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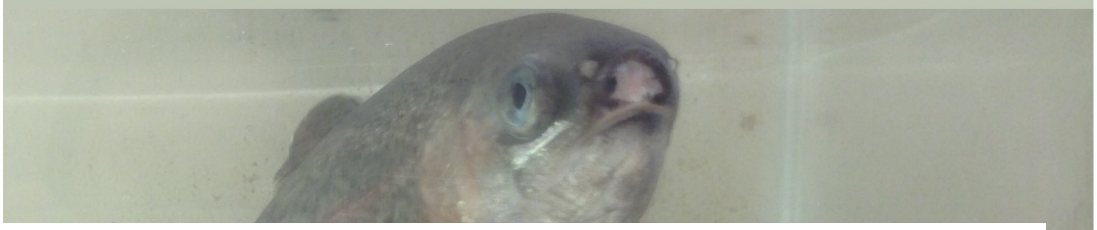

HANNA KORAJOKI

# *Effects of temperature acclimation on the molecular machinery of the cardiac sarcoplasmic reticulum in fishes*

PUBLICATIONS OF THE UNIVERSITY OF EASTERN FINLAND
*Dissertations in Forestry and Natural Sciences No 116*

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EASTERN FINLAND

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Number 116

Academic Dissertation
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**ABSTRACT**

Habitats with highly varying temperature can be challenging to the body functions of ectothermal animals, including contractility of the heart. The cardio-vascular system serves several vital body functions, in particular the transport and distribution of oxygen, $CO_2$, nutrients, metabolic wastes and hormones. For sustaining a proper rate of blood circulation under varying temperature regimes, temperature-dependent adjustments in heart function are necessary. At a given muscle length, changes in the concentration of cytosolic free $Ca^{2+}$ $[Ca^{2+}]_C$ around the myofibrils set the rate, force and duration of cardiac contraction, i.e. cardiac contractility. In vertebrate cardiac myocytes, changes in $[Ca^{2+}]$c are produced by concerted activity of the $Ca^{2+}$ transport systems of the sarcolemma (SL) and sarcoplasmic reticulum (SR). The functioning of the cardiac SR has been thoroughly studied in mammals, but is poorly known in fishes.

In this thesis, the expression of four proteins intimately involved in the SR $Ca^{2+}$ recycling of cardiac myocytes was studied in the atrium and ventricle of rainbow trout (*Oncorhynchus mykiss*), burbot (*Lota lota*), and crucian carp (*Carassius carassius*), three fish species adapted to different habitat temperatures and showing different activity patterns. Within cardiac myocytes, the 12 kDa FK-506 binding protein (FKBP12; a cytosolic regulator of the SR $Ca^{2+}$ release channel/ ryanodine receptor, RyR2), the sarco(endo)plasmic reticulum $Ca^{2+}$ ATPase (SERCA2) and its regulator phospholamban (PLN) are responsible for the release and restoring of SR $Ca^{2+}$, and calsequestrin (CASQ2) is the main $Ca^{2+}$-binding protein in the lumen of the SR. The protein and mRNA levels of these molecules were measured using western blotting and quantitative real-time polymerase chain reaction (qRT-PCR), respectively, from the hearts of fish acclimated to +4°C (cold-acclimation, CA) or +18°C (warm-acclimation, WA).

In the atrial myocytes of the rainbow trout heart, expression of FKBP12 and SERCA2 increased in cold-acclimation. This

explains the previously observed increase in the SR $Ca^{2+}$ uptake rate and the enhancement of $Ca^{2+}$-induced $Ca^{2+}$-release (CICR) from SR in the atrium of CA trout. Similarly to the case of the trout, CA promotes SERCA2 expression of the burbot heart, in both the atrial and the ventricular myocytes. Divergent from the trout heart, FKBP12 expression in burbot is not affected by thermal acclimation. The acclimatory responses of the burbot cardiac SR will probably also improve the CICR of the CA fish, in this case by increasing the SR $Ca^{2+}$ load. In the atrial muscle of the crucian carp heart cold-acclimation reduced FKBP12 expression, while SERCA2 expression remained unchanged. These changes should cause a decrease in $Ca^{2+}$ uptake into the SR, weaken CICR and reduce SR $Ca^{2+}$ leakage. Temperature acclimation did not have any effect on CASQ2 expression in rainbow trout. These findings increase our understanding of the molecular mechanism by which SR contribution to cardiac $Ca^{2+}$ transient is increased in the cold-active rainbow trout and burbot but decreased in the cold-dormant crucian carp.

*Universal Decimal Classification: 591.044, 591.112.1, 591.412, 591.543.1*

*CAB Thesaurus: acclimatization; heat adaptation; environmental temperature; fishes; Oncorhynchus; rainbow trout; Lota lota; Carassius carassius; heart; myocardium; endoplasmic reticulum; calcium; proteins; gene expression*

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# *Acknowledgements*

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First, I should like to thank my supervisor, Professor Matti Vornanen, for offering the interesting topic for my thesis and even more, for having confidence in the progress of the thesis, despite several setbacks.

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My thanks are due to several people who had an impact on my work. Juha Lemmetyinen assisted me in constructing the phylogenetic trees, and gave excellent tips for the laboratory work. My roommate, Maarit Mäenpää, gave a totally new perspective on my work and showed me a good example how to write a thesis. Nina Kekäläinen contributed many relaxing informal conversations in the lab and during dinners and coffee breaks. Katja Anttila was kind enough to listen to and understand my feelings, both positive and negative, concerning this thesis and life outside work.

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four-legged therapists. My own therapist helped me to appreciate the small, good moments in life.

I am particularly grateful to my parents, Elina and Jorma, who always encouraged me to study and told me that everything can be achieved through hard work. However, at the same time, they succeeded in this without exerting any pressure on me. My two brothers, Tuomas and Markus, taught me how to be strong and defend myself, and I value the moments spent with them as much today as in our childhood.

This thesis is dedicated to the most important persons in my life: my dear husband Veli-Pekka, who stands by me and encourages me to continue with my work, and my two sweet little girls, Manta and Elli, who every day remind me of the one and only important issue in my life: their best.

Joensuu, August 2013
Hanna Korajoki

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AP | action potential |
| CA | cold-acclimation/cold-acclimated |
| $[Ca^{2+}]_C$ | cytosolic free $Ca^{2+}$ concentration |
| CaMKII | Ca2+/calmodulin-dependent protein kinase II |
| $[Ca^{2+}]_{SR}$ | SR lumenal $Ca^{2+}$ concentration |
| CASQ | calsequestrin |
| cDNA | complementary DNA |
| CICR | $Ca^{2+}$ induced $Ca^{2+}$ release |
| c-SR | circular SR |
| DHPR | dihydropyridine receptor (L-type $Ca^{2+}$ channel) |
| EC | extracellular |
| e-c | excitation-contraction |
| FKBP | FK506-binding protein |
| IC | intracellular |
| JCN | junctin |
| j-SR | junctional SR |
| l-SR | longitudinal SR |
| MF | myofilament |
| mRNA | messenger RNA |
| MW | molecular weight |
| NCX | $Na^+/Ca^{2+}$ exchanger |
| nj-SR | nonjunctional SR |
| PAGE | polyacrylamide gel electrophoresis |
| PKA | cAMP-dependent protein kinase |
| PKC | protein kinase C |
| PKG | cGMP-dependent protein kinase |
| PLN | phospholamban |
| $P_o$ | open probability |
| qRT-PCR | quantitative RT-PCR |
| r-SR | reticular SR |
| RT | reverse transcription |
| RT-PCR | reverse transcription polymerase chain reaction |
| Ry | ryanodine |
| RyR | ryanodine receptor ($Ca^{2+}$ release channel ) |

| | |
|---|---|
| SDS | sodium dodecyl sulphate |
| SERCA | sarco(endo)plasmic reticulum $Ca^{2+}$ ATPase |
| SL | sarcolemma |
| SR | sarcoplasmic reticulum |
| TnC | troponin C |
| TRDN | triadin |
| WA | warm-acclimation/warm-acclimated |
| WB | western blotting |

## LIST OF ORIGINAL PUBLICATIONS

This thesis is based on data presented in the following articles, referred to by the Roman numerals I-IV.

**I** Korajoki H and Vornanen M. Species- and chamber-specific responses of 12 kDa FK-506 binding protein to temperature acclimation in fish heart. Manuscript.

**II** Korajoki H and Vornanen M. Expression of SERCA and phospholamban in rainbow trout (*Oncorhynchus mykiss*) heart: comparison of atrial and ventricular tissue and effects of thermal acclimation. *The Journal of Experimental Biology 215: 1162-1169, 2012.*

**III** Korajoki H and Vornanen M. Temperature dependence of sarco(endo)plasmic reticulum $Ca^{2+}$ ATPase expression in fish hearts. *Journal of Comparative Physiology B, 183: 467-476, 2013.*

**IV** Korajoki H and Vornanen M. Expression of calsequestrin in atrial and ventricular muscle of thermally acclimated rainbow trout. *The Journal of Experimental Biology 212: 3403-3414, 2009.*

## AUTHOR'S CONTRIBUTION

All the studies were planned by the present author (H.K.) together with her supervisor M. Vornanen. H.K. performed all the molecular studies and collected the data. The interpretation of the results and the writing of the first versions of the manuscripts were performed by H.K. and finalized together with M. Vornanen.

# *Contents*

# 1 Introduction

Fish are ectothermic vertebrates, i.e. the heat of the animal body originates from the immediate surroundings of the animal. In practice this means that the body temperature of the fish is equal to the water temperature. All the physiological processes and reaction rates of enzymes decelerate with decreasing temperature, and therefore, compensatory changes are required in order to maintain a proper rate of vital body functions and good physical performance in cold waters. Conversely, increases in ambient temperature accelerate the metabolism and catalytic rate of enzymes, which may overexploit the energy resources of the body and may thus threaten the thermal stability of enzymes and other proteins (Somero, 2011). Therefore, in ectothermic vertebrates, large seasonal temperature changes, which are typical of boreal climates, require acclimatization of the body functions to seasonal temperature regimes. The acclimation capacity of the animal is also important for its high temperature tolerance under the present threats of predicted climate warming (Somero, 2010).

