGAATGGAATAATTGAAGGATACCCTGCGGGAAT
GTAAAAATGACAAATGCCTGAGTTACCTGCCGCTTTTGTCACCTATGTACATTTGTGACATTCG
TGCTGGGTGG

Hsp70.1_Ca_1958bp (Acc. AB092839) **real-time RT-PCR-Product: 413-659**

TGGGGGTGTTTCAGCATGGAAAAGTAGAGATCATCGCCAACGACCAAGGAAACAGAACAACA
CCCAGCTATGTTGCCTTCACAGACACCGAGAGGCTCATTGGAGATGCAGCTAAAAACCAGGT
GGCCATGAACCCCAACAACACCGTGTTTGACGCCAAGAGGCTGATCGGTAGAAAGTTTGAGG
ACCCAGTTGTGCAGTCTGACATGAAGCACTGGTCTTTCCAAGTCATCAGTGATGGAGGGAAGC
CGAAAGTTCAAGTTGAATACAAGGGAGAAAACAAGACCTTTTATCCCGAAGAGATCTCCTCT
ATGGTCTTGGTGAAGATGAAGGAGATTGCTGAAGCTTATCTGGGGCAGAAGGTGACAAATGC
AGTTATCACAGTTCCTGCCTATTTCAATGACTCCCAGAGACAAGCGACTAAAGACGCTGGAGT
GATCGCTGGACTGAATGTCCTGAGAATCATCAACGAGCCCACAGCTGCAGCCATTGCCTACGG

CCTCGACAAAGGCAAAGCTTCAGAGCGCAACGTGCTGATCTTTGACCTGGGAGGAGGCACCT
TTGATGTGTCCATCCTGACCATTGAAGATGGCATCTTTGAGGTGAAGGCCACGGCTGGAGACA
CTCATCTGGGCGGAGAGGACTTCGACAACCGCATGGTGAATCACTTTGTTGAAGAATTCAAGA
GGAAGCACAAGAAGGACATCAGTCAGAACAAGAGAGCCCTGAGGAGGCTGAGGACAGCGTG
TGAGCGAGCCAAGAGGACCCTCTCGTCCAGCTCTCAGGCCAGCATTGAGATCGACTCGCTGTA
CGAGGGCATCGACTTCTACACGTCCATCACCAGAGCTCGCTTTGAAGAGATGTGCTCGGACCT
CTTCAGAGGAACACTTGAGCCTGTGGAAAAAGCCCTGAGAGACGCCCAGATGGACAAGTCTC
AGATCAATGACGTTGTGCTGGTCGGAGGATCGACAAGAATCCCAAAGATCCAGAAGCTTCTG
CAGGATTTCTTCAACGGCAGAGACTTGAACAAGAGCATCAACCCAGACGAGGCAGTGGCTTA
TGGTGCCGCGGTCCAAGCCGCCATCCTCATGGGAGACACATCTGGAAACGTCCAGGACCTGCT
GCTGCTGGATGTGGCTCGTCTGTCCCTGGGTATCGAAACCGCAGGCGGAGTCATGACGGCCCT
CATCAAACGCAACACCACCATCCCCACCAAACAGACCCAGACCTTCAGCACCTACTCAGACA
ACCAGCCCGGTGTCCTGATCCAGGTCTACGAGGGAGAGAGGGCCATGACAAAAGACAACAAC
CTGCTGGGTAAATTTGAGCTGACAGGAATCCCACCTGCTCCACGTGGAGTCCCGCAGATCGAA
GTCACCTTTGACATCGACGCCAACGGAATCCTAAATGTGTCGGCAGTGGACAAAAGCACCGG
AAAAGAGAACAAGATCACCATCACCAATGACAAGGGCAGACTGAGTAAAGACGAGATCGAG
AGGATGGTGCAGGAAGCAGACAAGTACAAAGCTGAAGACGATCTGCAAAGAGAGAAGATTG
CTGCCAAAAACTCTCTGGAGTCTTACGCCTTCAACATGAAGAACAGTGTGGAAGATGAGGAC
CTGAAGGGAAAGATCAGCGAGGACGACAAGAAGAAAGTTATTGAGAAATGTAACGAGGCCG
TCAGCTGGCTAGAAAACAACCAGCTGGCTGACAAAGAGGAGTATGAACATCATCTGAAGGAG
CTGGAGAAAGTCTGCAATCCAATCATCACTAAACTGTATCAGGGAGGGATGCCAGCTGGAGG
ATGTGGAGCTCAGACACGTGGAGGATCAGGGCCGGTGCTCAGGGGCCCAACTATTGAAGAAG
TGGATTAAAACACCTCATGAACTGAATGGTGAAGGGACTGATAAACCTCTTTCCTCATTGACT
CTTCCATTTATTTAAAAAAAAAAAAAAAA