The functions of the circulatory and respiratory systems are considered to be central factors in limiting the temperature tolerance of ectotherms (Pörtner, 2002). Temperature dependent changes in the cardiac output (the product of heart rate and stroke volume) of fishes are mainly regulated by heart rate, with only minor changes in stroke volume (Graham & Farrell, 1989). Thus heart rate and factors that affect it are considered to be particularly important for thermal resistance in fishes. During acclimation and acclimatization, physiological adjustments, such as an increase in heart size and heart rate, and a decrease in the duration of cardiac contraction, help to maintain the cardiac output and activity of the fish under changing temperature regimes (Graham & Farrell, 1989; Driedzic et al., 1996; Aho & Vornanen, 1999; Aho & Vornanen, 2001). Acclimation is found

to increase tolerance of low temperature more than tolerance of high temperature (Beitinger & Bennett, 2000).

The range of temperature tolerance limits is characteristic for each fish species or a group of species. Some species can live in a wide range of habitat temperatures and are designated as eurytherms, while other species tolerate a narrower temperature range and are designated as stenotherms. Between these two extremes are the mesothermic species, which tolerate moderate but narrower temperature ranges than eurythermal fish. At northern temperate latitudes all fishes tolerate freezing temperatures, and therefore classification of fishes into different thermal tolerance groups is based mainly on their upper thermal tolerance limits. Mesothermic rainbow trout (*Oncorhynchus mykiss* Walbaum) is one of the most commercially utilized and scientifically studied fish species, and cold-stenothermic burbot (*Lota lota* L.) is commercially harvested and used as a food fish in Eurasian countries (McPhail & Paragamian, 2000; Stapanian et al., 2010). Both rainbow trout and burbot are active throughout the year, and burbot even spawn during the winter season, when the temperature of the water is ca 0-4°C. Rainbow trout tolerate habitat temperatures ranging from 0 up to 25°C (Currie et al., 1998), while burbot prefer habitat temperatures below 13°C but can, however, survive at temperatures of over 20°C (Pääkkönen et al., 2003). Burbot are benthic fish that inhabit the cold hypolimnion, while rainbow trout occur mainly across the thermocline (Rowe & Chisnall, 1995). However, rainbow trout and burbot inhabit lakes and rivers where the water temperature may arise up to 15-18°C during the summer season (Pääkkönen et al., 2003).

Crucian carp (*Carassius carassius* L.) is a eurythermic freshwater fish species of the family Cyprinidae. Crucian carp tolerate a wide range of temperatures from 0 to 38°C, but prefer warm habitat temperatures with an optimum at around 27°C (Horoszewicz, 1973). On a north-south axis their distribution ranges from the Arctic Circle in Scandinavia to central France and the Black Sea, and in the west-east direction from Great Britain to Siberia. In northern Europe, crucian carp have an

exceptional over-wintering strategy; they inhabit seasonally anoxic lakes and ponds where other fish species are unable to survive (Blazhka, 1958). By this means, they avoid interspecies competition for common resources and can escape predation by carnivorous fish species (Holopainen & Hyvärinen, 1984). This adaptation strategy has stringent physiological requirements, since crucian carp must tolerate prolonged anoxia, which is a fairly exceptional characteristic among vertebrate animals. The wintering of crucian carp is characterized by inactivity or dormancy, which reduces the consumption of rather limited energy sources (Johnston & Bernard, 1983; Holopainen et al., 1986; Vornanen, 1994). In cardiac function, this lifestyle takes the form of inverse thermal compensation, where seasonal acclimatization to cold and anoxic winter causes decreases in the heart rate and in cardiac contractility (Matikainen & Vornanen, 1992; Tiitu & Vornanen, 2001).

### 1.1 MORPHOLOGY AND FUNCTIONING OF THE FISH HEART

The heart of teleost fishes consists of four chambers: the sinus venosus, the atrium, the ventricle and the bulbus arteriosus, of which the atrium and the ventricle are contractile muscle chambers (Yamauchi, 1980; Farrell & Jones, 1992). Venous blood from the Cuverian ducts and the hepatic vein first enters the sinus venosus, and contractile activity of the atrium and the ventricle propels the blood into the vasculature. It is good to note that when discussing the two chambers of the fish heart in this thesis, I refer to the atrium and the ventricle.

Similarly to the mammalian heart, filling of the teleost heart occurs during the relaxation phase of the heart (diastole) by venous return (*vis-a-tergo*), i.e. due to low "positive" pressure of the venous blood. In some fish species with a rigid cartilaginous pericardium, ventricular filling may occur by suction (*vis-a-fronte*), i.e. through "negative" pressure of the enlarging ventricular chamber (Johansen & Burggren, 1980; Farrell & Jones, 1992). The role of atrial contraction (systole) is to provide

additional force for ventricular filling in the later phase of the ventricular diastole; this will increase the end-diastolic volume of the ventricle prior to the ventricular systole and thereby increases the force of ventricular contraction *via* the Frank-Starling mechanism (Lai et al., 1998). During the ventricular systole, the blood flows from the ventricle through the elastic *bulbus arteriosus* into the ventral aorta.

In the sinoatrial junction between the sinus venosus and the atrium (Saito, 1969; Haverinen & Vornanen, 2007; Arrenberg et al., 2010; Tessadori et al., 2012) are the cardiac pacemaker cells that provoke and control the rate and rhythm of the heartbeat. A transient change of membrane potential, i.e. action potential (AP), spontaneously arises in the pacemaker cells and is propagated *via* gap junctions of the intercalated discs into the atrial myocytes and, after a short delay, into the ventricular myocytes, thereby triggering a sequential contraction of the whole heart.

The heart musculature is mainly composed of muscle and connective tissues. In the majority of fish species the ventricular wall consist exclusively of a spongy or trabecular muscle layer, while in the hearts of highly active species, e.g. trout and tuna, the spongy myocardium is surrounded by a variable thickness of compact myocardium (Farrell & Jones, 1992). The cardiac muscle cells, i.e. the atrial and ventricular myocytes of the fish heart are spindle-shaped: roughly equal in length to rat ventricular myocytes (100–170 μm vs. 142 μm), but over 75% narrower (6–8 μm vs. 32 μm) than the rat ventricular cell (Satoh et al., 1996; Vornanen, 1998; Vornanen et al., 2002; Tiitu & Vornanen, 2002a). The volume of fish ventricular myocyte is less than 10% of the volume of the mammalian ventricular myocyte. The myofibrils are cortically located beneath the sarcolemma (SL) and occupy 40-65% of the cell volume in the ventricular myocyte (Santer, 1985; Vornanen, 1998; Tiitu & Vornanen, 2002a). The remaining cell volume is occupied by the nucleus, mitochondria and glycogen, which are centrally located in the cell. The T-tubules are missing, but variable amounts of sarcoplasmic reticulum (SR) surround the myofilaments. It

should be noted that significant interspecies variation exists in both the size and the subcellular composition of fish cardiac myocytes (Table 1).

The SR of the fish cardiac myocyte is generally considered to be less developed than in the mammalian myocyte, but more extensive than e.g. in the frog heart (Santer, 1985; Driedzic & Gesser, 1994). Morphologically, the cardiac SR is divided into free or nonjunctional SR (nj-SR), which wraps around the bundles of myofilaments demarcating myofibrillar domains, and junctional SR (j-SR), which forms couplings with the SL. The free SR consists of three types of SR structures. Reticular (r-)SR forms tight hexagonal lattices of SR network on the surface of myofibrils (Figure 1). Longitudinal (l-) and circular (c-) tubules of SR membrane run parallel and perpendicular, respectively, with regard to the myofibrils and connect the r-SR sheets and subcellular cisternae of the j-SR to form a functional entity (Santer, 1985)(merged). The cisternae of the j-SR are filled with the calsequestrin (CASQ), and the thin gaps between the SL and j-SR are often occupied by 25-nm-wide and 10-nm-high foot particles that represent ligand-gated $Ca^{2+}$ release channels or ryanodine receptors (RyR) (Vornanen et al., 2002).

***Table 1.*** *Morphometric data of the ventricular myocyte of rainbow trout, crucian carp, burbot, and perch (Perca fluviatilis).*

| | **Rainbow trout** | **Crucian carp** | **Burbot** | **Perch** |
|---|---|---|---|---|
| Cell length | 197 [a] | 110 [a] | 147 [b] | |
| Cell width | 7.42 [a] | 5.78 [a] | 6.3 [b] | |
| Cell volume | 2.53 [a] | 1.38 [a] | 2.36 [b] | |
| Proportion of: | | | | |
| • Myofilaments | 40 % [a] | 40 % [a] | 65 % [b] | |
| • Mitochondria | 45 % [a] | 22 % [a] | 27 % [b] | 22 % [d] |
| • Glycogen | Little [a] | Plenty [a] | 6 % [b] | |
| • Fat droplets | Abundant [a] | 0 % [a] | 0 % [b] | |
| • SR | Extensive [c] | Some [c] | Extensive[b] | 4.5 % [d] |

[a] (Vornanen, 1998)
[b] (Tiitu & Vornanen, 2002a)
[c] (Vornanen et al., 2002)
[d] (Bowler & Tirri, 1990)

## 1.2 CONTRACTION OF THE FISH CARDIAC MYOCYTE

Contraction of the cardiac myocyte is set in motion by a transient rise in the cytosolic free $Ca^{2+}$ concentration ($[Ca^{2+}]_C$) around the myofilament proteins actin and myosin. $Ca^{2+}$ ions bind to the troponin C (TnC) molecule in the thin filament, causing a change in the position of the tropomyosin, which leads to uncovering of the myosin-binding sites of the actin molecule (Katz, 1983). $Ca^{2+}$-related events, from the depolarization of the cell membrane to the contraction of the myocyte, are termed excitation-contraction (e-c) coupling.

$[Ca^{2+}]_C$ near the myofilaments varies from 0.1 μmol $l^{-1}$ in the diastolic state to about 1.0 μmol $l^{-1}$ during maximal contraction (Bers, 2001). In the cardiac myocytes of several fish species, the rise in $[Ca^{2+}]_C$ ensues mainly as a result of sarcolemmal $Ca^{2+}$ entry from the extracellular space into the cell (Figure 1). By depolarization of the SL, $Ca^{2+}$ enters the cell via voltage-dependent openings of L-type $Ca^{2+}$ channels (dihydropyridine receptors, DHPRs) and through the reverse-mode function of the cardiac $Na^+/Ca^{2+}$ exchanger (NCX) (Vornanen, 1997; Hove-Madsen & Tort, 1998; Vornanen, 1999; Shiels et al., 2000; Hove-Madsen et al., 2000) Trans-sarcolemmal influx of $Ca^{2+}$ is regarded as sufficient to activate a major part of the contraction in fish hearts. Since fish cardiac myocytes have a higher surface-to-volume ratio than do mammalian cardiac myocytes, and the myofilaments of fish myocytes are located next to the cell membrane, a similar $Ca^{2+}$ flux density causes a greater increase of $[Ca^{2+}]_C$ in piscine than in mammalian cardiomyocytes (Vornanen, 1998). However, in a number of fish species, SR $Ca^{2+}$ stores make a significant but largely variable contribution to cytosolic $Ca^{2+}$ transient.

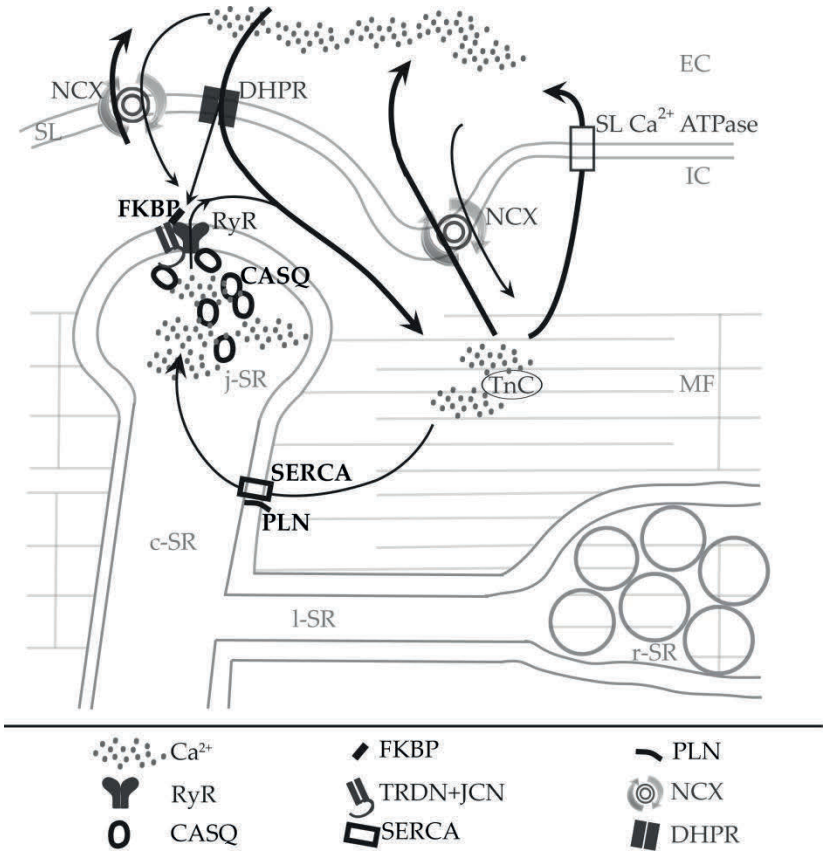


***Figure 1.*** *Excitation-contraction (e-c) coupling in a fish myocyte. Sarcolemmal (SL) $Ca^{2+}$ influx via the L-type $Ca^{2+}$ channel/dihydropyridine receptor (DHPR) and $Na^{+}/Ca^{2+}$ exchanger (NCX) activates contraction of myofilaments (MF) by increasing the intracellular (IC) free $Ca^{2+}$ concentration ($[Ca^{2+}]_C$) and $Ca^{2+}$ binding to troponin C (TnC) in the thin filament. Binding of $Ca^{2+}$ to the cytosolic site of the calcium release channel/ryanodine receptor (RyR) of the sarcoplasmic reticulum (SR) induces $Ca^{2+}$ release from the junctional (j-) SR. NCX and SL $Ca^{2+}$ ATPase return $Ca^{2+}$ to the extracellular space (EC), and sarco(endo)plasmic reticulum $Ca^{2+}$ ATPase (SERCA) into the non-junctional SR under the regulation of phospholamban (PLN). Within the SR, $Ca^{2+}$ diffuses to the j-SR, where most of the $Ca^{2+}$ binds to the store protein calsequestrin (CASQ). CASQ regulates RyR activity via two small auxiliary proteins triadin (TRDN) and junctin (JCN). The activity of RyRs is also modulated by FKBPs. This thesis focuses on the expression levels of FKBP, SERCA, PLN, and CASQ (marked in bold). Three morphologically different compartments of the non-junctional (nj-)SR can be separated: circular SR (c-SR), longitudinal SR (l-SR), and reticular SR (r-SR).*

## 1.3 FUNCTION OF THE SR IN CARDIAC CONTRACTION AND RELAXATION

Although the SR of the fish heart is not as extensive as that of the mammalian heart, it may still have an important role in the regulation of cardiac contraction and possibly also in maintaining the stability of cardiac e-c coupling.

### 1.3.1 $Ca^{2+}$-induced $Ca^{2+}$ release (CICR)

E-c coupling of the mammalian cardiac myocyte is characterized by the $Ca^{2+}$ induced $Ca^{2+}$ release (CICR) process, where binding of a small amount of cytosolic $Ca^{2+}$ to the cardiac RyR2 of the SR membrane opens the channels and causes a large release of $Ca^{2+}$ from the lumen of the SR into the cytosol. The trigger $Ca^{2+}$ comes from the EC space mainly *via* DHPRs (Bers, 2002). In contrast to mammals, in the cardiac myocytes of several ectothermic vertebrates, including most fish species, SL $Ca^{2+}$ influx is large but able to induce only a relatively limited $Ca^{2+}$ release from the SR (Fabiato & Fabiato, 1978; Hove-Madsen, 1992; Keen et al., 1994).

An acute drop in temperature depresses the CICR of fish cardiac myocytes. However, the reduced efficiency of CICR is compensated in long-term acclimation to cold by proliferation of the SR (Bowler & Tirri, 1990; Shiels et al., 2011) and possibly by changes in the abundance of SR proteins involved in $Ca^{2+}$ cycling (the present thesis). It should also be noted that there are quantitative differences between the fish cardiac chambers regarding the contribution of the to contractile activation: the role of CICR is generally more prominent in atrial than in ventricular myocytes (Gesser, 1996; Aho & Vornanen, 1999; Tiitu & Vornanen, 2001; Tiitu & Vornanen, 2002b)

Considerable variation exists between fish species regarding the role of SR $Ca^{2+}$ stores in cardiac e-c coupling, and this seems to be partially associated with the activity-level of the species. The contribution of SR $Ca^{2+}$ stores to contractile activation is often assessed in the intact cardiac muscle or isolated cardiac myocytes as the size of the ryanodine (Ry)-sensitive component

of contraction. In the rainbow trout heart, functional impairment of the cardiac RyRs has relatively little effect on ventricular contraction. By contrast, Ry has a pronounced negative effect on atrial contraction, in particular in cold-acclimated (CA, 4°C) fishes (Aho & Vornanen, 1999). The response of the crucian carp heart to RyR blocking is different from that of the rainbow trout. Generally, the contractility of the crucian carp heart is insensitive to Ry with the exception of the atrial muscle of warm-acclimated (WA, 18°C) fishes (Tiitu & Vornanen, 2001) These findings suggest that, compared to the crucian carp heart, the trout heart relies more on SR $Ca^{2+}$ stores for contractile activity, and that thermal acclimation has opposite effects on the function of the cardiac SR in the two teleost species. In the heart of cold-adapted burbot, cardiac myocytes rely more heavily on the SR $Ca^{2+}$ stores than in many other fish species. However, similarly to the case with other fishes, contraction of the atrial muscle is more dependent on intracellular $Ca^{2+}$ stores than is ventricular contraction (Tiitu & Vornanen, 2002b)

The $Ca^{2+}$ sensitivity of cardiac RyR determines how effective the sarcolemmal $Ca^{2+}$ influx is in inducing $Ca^{2+}$ release from the SR (Stern & Cheng, 2004) The significance of intracellular $Ca^{2+}$ stores for cardiac e-c coupling is reflected in the $Ca^{2+}$-binding affinity of cardiac RyRs. In the burbot ventricular muscle, where the SR plays a significant role in cardiac contraction, RyRs have high sensitivity to $[Ca^{2+}]_C$. Higher concentrations of $[Ca^{2+}]_C$ are needed to activate RyRs in the ventricular muscle of rainbow trout, which is less dependent on SR $Ca^{2+}$ release for contractile activation. The $Ca^{2+}$ sensitivity of RyRs is lowest in the crucian carp heart, which relies mainly on transsarcolemmal $Ca^{2+}$ influx in e-c coupling (Vornanen, 2006). The $Ca^{2+}$ sensitivity of burbot RyR is similar to that of the mammalian ventricular myocyte, where the role of SR $Ca^{2+}$ as an initiator of contraction is more important than that of the extracellular $Ca^{2+}$. This suggests that CICR has an important role in the e-c coupling of the burbot heart, but less so in rainbow trout and least of all in crucian carp myocytes.

### 1.3.2 Molecular aspects of the SR CICR

RyRs are large homotetrameric molecular complexes (ca. 2 200 kDa) and belong to the superfamily of ligand-gated intracellular channels (Zalk et al., 2007). RyR monomers (ca. 565 kDa) consist of cytosolic and trans-membrane domains, the latter forming the $Ca^{2+}$ channel pore. The cytosolic domains form approximately 80 % of the protein mass, and are seen in electron micrographs as the foot particles in the gaps between the SL and j-SR of the vertebrate myocyte (Inui et al., 1987a; Inui et al., 1987b; Vornanen et al., 2002; Tiitu & Vornanen, 2002a).

Temperature acclimation affects SR $Ca^{2+}$ release in fish hearts, but the change in SR function is not associated with alterations in the number of RyR $Ca^{2+}$ release channels (Tiitu & Vornanen, 2003; Birkedal et al., 2009). This suggests that the increased/decreased contribution of SR $Ca^{2+}$ stores to contractile activation following thermal acclimation is due to changes in activity and/or regulation of the release channel. The regulation of the function of cardiac RyR2 is a highly complicated process that involves several molecular entities in both the membrane and the lumen of the SR.