Hsp70.2_Cc_684bp real-time RT-PCR-Product: 510-679

ATCCTGACGATTGAGGATGGGATCTTTGAGGTCAAAGCCACAGCAGGAGACACTCACCTGGG
TGGGGAGGACTTCGACAACCGGATGGTGAAGCACTTTGTTGAAGAGTTTAAGAGGAAGCACA
AGAAGGACATCAGTCAGAATAAGAGAGCAGTGAGAAGGCTCCGCACGGCCTGCGAAAGAGC
TAAAAGGACCCTCTCATCTAGCACTCAGGCCAGTATTGAGATCGATTCCCTGTTTGAAGGCAT
CGATTTCTATACTTCCATCACAAGGGCTCGATTTGAAGAGCTTAACGCTGAGCTCTTTAGAGG
CACGCTGGAACCAGTAGAGAAGGCTTTGGGAGATGCTAAAATGGACAAGTCCCAGATCCATG
ACATTGTTCTGGTTGGCGGCTCCACCAGGATTCCCAAAATCCAAAAGCTTTTGCAGGATTTCTT
CAATGGCAGGGATCTCAACAAGAGCATCAACCCAGATGAGGCGGTGGCATATGGAGCCGCGG
TGCAGGCCGCCATCCTGATGGGCGACACCTCGGAAAACGTTCAGGACTTGTTGCTTCTGGACG
TGGCTCCTTTGTCCTTGGGTATCGAGACCGCAGGAGGAGTCATGACTGCTCTGATCAAACGAA
ACACCACCATCCCCACTAAGCAAACGCAGATCTTCTCCACATATTCCGATAACCAGCCG

Hsc70_Ca_2208bp (Acc. AB092840) real-time RT-PCR-Product: 486-690

TAGGTGTTTTCCAACATGGAAAAGTTGAAATCATTGCTAATGACCAAGGGAACAGGACCACTC
CAAGCTATGTAGCTTTCACAGATACTGAGAGATTGATTGGAGATGCTGCAAAAAATCAGGTCG
CAATGAACCCCACCAACACAGTCTTTGATGCCAAGCGTCTGATTGGCCGCAAGTTTGATGATG
GCGTTGTTCAGTCTGACATGAAGCACTGGCCTTTTAATGTCATCAATGACAATTCCCGTCCCAA
GGTCCAGGTTGAATACAAGGGTGAAGCCAATTCCTTCTACCCTGAAGAGATTTCCTCCATGGT
TCTTACCAAGATGAAGGAAATTGCAGAGGCCTACCTGGGAAAGACTGTTTCCAACGCTGTCGT
CACCGTGCCTGCCTACTTCAACGATTCTCAGCGACAGGCCACCAAGGATGCTGGAACCATCTC
TGGCTTGAATGTTCTGCGTATCATCAATGAACCAACTGCTGCTGCTATTGCTTACGGTCTGGAC
AAAAAGGTTGGTGCTGAGAGAAATGTCCTCATTTTCGATCTTGGTGGTGGCACTTTCGATGTG
TCTATCCTCACCATTGAGGATGGCATCTTCGAGGTCAAATCTACTGCTGGAGACACTCACTTG
GGTGGAGAAGACTTTGACAACCGCATGGTGAACCACTTCATCACAGAGTTCAAGCGCCAGCA
CAAGAAGGACATCACCGACGACAAGAGAGCCGTTCGCCGTCTCCGCACTGCCTGCGAGAGGG
CCAAGCGTACCCTATCCTCCAGCACTCAGGCCAGTATTGAGATCGACTCTCTCTATGAGGGTA
TCGATTTCTATACCTCAATCACCAGGGCCCGTTTTGAGGAGCTCAATGCTGACCTCTTCCGTGG
CACCTTGGACCCAGTTGAGAAGTCTCTTCGTGATGCCAAGATGGACAAGGCTCAGATCCACGA
CATTGTCTTGGTTGGTGGCTCCACTCGCATTCCCAAAATCCAGAAGCTGCTCCAAGACTACTTC