Cytoplasmic $Ca^{2+}$ is the physiological agonist of cardiac RyRs; increases in $[Ca^{2+}]c$ , following excitation of SL, result in proportional increases in $Ca^{2+}$ binding to RyRs and gradual opening of the RyRs (Meissner & Henderson, 1987; Laver, 2007; Laver & Honen, 2008). Although a change in $[Ca^{2+}]c$ is a necessary prerequisite for the activation of cardiac contraction, the CICR process is also regulated by the lumenal $Ca^{2+}$ concentration of the SR ($[Ca^{2+}]_{SR}$). $[Ca^{2+}]_{SR}$ affects the functioning of the RyR2 directly (Xu & Meissner, 1998; Gyorke & Gyorke, 1998) and indirectly *via* CASQ, the main $Ca^{2+}$-binding protein of the cardiac SR (Zhang et al., 1997; Gyorke et al., 2004). In addition to CASQ there are several small proteins, either in the cytosol or within the cardiac SR, that modulate the RyR function. FK506-binding proteins (FKBPs), calmodulin (Meissner & Henderson, 1987) and sorcin (Meyers et al., 1995), bind to the cytosolic side of the cardiac RyRs and regulate their activity. Finally, phosphorylation of the RyR2 channel increases the open

probability ($P_o$) and $Ca^{2+}$ sensitivity of the $Ca^{2+}$ release channel in the mammalian heart. The phosphorylation sites of cAMP-dependent protein kinase (PKA) and $Ca^{2+}$-calmodulin-dependent kinase (CaMKII) are also located in the cytosolic domain of the RyR2 (Marx et al., 2000; Bultynck et al., 2001; Wehrens et al., 2004). By contrast, the interaction sites of triadin (TRDN) and junctin (JCN), which form complexes with the CASQ2, are located on the luminal side of the RyR protein (Lee et al., 2004).

### 1.3.3 Regulation of the CICR by FK506-binding proteins

FKBPs are a family of cytosolic RyR regulators that possess peptidyl-prolyl cis-trans isomerase activity (Göthel & Marahiel, 1999). The first member of this family was isolated and cloned from human T-cells, and was named FKBP12 according to its molecular weight (12 kDa). It blocks T-cell activation when bound to the immunosuppressant drug FK-506 (Standaert et al., 1990). Later on, FKBP12 was found to occur in most eukaryotic cells, including fish skeletal and cardiac myocytes (Qi et al., 1998; Jeyakumar et al., 2001). The binding of FKBP12 to the mammalian skeletal RyR1 leads to enhanced co-operation of the RyR monomers (Marks, 1996) and to more stable functioning of the tetrameric $Ca^{2+}$ release channel. As a result, only fully closed or fully open states of the $Ca^{2+}$ channel exist (Brillantes et al., 1994), and the channel has longer mean open time but smaller $P_o$ (Ahern et al., 1997). In addition, FKBP12 regulates the group behaviour of the RyRs, known as coupled gating, i.e. it coordinates opening and closing of the RyRs in the closely packed arrays in the SR membrane (Marx et al., 1998). In the mammalian cardiac muscle, coupled gating ensures the systolic opening of all RyRs in a given SR/T-tubule junction, and prevents the diastolic openings of the RyRs which may lead to SR $Ca^{2+}$ leak (Marx et al., 2001). Although the physical coupling of RyRs does not require FKBPs, the functional coupling requires (Marx et al., 2001). Thus far, the function of FKBPs is poorly known in fish muscles.

Besides binding FKBP12, the mammalian cardiac RyR2 is capable of binding FKBP12.6, another member of the FKBP family. FKBP12.6 has a slightly slower relative mobility in the SDS-PAGE gel than does FKBP12, i.e. it has a higher molecular mass (Timerman et al., 1994; Lam et al., 1995). The binding affinity of the cardiac RyR2 for FKBP12.6 is about seven times higher than for FKBP12. Both isoforms have been found in the mammalian cardiac muscle, but the abundance of the two FKBPs varies greatly between species (Jeyakumar et al., 2001; Zissimopoulos et al., 2012). In rat cardiac myocytes, the concentration of FKBP12 is more than 10 times higher than the concentration of FKBP12.6 (Guo et al., 2010). The effects of the two FKBPs on cardiac RyR function are antagonistic: FKBP12 activates the RyR2 and FKBP12.6 inhibits the functioning of the RyR2 channel by competing with FKBP12 for the same binding site on the RyR2 (Galfre et al., 2012). FKBP12 increases the $P_o$ of the RyR2 by increasing the channel openings and increasing the $Ca^{2+}$ sensitivity of the channel. This can cause spontaneous openings of RyRs and $Ca^{2+}$ leakage from the SR, consequently reducing the $Ca^{2+}$ content of the SR. The binding of FKBP12.6 to cardiac RyR2 diminishes the number of spontaneous $Ca^{2+}$ releases ($Ca^{2+}$ sparks) and therefore increases the $Ca^{2+}$ content of the SR (Guo et al., 2010). The binding of FKBPs to RyRs is prevented by phosphorylation of the RyR channel (Marx et al., 2000), thus diminishing the regulatory activity of the FKBPs.

### 1.3.4 Regulation of the CICR by calsequestrin

In fish cardiac myocytes, the $[Ca^{2+}]_{SR}$, releasable by caffeine-induced opening of the RyR2 channels, varies between 0.1 and 1.0 mmol $l^{-1}$ myocyte (Hove-Madsen et al., 1998; Hove-Madsen et al., 1999; Shiels et al., 2006; Haverinen & Vornanen, 2009). In general, the steady-state $Ca^{2+}$ load seems to be higher in atrial than in ventricular myocytes of the fish heart (Haverinen & Vornanen, 2009; Galli et al., 2011). Interestingly, in spite of the minor participation of SR $Ca^{2+}$ stores in the e-c-coupling of fish cardiac myocytes, SR $Ca^{2+}$ stores are 2-14 times larger in fish than in mammalian cardiac myocytes (Negretti et al., 1995;

Delbridge et al., 1996), suggesting important differences between piscine and mammalian hearts regarding the regulation of SR $Ca^{2+}$ content and CICR.

As mentioned above, $[Ca^{2+}]_{SR}$ seems to be an important regulator of cardiac CICR. $[Ca^{2+}]_{SR}$ regulates CICR directly by binding to the activation and inhibition sites of the RyR2 (Xu & Meissner, 1998; Gyorke & Gyorke, 1998) and indirectly *via* CASQ2. According to general understanding, acidic residues on the surface of CASQ2 bind lumenal $Ca^{2+}$ with moderate affinity, but high capacity (Park et al., 2004; Beard et al., 2004; Sanchez et al., 2012). In low $[Ca^{2+}]$, CASQ2s exist as monomers, and the increase in $[Ca^{2+}]$ leads to the formation of front-to-front dimers, followed by the back-to-back stacking of the dimers to form higher polymers of the CASQ2 (Wang et al., 1998). In addition, CASQ2s undergo depolymerization, but binding of CASQ2 to JCN may be needed in order to initiate the process (Lee et al., 2012). It is suggested that in low cardiac $[Ca^{2+}]_{SR}$, either monomerized or dimerized CASQ2s bind to TRDN and JCN, inhibiting the function of the RyR2 (Gyorke et al., 2004; Wei et al., 2009), and that polymerization of CASQ2 leads to dissociation of CASQ2 from the complex in high $[Ca^{2+}]_{SR}$. At physiological $[Ca^{2+}]_{SR}$, the cardiac CASQ2 is assumed to exist mainly in the form of monomers (Park et al., 2003; Wei et al., 2009), but evidence for the polymerization of CASQ2 also exists (Franzini-Armstrong et al., 2005). Although CASQ2 seems to be an essential SR $Ca^{2+}$ buffer and an important regulator of RyRs, the $Ca^{2+}$ storing capacity of the cardiac SR is not altered in myocytes from the knock-down mice for CASQ2 (Knollmann et al., 2006). The total absence of CASQ2 is compensated by the increased volume of the SR (Knollmann et al., 2006). Thus, the exact role of CASQ2 in the regulation of cardiac CICR is still largely unresolved.

## 1.4 RELAXATION OF THE FISH CARDIAC MYOCYTE

A decrease in $[Ca^{2+}]_C$ to the diastolic level causes a release of $Ca^{2+}$ from TnC, and the result is dissociation of myosin heads from the actin and relaxation of the myocyte. Thus, recycling of $Ca^{2+}$ ions back into the extracellular space and into the lumen of the SR is critical for the relaxation of the myocyte. SL $Ca^{2+}$ extrusion occurs due to the activity of the $Na^+/Ca^{2+}$ exchanger (NCX) and the sarcolemmal $Ca^{2+}$ ATPase, while the SERCA2 pump restores the $Ca^{2+}$ content of the SR.

### 1.4.1 SR $Ca^{2+}$ uptake

$Ca^{2+}$ uptake into the SR is mediated by the sarco(endo)plasmic reticulum $Ca^{2+}$ ATPase (SERCA). There are marked species specific differences in the rate of $Ca^{2+}$ uptake and in the activity of cardiac $Ca^{2+}$-ATPase. Both $Ca^{2+}$ uptake rate and ATPase activity are higher in the fish species, such as tunas, that rely significantly on the SR $Ca^{2+}$ stores as a source of activator $Ca^{2+}$, while they are lower in the hearts of species, such as rainbow trout, crucian carp, or mackerel, that mainly utilize extracellular $Ca^{2+}$ for contractile activation (Aho & Vornanen, 1998; Landeira-Fernandez et al., 2004; Castilho et al., 2007). In addition, thermal compensation during CA produces an increase in the rate of $Ca^{2+}$ uptake and in the ATPase activity of SERCA2 in the heart of cold-active rainbow trout, but causes a decrease in the cold-dormant crucian carp (Aho & Vornanen, 1998). The $Ca^{2+}$ uptake rate is higher in atrial myocytes, which are thought to exploit the intracellular $Ca^{2+}$ stores more than the ventricular myocytes. Thus, the rate of $Ca^{2+}$ uptake and the ATPase activity of SERCA seem to be good indicators for the role of SR $Ca^{2+}$ release in e-c coupling.