AATGGCAAGGAGCTCAATAAGAGCATCAATCCCGATGAGGCTGTGGCCTACGGAGCAGCGGT
TCAGGCTGCCATCCTGTCTGGTGACAAGTCTGAGAATGTTCAGGACTTGCTGTTGCTAGATGT
CACTCCTCTGTCCCTTGGAATTGAGACCGCTGGTGGAGTCATGACTGTCCTCATCAAGCGTAA
CACCACTATCCCAACCAAACAGACTCAGACTTTCACCACCTATTCTGACAACCAGCCCGGTGT
GCTCATTCAGGTCTATGAGGGCGAGCGTGCAATGACAAAGGATAACAACTTGCTGGGCAAGT
TTGAGCTTACTGGAATCCCCCCTGCACCTCGTGGTGTTCCCCAGATTGAGGTCACCTTTGACAT
TGATGCCAATGGCATCATGAATGTTTCAGCTGCAGATAAGAGCACTGGCAAGGAGAACAAAA
TAACCATCACCTACGACAAGGGTCGTCTCAGCAAGGAGGACATTGAGCGCATGGTGCACGAG
GCAGAGAAGTACAAGTCCGAGGATGATGTGCAGCGTGAAAAGGTGTCTGCCAAGAATGGTCT
GGAGTCGTACGCCTTCAACATGAAGTCCACTGTTGAGGATGAGAAACTGAAGGGCAAGATCA
GTGATGAGGACAAGCAGAAGATCCTTGACAAGTGCAATGAAGTCATCAGTTGGCTTGACAAG
AATCAGACTGCTGAGAAGGAAGAGTTTGAGCACCAGCAGAAGGAGCTGGAGAAGATCTGCA
ACCCCATCATCACCAAGCTGTACCAGAGTGCTGGAGGCATGCCAGGTGGAATGCCTGATGGT
ATGCCCGGTGGCTTCCCAGGGGCCGGCTCCGCTCCAGGAGGTGGATCCTCTGGCCCAACCATT
GAGGAGGTCGACTAAGACATTCCAAAGCCACTGTGCTGCCTCCATAGCAATATTTACTGTTGC
CCTCTATAGTTGGACTCCCCTAAAATGGTTACTTTGACGACGTTTTGGACGACTTCAACTTGCA
GAGTGATTGTTGCAATTCTAAAAAAGGGGATTAACGGGTCACATTTCTAGATCACTGGGAACA
GACTTTCTACCTAGATTGCACAACCTATTTATTGACTTGTGAACTAAGTTCATAAGCCTTATCA
AACCACCTCAATGCTTTAAAGAAAACGAATAAAATGGTGACTTGTTCTCAATGAAAAAAAAA
AAAAAAAAA

Hsp90_Cc_707bp **real-time RT-PCR-Product: 423-570**

TTCCAGGCTGAGATTGCTCAGCTGATGTCTCTCATCATCAACACTTTCTATTCCAACAAAGAGA
TCTTCCTCAGGGAGCTCATCTCCAACTCTTCAGATGCTCTGGATAAAATCCGCTATGAGAGTCT
CACAGACCCGAGCAAGCTGGACTCAGGAAAAGACCTTAAAATCGAAATCATTCCCAACAAAC
AAGAGCGCACGCTGACCCTCGTCGACACCGGCATCGGCATGACCAAAGCTGACCTCATCAAC
AACCTGGGAACCATCGCCAAATCCGGCACCAAGGCCTTCATGGAGGCTCTGCAGGCCGGAGC
GGACATCTCCATGATCGGTCAGTTCGGAGTGGGCTTCTATTCCGCCTACTTAGTGGCCGAGAA
AGTGACGGTCATCACCAAACACAACGATGACGAGCAGTACGCCTGGGAATCTTCCGCTGGAG
GATCCTTCACCGTTAAAGTGGACAGCTCCGAGCCGATCGGTCGAGGAACTAGAGTGATCCTGC
ATCTGAAGGAGGATCAGACGGAGTACATCGAAGAGCGACGGATCAAAGAGATCGTCAAGAA
GCACTCGCAGTTCATTGGCTACCCTATTACGAATCACTAGTGAATTCGCGGCCGCCTGCAGGT
CGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCT
AAATAGCTTGGCGTAATC

Hsp30_Ca_865bp (Acc. AB177389) **real-time RT-PCR-product: 324-561**

CCAAATCTAAAAGCCTTACGACGCCACCAGAGAGACAACTCGACTGAGGAGCAAAACTAAAA
GCCATTGAAAATGTTGAGCCTGCATGGATTCCAGCCTTCCCTCAGCTCATTCATGGGAATGGA
CTGGCCAGTGCGCAGTCTCTGGCCGGAGATCACATCTCTTTACAGACATGGAAAAGAACTGCA
GGAGCTGAAGAGAAGCCTAGAGCAGCTGGGGAAATTTCAGGATAAGATCCTTGAGGAGATCC
AGCTGATGCCACCCTCACAGGAGATCTACCCAGTGGCCTGCACAATGGAGAAAGAAGGAAGC
GGCTTTGCTTTGACGCTGGACACTAAAGACTTTTCCCCAGAAGAACTGTCAGTCAGACAGGTG
GGCAGGAAGCTGCATGTCAGCGGAAAGAGTGAGAAGAAGCAGGAGGATGGGAAGGGCTCGT
ACTCTTTCAGAACCCAAGAGTTCAGGCGGGTGTTTGATCTTCCTCAAGGAGTGAATCCTGAGG
CGGTGACCTGCTCCATGGCTGATGGAAAGCTCTATATACAGGCACCAGTAAATCAGCCGTCAG
ATGCTGCTGAGAGGATGCTGCCCATTGACTGTCAAACTGTGAAGACAACGCAGCCGGAGACA
GCCGACACCAGCACCACCCAGACGACACTCAACAATCCTGAAAGCACTGAGCACAGAGTACA
ACCCTCTGCTGGACAATCTTAAATAATGATGTTTTGTTTTATTTAAAAACATTGTGTTGTACTT
AATTGTTTTCTGGTCTTTAAGTAATCTAAATGAACAAAAAGATGGATTTTTTATTTTTGAATAT
ATACATGGAATTAAATAAAATAAGAACAGCAAAAAAAAAAAAAAAAAAAAA

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