Cardiac SERCA2 is a 110 kDa protein that includes ten hydrophobic transmembrane helices and two cytosolic loops (Brandl et al., 1986). The $Ca^{2+}$-binding domain, or the stalk area, is located on the cytosolic face of the transmembrane domain, and includes the sections from the N-terminal end as well as both cytosolic loops (MacLennan et al., 1985). The

transmembrane domains constitute the channel, which is closed by the barrier formed by the helices of the stalk. The second cytosolic loop includes the phosphorylation domain and the ATP-binding domain.

### 1.4.2 Regulation of $Ca^{2+}$ uptake by phospholamban

Phospholamban (PLN) is an important regulator of cardiac muscle relaxation in all vertebrates (Cerra & Imbrogno, 2012). The PLN monomer (7 kDa) consists of a C-terminal transmembrane domain and an N-terminal cytoplasmic domain, the latter including three phosphorylation sites. The dephosphorylated form of PLN binds to SERCA and reduces its affinity for cytosolic $Ca^{2+}$ (Kimura et al., 1996). At low diastolic $[Ca^{2+}]_C$, the dephosphorylated PLN inhibits the activity of SERCA2. When $[Ca^{2+}]_C$ increases in the systole, CaMKII phosphorylates PLN, thereby reactivating the SERCA pump (Wegener et al., 1989). PLN is also phosphorylated by PKA in the presence of β-adrenergic tone (Tada & Kadoma, 1989). In its phosphorylated form, the PLN monomer is separated from SERCA2, thus increasing the $Ca^{2+}$ affinity of the SERCA2. PLN is stored in the myocyte in the form of 22 kDa homopentamers with little or no regulator activity.

The increase in the activity of SERCA may be a result of the increased expression of SERCA or decreased inhibition of the SERCA pump caused by PLN. In the mammalian heart, increased SERCA2 expression, or the ratio of the PLN and SERCA2 expressions, has been shown to impact the contractile properties of the heart (Luo et al., 1996; He et al., 1997; Koss et al., 1997; Baker et al., 1998; Meyer et al., 1999). However, the significance of the PLN/SERCA2 ratio for cardiac contractility has not been examined in fish hearts.

## 1.5 OBJECTIVES OF THE THESIS

Species-specific differences in the thermal acclimation of fish cardiac contractility have raised questions regarding the molecular mechanisms by which acclimation may affect the e-c coupling of fish cardiac myocytes. The role of SR $Ca^{2+}$ stores as an activator of myocyte contraction is greater in the heart of two cold-active fish species, rainbow trout and burbot, after acclimation to cold temperatures, while on the other hand, the intracellular $Ca^{2+}$ stores are less important in the myocytes of the cold-dormant crucian carp, although they do play a minor role in the atrium of crucian carp acclimated to warm temperatures (Tiitu & Vornanen, 2001). Since the temperature-induced changes in $Ca^{2+}$ release from the SR are not associated with alterations in the number of RyR $Ca^{2+}$ release channels (Tiitu & Vornanen, 2003; Birkedal et al., 2009), acclimation-related changes in the regulation of RyR activity are probably involved. To establish this, the expression of theRyR2 activator FKBP12 was measured (Paper I). Specifically, it was hypothesized that the expression of FKBP12 is higher in the atrium than in the ventricle, and that it increases in the heart of rainbow trout and burbot after acclimation to cold, while decreasing in the heart of cold-dormant crucian carp.

The role of intracellular $Ca^{2+}$ stores in thermal acclimation is also evident in the increased rate of SR $Ca^{2+}$ uptake in the heart of rainbow trout and in the decreased rate of $Ca^{2+}$ uptake in the heart of crucian carp (Aho & Vornanen, 1998). Paper II considers whether the increase in the rate of $Ca^{2+}$ uptake is a consequence of increased expression of SERCA2 or of decreased expression of PLN, the regulator of SERCA2. It was hypothesized that the ratio of SERCA and PLN is lower in the atrium than in the ventricle, and decreased in the heart of cold-acclimated rainbow trout. Paper III clarifies SERCA2 expression in the hearts of thermally acclimated burbot and crucian carp. The hypothesis was that the expression of SERCA2 is higher in the atrium than in the ventricle, increased in the heart of cold-active burbot after

acclimation to cold and decreased or independent of temperature in the heart of cold-dormant crucian carp.

The third origin of the increased role of intracellular $Ca^{2+}$ stores in cold-acclimation may be the increased expression of CASQ, the main SR $Ca^{2+}$ storage protein and the essential regulator of RyR. Paper IV debates the effect of temperature on the expression of the main SR $Ca^{2+}$ storage protein, CASQ2, in the heart of rainbow trout. The hypothesis was that the expression of CASQ2 is elevated in the atrium in comparison to the ventricle of the rainbow trout, and is increased after acclimation to cold. Together these papers offer an insight into the effects of thermal acclimation on $Ca^{2+}$ handling in the SR of the myocyte of ectothermal fish.

# 2 Synopsis of the methods

Rainbow trout were obtained year-round from Kontiolahti fish farm, while burbot were captured in February from Lake Orivesi and crucian carp in January and June-July from a local pond, Mustalampi. Acclimation of the fish was performed in 500-L stainless steel tanks (Figure 2) by changing the water temperature at a rate of 3°C per day, up or down, from the temperature at the fish´s place of origin, until the acclimation temperature was obtained. The fish were maintained at a constant acclimation temperature (+4°C for cold-acclimation and +18°C for warm-acclimation) for a minimum of 4 weeks before sampling.


***Figure 2.*** *The fish were acclimated in 500-L tanks under oxygen-saturated conditions.*

The expression of FKBP12, SERCA2, and CASQ2 were studied at both protein and mRNA level, in order to find out not only the relative amount of the proteins expressed in the atrial and

ventricular myocyte after thermal acclimation, but also to examine the effect of acclimation on gene transcription in each cardiac chamber and to clarify the regulation of protein expression in the myocytes of different fish species. The methods used for studying each protein in each species are listed in Table 2. The protein expression studies were carried out using the western blotting (WB) method, and the transcription levels were quantified by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) after reverse transcription of the messenger RNA (mRNA) to the complementary DNA (cDNA). Before the quantification studies, it was necessary to solve, at least partially, the nucleotide sequences of the genes coding the target protein by cloning and sequencing. The mRNA expression of PLN in the rainbow trout and FKBP12 in the burbot heart could not be measured due to the lack of specific primers, which was a consequence of unsuccessful cloning.

***Table 2.*** *Summary of the species studied and methods used in the original papers I-IV.*

| **Paper** | **I** | **II** | | **III** | **IV** |
|---|---|---|---|---|---|
| | **FKBP12:** | **SERCA2:** | **PLN:** | **SERCA2:** | **CASQ2:** |
| Rainbow trout | WB, qRT-PCR | WB, qRT-PCR | WB | - | WB, qRT-PCR |
| Crucian carp | WB qRT-PCR | - | - | WB, qRT-PCR | - |
| Burbot | WB | - | - | WB, qRT-PCR | - |

## 2.1 CLONING

The whole genome duplication (3R) in the teleost lineage may have multiplied the number of genes encoding the SR $Ca^{2+}$-cycling proteins, thus generating complexity in protein expression and analysis. Transcript expression provides an

alternative way of examining gene activity, which also partly avoids the problems associated with protein expression. By cloning and sequencing all putative gene paralogs and measuring their expression by qRT-PCR insight is provided into gene expression at the transcriptional level. However, quantification of gene transcription by qRT-PCR requires the use of specific primers, which cannot be designed without knowing the exact mRNA sequence of the gene in each species. This causes some difficulties in transcript analysis. Gene cloning is not always successful, since interspecies variance in nucleotide sequences, together with the relatively low mRNA expression levels in fish hearts, often leads to mispriming.

When I started my thesis work, the nucleotide sequences of the studied genes in rainbow trout, crucian carp or burbot were all unknown. The cDNA was reverse transcripted from the mRNA extracted from the hearts of each studied fish species, and used as a template in the reverse transcription PCR (RT-PCR) in order to partially clone the mRNA of the target gene. Degenerative primers were designed after aligning the known cDNA sequences of several vertebrate species. In order to obtain the best results, the primers were designed for the most conserved regions of the target gene. The sequences of the primers are presented in the original papers. In order to minimize the nucleotide substitutions derived from the mistakes made by a DNA polymerase, we chose the DNA polymerase with a proofreading activity (Phusion DNA Polymerase, Finnzymes, Espoo, Finland) or a mixture (DyNAzyme EXT, Finnzymes) that included an enzyme with the proofreading activity. PRC products ligated into vectors were further transferred into E*scherichia coli* bacteria before sequencing. The identity of the cloned sequence was verified by comparing the sequence with the sequences of the corresponding region in other vertebrates.

## 2.2 QUANTIFICATION OF mRNA EXPRESSION BY QUANTITATIVE RT-PCR

Real-time qRT-PCR is a highly sensitive method for the study and quantification of mRNA expression in the cell (Heid et al., 1996; Bustin, 2002). In the present work, the SYBR Green method was used for qRT-PCR. The starting amount of the template cDNA is back-calculated from the intensity of the SYBR Green fluorescent dye that binds to double-stranded DNA, thus increasing the emission of the sample after every PCR amplification cycle. The expression level of the studied gene is compared with the expression level of a carefully chosen reference gene, the expression of which is known to remain relatively constant in the studied treatment. The endogenous reference gene DnaJ subfamily A member 2 (DnaJA2) was chosen on account of its less temperature-sensitive expression pattern compared with that of other commonly used reference genes (Vornanen et al., 2005) (for a general discussion see (Rytkonen et al., 2010)).

In order to obtain reliable results, attention was paid to the accuracy of the reverse transcription (RT) step prior to PCR amplification. The sensitive M-MuLV RNase $H^+$ RT enzyme used in this study possesses at RNase H activity that degrades the RNA after cDNA synthesis and is not affected by the RNase inhibitor added to the reaction mixture for the inhibition of external RNases. In addition, control reactions without the RT enzyme were performed on each sample in order to verify the absence of contaminating genomic DNA.

Specific gene primers were separately designed for each studied fish species to avoid mispriming. The number of amplicons was confirmed from the melting curve and by separating the product in agarose gel. The efficiency of the amplification reaction was verified from the slope of the standard curve generated from the dilution series of the standard sample, and the reactions with an efficiency matching the limits set by the manufacturer were accepted.

## 2.3 QUANTIFICATION OF PROTEIN EXPRESSION BY WESTERN BLOTTING

Western blotting is based on the identification and quantification of the studied protein by the protein-specific antibody from the nitrocellulose membrane. Before being transferred to the membrane (Towbin et al., 1979), the proteins were separated according to their MW by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described in (Laemmli, 1970).

The antibodies used in the studies were polyclonal and raised against the purified target protein or short peptide synthesized according the amino acid sequence of the protein (Table 3). The antibodies used for the detection of FKBP12 and CASQ2 were raised against the protein sequences of the rainbow trout, while detection of SERCA2 and PLN was based on antibodies raised against zebra fish sequences. Additionally, some commercial antibodies to mammalian protein sequences were used in the detection of SERCA2 and CASQ2. In the CASQ2 studies, the commercial anti-CASQ2 antibody was used to confirm the identity of the protein recognized by the anti-trout CASQ2 antibody. SERCA2 expression in trout was measured by using the custom-made antibody to the zebra fish sequence. For crucian carp and burbot SERCA2, a commercial antibody to human SERCA2 was used, since the custom-made antibody was had almost run out. When the anti-zebra fish antibody was changed for the commercial anti-SERCA2 antibody, we verified that the two antibodies recognized the same protein.

Polyclonal antibodies are purified from the serum of the host animal after induction of antibody production by frequent exposure to the antigen. In comparison to the monoclonal antibodies that recognize a single epitope in the antigen, the polyclonal antibodies are a heterogenous combination of antibodies with an affinity for several distinct epitopes. This results in more sensitive detection of the target protein on the one hand, but a minor reduction in accuracy on the other. Since the expression levels of the studied proteins are relatively low,

the use of polyclonal antibodies may give more reliable results than the use of monoclonal antibodies.

***Table 3.*** *Antibodies used in the thesis.*

| **Antibody** | **Antigen ($NH_2$ – COOH)** | **Paper** |
|---|---|---|
| anti-omFKBP12[a] | TPGDGQTFPKKGQTC | I |
| anti-drPLN[a] | CHMTRSAIRRASNIE | II |
| anti-drSERCA1[a] | KYGFNELPAEEGKS | II |
| anti-hsSERCA2 (ab91032)[b] | Residues 1 – 100 of human SERCA2 | III |
| anti-omCASQ2[c] | Residues 173 – 316 of trout CASQ2 | IV |
| anti-clfCASQ2 (ab3516)[b] | Purified canine CASQ2 | IV |

[a] Manufactured by CovalAb Ltd., Villeurbanne, France
[b] Manufactured by Abcam Plc., Cambridge, UK
[c] Manufactured by Inbio Ltd., Tallinn, Estonia

In sample preparation, special attention was paid to the rapid cooling of the samples in order to minimize protein degradation by endogenous proteases. In addition, a protease inhibitor cocktail (Sigma Chemicals Co., St Louis, USA) was included in the sample buffers. Crude homogenates rather than purified SR preparations were used in the experiments in order to prevent errors caused by the loss of SR proteins in prolonged isolation procedures (Murphy et al., 2011).

# 3 Results and Discussion

The main findings of the original publications (I-IV) are collected in Table 4. The expression levels of the four proteins involved in SR $Ca^{2+}$ cycling are compared between the two muscular chambers of the heart and between the two acclimation groups in each species. The protein expression of FKBP12 (I) and the SR $Ca^{2+}$ pump SERCA2 (II,III) were studied in the hearts of all three fish species acclimated to cold or warm temperatures, while the expression of the SERCA2 inhibitor PLN (II) and SR $Ca^{2+}$ store protein CASQ2 (IV) were studied only in the hearts of CA and WA rainbow trout. The number of SERCA2 transcripts was measured in all three fish species, FKBP12 transcripts in rainbow trout and crucian carp, and CASQ2 in rainbow trout.

It should noted that absolute levels of protein expression cannot be compared between fish species in a straightforward manner on the basis of western blot data, since the polyconal antibody raised against zebra fish or mammalian proteins may have a different number of epitopes in the proteins originating from different fish species. A decrease in signal magnitude, despite elevated antibody concentrations and prolonged incubation times, may indicate a lesser degree of similarity between the proteins of two different species, rather than a true difference between the species as regards expression levels. For instance, the intensity of rainbow trout FKBP12 was observed to be clearly higher than the intensity of burbot and crucian carp FKBP12s, but this does not necessarily indicate a higher expression level in the trout heart, since the antibody was raised against trout FKBP12. Thus, in order to compare expression levels between species, the exact quantities of the proteins need to be determined e.g. by using the purified protein as a standard.

***Table 4.*** *Main results of the original articles I-IV.*

| Article | Main results |
|---|---|
| I | Under CA, the expression of FKBP12 was increased in the atrium of rainbow trout, but decreased in the atrium of crucian carp.<br>No acclimatory changes were seen in the heart of burbot, nor in the ventricles of rainbow trout and crucian carp.<br>Contrary to protein expression, the number of FKBP12 transcripts was higher in the ventricle than in the atrium of both crucian carp and rainbow trout, but was increased by CA only in the heart of rainbow trout. |
| II | In the rainbow trout heart, SERCA2 expression was higher in the atrium than in the ventricle.<br>CA increased SERCA2 expression in the atrium but not in the ventricle of the trout heart.<br>The number of SERCA2 transcripts was higher in the atrium than in the ventricle of the trout heart, and was increased in both chambers under CA.<br>CA induced an increase in the expression of PLN in the trout atrium, but to a lesser degree than for SERCA2.<br>The PLN/SERCA2 ratio was lower in the atrium than in the ventricle of the trout heart. |
| III | Under CA, SERCA2 expression was increased in both chambers of the burbot heart. No differences existed between the two chambers as regards SERCA2 expression.<br>No acclimatory changes in SERCA2 expression were observed at the transcript level in the burbot heart.<br>The expression of SERCA2 in the crucian carp heart showed no responses to thermal acclimation. However, the SERCA2 isoform composition differed between the atrium and the ventricle.<br>Two SERCA2 transcripts were found in the crucian carp heart. CA induced an increase in the number of dominating SERCA2 transcripts in the atrial tissue but not in the ventricle. |
| IV | Two CASQ2 isoforms were expressed in the heart of rainbow trout.<br>The total CASQ2 content was similar in both chambers of the rainbow trout heart.<br>Expression of the 2 CASQ2 isoforms was not changed by thermal acclimation.<br>Several splice-variants of CASQ2 existed in the trout heart. The expression levels of these transcripts were higher in the atrium than in the ventricle, and increased in both chambers under CA. |

### 3.1 THERMAL ACCLIMATION EFFECTS ON THE EXPRESSION LEVELS OF FKBP12, SERCA2 AND PLN IN THE HEART OF RAINBOW TROUT AND CRUCIAN CARP

Contraction and relaxation of the cardiac muscle are roughly two times faster in the atrium than in the ventricle of both rainbow trout and crucian carp. The faster cardiac cycle of the atrial muscle is associated with the higher SR $Ca^{2+}$ uptake rate in the atrial than in the ventricular muscle of the trout heart (Aho & Vornanen, 1999; Tiitu & Vornanen, 2001). Thus, it was unsurprising to observe a higher SERCA2 expression and a lower PLN/SERCA2 ratio in the atrium than in the ventricle of the trout heart (II). Similarly to the case with rat, mouse and rabbit hearts (Luss et al., 1999), the faster $Ca^{2+}$ uptake in the trout atrial muscle seems to be a result of the increased number, and probably reduced inhibition, of the SERCA2 pump. Only one SERCA2 isoform with a MW of 110 kDa was present in the trout heart.

In the heart of crucian carp, two SERCA2 isoforms were recognized, and their relative abundance differed between the atrium and the ventricle (III). Expression of the SERCA2 isoform with an equal MW to the SERCA2 isoform of the trout heart (110 kDa) was 2-3 times more abundant in the atrial than in the ventricular muscle of the crucian carp heart. This isoform formed approximately half of the total SERCA2 proteins in the atrium. In the ventricle, the decrease in the 110 kDa isoform was compensated by the increased expression of a smaller (93 kDa) SERCA2 isoform, since total SERCA2 expression did not vary between the two cardiac chambers. Although total SERCA2 expression is similar in both cardiac chambers of the crucian carp heart, the presence of the 110 kDa SERCA2 isoform in the atrium may contribute to the two times faster relaxation of contraction in the atrial tissue (Tiitu & Vornanen, 2001). Further studies are needed to characterize the $Ca^{2+}$ uptake rates, $Ca^{2+}$-ATPase activities and temperature sensitivities of the two SERCA2 isoforms.

There were apparent differences between different cardiac chambers and between fish species in terms of their responses to thermal acclimation. First, the effect of thermal acclimation on the expression of SERCA2 and FKBP12 was observed mainly in the atrial muscle. Secondly, opposite changes in the expression of FKBP12 (I) were observed between the two species: FKBP12 expression increased in the atrium of CA rainbow trout, but decreased in the atrium of CA crucian carp. Moreover, the expression of SERCA2 (II) was observed to increase in the atrium of rainbow trout, while the expression remained unchanged (III) in the atrium of crucian carp. The expression of the SERCA2 inhibitor PLN was also increased in the atrium of CA rainbow trout, but to a lesser degree than that of SERCA2. However, thermal acclimation did not affect on the PLN/SERCA2 ratio in either cardiac chamber (II).

According to these results, the expression of SR proteins seems to be relatively independent of temperature in the ventricle. This is consistent with the findings that temperature acclimation has a stronger effect on the atrial than on the ventricular contractility of the trout heart (Aho & Vornanen, 1999). However, temperature acclimation has also been observed to change $Ca^{2+}$ uptake in the ventricular muscle of both rainbow trout and crucian carp heart (Aho & Vornanen, 1998; Aho & Vornanen, 1999). This is at odds with the absence of thermal response in FKBP12 (I), PLN (II) and SERCA2 expression (II, III) in ventricles. There are two possible explanations for this apparent discrepancy. It is possible that temperature-induced changes in the expression of these $Ca^{2+}$-recycling proteins are minor in the ventricular muscle and will therefore go undetected by the western blotting. On the other hand, differences in isoform composition (e.g. crucian carp SERCA2) may cause differences between CA and WA hearts as regards the temperature dependence of $Ca^{2+}$ uptake rate. Nevertheless, the expression of the SERCA2 and PLN/SERCA2 ratio are regarded as good indicators of contraction kinetics in both mammals and fish hearts (He et al., 1997; Koss et al., 1997; Baker et al., 1998; Luss et al., 1999; Landeira-Fernandez et al.,

2004; Castilho et al., 2007). Furthermore, the differences found between atrial and ventricular tissue regarding the thermal response of FKBP12, SERCA2 and PLN protein expressions are in agreement with previous results showing that the blocking of the CICR with Ry affects contraction force only in the atrial muscle of CA rainbow trout and WA crucian carp (Aho & Vornanen, 1999; Tiitu & Vornanen, 2001). The present results agree with those of earlier studies indicating that the SR $Ca^{2+}$ stores may play a more important role in thermal compensation in the atrial than in the ventricular myocytes of rainbow trout.

### 3.2 THERMAL RESPONSE OF SERCA2 IS OBSERVED IN BOTH CARDIAC CHAMBERS OF COLD-ADAPTED BURBOT, WHILE FKBP12 SHOWS NO RESPONSE

In contrast to the hearts of rainbow trout and crucian carp, where the effect of thermal acclimation appeared only in atrial SERCA2 expression, in the burbot heart temperature-induced changes in SERCA2 were observed in both the atrial and the ventricular muscle. Expression of SERCA2 was over four times higher after CA in both cardiac chambers of the burbot heart (III), which may underlie the observed increase in the steady-state $Ca^{2+}$ load of the atrial and ventricular myocytes of CA burbot in comparison to that of WA burbot (Haverinen & Vornanen, 2009). As mentioned earlier, the RyR2 channels of the burbot ventricle are more sensitive to $Ca^{2+}$ than are the RyR2s in rainbow trout and crucian carp hearts (Vornanen, 2006). Furthermore, Ry inhibits a major part of contraction in both the atrium and the ventricle of the CA burbot heart (Tiitu & Vornanen, 2002b). The present findings are completely consistent with the functional data in that they show up-regulation of SERCA2 pumps in both the atrium and the ventricle of the CA burbot heart. Thus, there seem to be genuine interspecies (trout, crucian carp, burbot) differences in temperature acclimation both at the functional and at the molecular level.

The thermal insensitivity of FKBP12 expression may be explained by evolutionary adaptation of the burbot heart to cold habitat temperatures. During cold adaptation the expression of FKBP12 may have increased to a level that is sufficient for the cardiac function at cold temperatures, and there is no need for seasonal temperature acclimatization, or alternatively the adaptation may have generated other mechanisms for increasing the role of CICR in contraction initiation. High $Ca^{2+}$-sensitivity of the cardiac RyRs is indicative of such an adaptation. Also morphological cold adaptation may be involved. In several teleost species thermal compensation is achieved by increasing the heart size (Hiroko et al., 1985; Goolish, 1987; Graham & Farrell, 1989; Klaiman et al., 2011). In this respect it is notable that ventricular mass relative to body mass is higher in cold-adapted burbot than in rainbow trout or crucian carp (Tiitu & Vornanen, 2002a). The large ventricular mass of the burbot heart is achieved by increasing the number of cardiac myocytes, i.e. by hyperplasia, while acclimatory increase in heart size is mainly produced by hypertrophy, i.e. by an increase in myocyte size (Vornanen, 1998). It is premature to say whether the more pronounced role of CICR in the atrium and ventricle of burbot is due to the adaptation of the heart to cold habitat temperatures or whether it is a general phylogenetic feature of the cod fish family. Future studies comparing fishes from different phylogenetic groups would clarify this issue.

Similar to the case with other teleost species, in the burbot heart the force of atrial contraction is reduced more than the force of ventricle contraction by Ry treatment (Tiitu & Vornanen, 2002b). This is apparently inconsistent with the present results in that the expression of SERCA2 (III) and FKBP12 (I) did not differ between the atrium and the ventricle of the burbot heart. This could simply be due to tissue-specific differences in the number of RyRs or in PLN regulation of the SERCA2. However, other factors could also be involved, such as differences in the concentration of cytoplasmic of $Ca^{2+}$, $Mg^{2+}$, ATP and the SR lumenal $Mg^{2+}$ and $Ca^{2+}$ (Meissner & Henderson, 1987; Xu & Meissner, 1998; Gyorke & Gyorke, 1998; Laver, 2007; Laver &

Honen, 2008). FKBP12.6 antagonizes FKBP12 and, due to the high affinity of FKBP12.6 for RyR2, even small decreases in concentrations of FKBP12.6 may be sufficient to increase the stimulating effect of FKBP12 (Galfre et al., 2012). Sorcin may reduce the $Ca^{2+}$ sensitivity of the RyR2 channel and also influence $Ca^{2+}$ entry *via* DHPR (Meyers et al., 2003). Calmodulin, which is 100% conserved in vertebrates (Nyegaard et al., 2012) may have essential functions in the cardiac myocytes, but the slow binding and dissociation rates may remain insufficient for beat-to-beat regulation of the RyR2 (Balshaw et al., 2001; Meissner, 2004). Additionally, the different role of β-adrenergic regulation in the contraction of the atrial and ventricular myocytes (Gesser, 1996) may underlie the atrio-ventricular differences in the Ry sensitivity of contraction. Further studies are needed to clarify the molecular mechanism of SR functioning in the atrial and ventricular myocytes of burbot, as well as in other fish species.

### 3.3 TWO CASQ2 ISOFORMS ARE EXPRESSED IN THE HEART OF RAINBOW TROUT

In the mammalian cardiac myocytes, 50-90% of the total $Ca^{2+}$ inside the SR is bound to CASQ2 (Shannon & Bers, 1997; Shannon et al., 2000). In the rainbow trout heart, the maximal and steady-state $Ca^{2+}$ content of the SR is higher in the atrial than in the ventricular myocytes and is unresponsive to temperature acclimation in both cardiac chambers (Haverinen & Vornanen, 2009). Moreover, the atrial SR $Ca^{2+}$ uptake rate is higher than the ventricular uptake rate, and CA induces increases in the uptake rates of the rainbow trout heart (Aho & Vornanen, 1999). Two isoforms of the SR $Ca^{2+}$-binding protein, CASQ2, (54 and 59 kDa) were found in both cardiac chambers of the trout heart, but no significant differences were detected between the atrium and the ventricle, or between the acclimation groups, as regards the expression of CASQ2 (IV). However, expression of both isoforms was characterized by

strong interindividual variation, which might have concealed any minor differences between the atrium and the ventricle, or the effects of thermal acclimation.

CA has been observed to induce an approximately 30% increase in SR volume in the heart of perch (*Perca fluviatilis*) and a 40% increase in the heart of bluefin tuna (*Thunnus orientalis*) (Bowler & Tirri, 1990; Shiels et al., 2011). If similar increases in the volume fraction of SR were to occur in the heart of rainbow trout, burbot, and crucian carp, this might also appear in the SERCA2 content. It is conceivable that the over four-fold increase in the expression of SERCA2 in the atrium of rainbow trout and in both cardiac chambers of burbot could be explained by a cold-induced increase in SR volume. However, if the volume of j-SR were to be increased by that amount, it would result in dilution of the SR CASQ2 content in the CA fish. Obviously, more studies on quantitative aspects of SR proteins and SR membranes are needed in order to resolve the effects of temperature acclimation on the functioning of fish cardiac SR.

Contradictory opinions exist on the molecular arrangement of CASQ2 in monomeric, dimeric, or polymeric forms within the cardiac SR (Park et al., 2003; Franzini-Armstrong et al., 2005; Wei et al., 2009). The same applies to the CASQ2 form, which binds to TRDN and JCN, forming an inhibitory complex with RyR2 (Gaburjakova et al., 2012). The structure of monomeric CASQ2 is modified by inorganic atoms, even though the affinity of the CASQ2 monomer for $Ca^{2+}$ is higher than for other cations (Bal et al., 2011). Although several physiologically important monovalent and divalent cations, e.g. $Na^{+}$, $K^{+}$, and $Mg^{2+}$, cannot induce polymerization of CASQ2, $Zn^{2+}$ may cause polymerization (Bal et al., 2011). Since the SR may contain physiologically significant amounts of $Zn^{2+}$ (Palmer et al., 2006), the possibility that CASQ2 functions as a storage protein for intracellular ions other than $Ca^{2+}$ (e.g. $Zn^{2+}$) cannot be excluded.

The present results suggest that the amount of CASQ2 is not a factor involved in the thermal response of the atrial and the ventricular myocytes of the rainbow trout. However, several casein kinase II (CKII) phosphorylation sites were found on the

cloned CASQ2, and the possibility that the regulator activity of CASQ2 may be dependent on the phoshorylation of CASQ2 cannot be excluded.

### 3.4 PUTATIVE EFFECTS OF B-ADRENERGIC REGULATION

β-adrenergic regulation is crucial for the proper functioning of the fish heart in exercising fish and when subject to temperature acclimation (Graham & Farrell, 1989; Keen et al., 1993). The heart rate and the strength of cardiac contraction are under tonic β-adrenergic control (1-5 nmol $L^{-1}$), and this stimulus is augmented under stressful conditions (Graham & Farrell, 1989; Farrell & Jones, 1992). Moreover, the strength of the β-adrenergic response is increased after acclimation to cold, seemingly as a result of the increase in the number of β-adrenergic receptors (Graham & Farrell, 1989; Keen et al., 1993; Aho & Vornanen, 2001). The binding of adrenalin, the predominant neurotransmitter of the fish autonomic nervous system, to the β-adrenoreceptors of cardiac myocytes leads to activation of the cAMP-PKA cascade and phosphorylation of various target proteins by the PKA. A serine-threonine kinase, CaMKII, is activated by the $Ca^{2+}$-calmodulin complex during systolic increase in $[Ca^{2+}]_C$. This kinase also phosphorylates several SR proteins (Couchonnal & Anderson, 2008).

Unlike endothermic vertebrates, in the fish heart β-adrenergic regulation does not affect the $Ca^{2+}$ sensitivity of the force generation of the contractile element (Gillis & Klaiman, 2011). This is due to the small number of PKA-phosphorylation sites in the piscine troponin I (TnI) and the cardiac myosin-binding protein C (cMyBP-C) (Shaffer & Gillis, 2010). The stimulatory effect of adrenalin therefore results mainly from changes in $[Ca^{2+}]_C$. The L-type $Ca^{2+}$ release channel, RyR2, PLN and CASQ2 possess PKA and/or CaMKII phosphorylation sites, and they are regulated by β-adrenergic stimulus. Since a major part of the activator $Ca^{2+}$ in the fish heart comes from the extracellular space, the L-type $Ca^{2+}$ channel may be one of the

main targets of β-adrenergic activation (Vornanen, 1998; Shiels et al., 2003). The strength of the adrenalin effect on the L-type $Ca^{2+}$ channels varies among teleost species. In the ventricle of rainbow trout, the maximal β-adrenergic stimulation induces an over 100% increase in $I_{Ca}$, while in the ventricle of crucian carp, the increase is only 40% (Vornanen, 1998). This seems to be due to the differences in the basal phosphorylation level of the L-type $Ca^{2+}$ channels in the two species. In the crucian carp heart the $Ca^{2+}$ channels seem to be almost maximally phosphorylated under basal conditions, thus leaving little room for further phosphorylation (Vornanen et al., 2010). In the rainbow trout there seems to be a greater regulatory reserve for $I_{Ca}$, which may be needed to support the active life-style of the trout. $I_{Ca}$ is also increased by beta-adrenergic stimulation in the ventricle of burbot, although a major portion of the activator $Ca^{2+}$ enters the cell *via* NCX and not *via* the L-type $Ca^{2+}$ release channel (Shiels et al., 2006).

Despite the lesser role of CICR in the e-c coupling of the fish myocyte, many of the $Ca^{2+}$-cycling proteins are also subject to β-adrenergic regulation. Phosphorylation of the RyR2 by PKA and/or CaMKII may inhibit the binding of FKBPs to the RyR2 (Marx et al., 2000; Wehrens et al., 2005). This may lead to a decrease/increase in the ratio of FKBP12.6/FKBP12 bound to the RyR2, and consequently to an enhancement/decrease of CICR (Marx et al., 2000; Jiang et al., 2002; Maier et al., 2003; Wehrens et al., 2004; Wehrens et al., 2005; Kohlhaas et al., 2006; Yang et al., 2007).

In addition to increased SR $Ca^{2+}$ release, β-adrenergic stimulation can increase SR $Ca^{2+}$ uptake *via* phosphorylation of PLN. Piscine PLN has phosphorylation sites for both PKA and CaMKII, and phosphorylation of PLN (Kranias, 1985; Tada & Kadoma, 1989) dissociates the PLN from the SERCA2 with a resultant increase in $Ca^{2+}$ sensitivity and the $Ca^{2+}$ uptake rate of the SERCA pump (Hicks et al., 1979). The stronger effect of adrenalin on the contractility of the CA trout heart may be a result of more extensive phosphorylation of PLN in the CA fish due to up-regulation of the β-adrenergic cascade in the CA trout

(Keen et al., 1993). The previously observed changes in the SR $Ca^{2+}$ uptake rate without any changes in the expression of PLN or SERCA2 may be explained by differences in the adrenalin response. This may underlie the observed lack of thermal compensation in ventricular SERCA2 expression or in the PLN/SERCA2 ratio (II).

### 3.5 EXPRESSION OF SR $CA^{2+}$-CYCLING PROTEINS DOES NOT ALWAYS CORRELATE WITH TRANSCRIPT EXPRESSION

The abundance of SERCA2 transcripts was measured from the atrium and the ventricle of all three fish species (II, III), while FKBP12 transcripts were measured from the hearts of rainbow trout and crucian carp (I) and CASQ2 transcripts only from the heart of rainbow trout (IV). The effects of CA on transcript expression and their protein products are presented in Table 5.

***Table 5.*** *Responses of SERCA2, PLN, FKBP12, and CASQ2 protein (black) and mRNA (red) in the atrium and the ventricle of rainbow trout, crucian carp, and burbot, induced under acclimation to cold (2°C or 4°C) .*

| | **Rainbow trout** | | **Crucian carp** | | **Burbot** | |
|---|---|---|---|---|---|---|
| | Atrium | Ventricle | Atrium | Ventricle | Atrium | Ventricle |
| FKBP12 | ↑↑ | ↑± | ±↓ | ±± | ± | ± |
| SERCA2 | ↑↑ | ↑± | ↑± | ±± | ±↑ | ±↑ |
| PLN | ↑ | ± | | | | |
| CASQ2 | ↑± | ↑± | | | | |

↑: increased expression, ↓: decreased expression, ±: constant expression

There was an apparent mismatch between transcript and protein expression levels suggesting that expression of SR proteins is not regulated purely by transcript production, but may involve differences in the stability of transcripts, or post-transcriptional regulation.

Contrary to protein expression, transcripts of FKBP12 were higher in the ventricle than in the atrium of both rainbow trout and crucian carp hearts. By contrast, SERCA2 transcripts

showed a similar expression pattern to that of SERCA2 proteins, both being higher in the atrium than in the ventricle of the rainbow trout heart. CA affected SERCA2 transcripts strongly in both cardiac chambers of the trout heart, while SERCA2 proteins were significantly increased only in the atrial tissue. It seems, however, that changes in transcript expression can explain most of the compensation responses in the heart of the cold-active rainbow trout.

A more prominent mismatch between protein and transcript expression was present in the crucian carp heart. An increase of SERCA2 transcripts in the atrium of CA crucian carp had no influence on protein expression, and a decrease in the FKBP12 protein of the CA atria was not associated with decreased transcript expression. Discrepancies between protein and transcript levels could be attributed partly to the presence of multiple isoforms (SERCA2), which are translated into proteins with different efficiency.

The increased expression of SERCA2 protein in the CA burbot heart was not observed at the transcript level. Even though the physiological response to CA is the same in burbot and rainbow trout hearts, i.e. an increase in SERCA pump expression and activity, the response seems to be achieved by different mechanisms in the two fish species. In trout it is mainly produced by increased transcript expression, while post-transcriptional mechanisms seem to be involved in the case of burbot.

Comparing mRNA and protein levels involves several methodological limitations that need to be considered when interpreting the results (Greenbaum et al., 2003). Western blotting may not be sensitive enough to detect proteins that are expressed at very low levels, and SDS-PAGE may not separate similar-sized proteins into different bands. On the other hand, qRT-PCR is extremely sensitive, but can pick up only those transcripts for which specific primers have been designed. Thus, a protein with a low expression level may go undetected by western blotting, but may be quantified by qRT-PCR at the transcript level. Analogously, gene paralogs of unknown

sequence cannot be detected at the transcript level, but may give a signal at the protein level.

Several whole-genome duplications have occurred in the teleost lineage (Venkatesh, 2003; Taylor et al., 2003; Jaillon et al., 2004; Volff, 2005; Infante et al., 2011). Many of the duplicates may have been lost, may be silent (pseudo genes) or may have evolved for other functions (neofunctionalization). Two paralogs of the genes of SERCA2, CASQ2 and FKBP12 with 1-3 protein coding transcripts have been cloned from several fish species, but the total number of the functional genes or protein isoforms in teleost fish may not have been found. It is possible that the number of genes encoding the SR $Ca^{2+}$-cycling proteins has been multiplied, which may generate the complexity of the transcript expression. Similarly to the two SERCA2 isoforms present, two SERCA2 transcripts were cloned from the heart of crucian carp. Additionally, several CASQ2 transcripts were cloned from the heart of rainbow trout, although two isoforms were found at the protein level. Some CASQ2 transcripts may be splice variants of the same CASQ2 gene, but the differences in the nucleotide sequences of crucian carp SERCA2 transcripts and in some of the rainbow trout CASQ2 transcripts suggest at different origin of the transcripts (nonfunctional/functional alleles).

The results of this thesis reveal that the increase in the mRNA expression does not straightforwardly mean the higher protein expression. The increased mRNA expression may be needed to maintain the sufficient number of highly active proteins with a short life-time, or maintain the efficient translation, in cold temperatures. Additionally, it is possible that the changes in transcript levels are transient in nature before a new steady-state level is achieved. In this study protein and transcript expression were measured at only one time point, i.e. in the steady-state situation of thermal acclimation. In conclusion, since protein represents the functional equivalent of the gene, protein expression may be a better measure of the physiological functioning of the gene.

# 4 Conclusions

This thesis shows that the temperature-induced changes in the expression of SR $Ca^{2+}$-cycling proteins are more prominent in the atrial than in the ventricular muscle of the fish heart. A decrease in temperature reduces the contractility of the cardiac muscle, and therefore ventricular filling could be compromised without thermal compensation of the atrial function. Thermal compensation is associated with several molecular changes in the cardiac SR. In atrial but not in ventricular myocytes of the rainbow trout heart, increases in FKBP12 and SERCA2 expression will enhance CICR and SR $Ca^{2+}$ uptake in the CA fish (Figure 3a): CICR is increased without changes in SR $Ca^{2+}$ content. Similar to the case with rainbow trout atrial myocytes, CA induces an increase in SERCA2 expression in both the atrial and the ventricular myocytes of the burbot heart, but unlike the trout atrium, FKBP12 expression in the burbot heart is not affected by thermal acclimation (Figure 3b). This is an alternative way to increase the contribution of SR $Ca^{2+}$ stores to contractile activation: $Ca^{2+}$ uptake into the SR is increased in the CA fish and results in enhanced CICR via the elevated SR $Ca^{2+}$ load (Bassani et al., 1995). In the atrial but not in ventricular cardiomyocytes of the cold-dormant crucian carp, absence of thermal compensation in SERCA2 expression probably slows down the rate of SR $Ca^{2+}$ uptake in the CA fish (Figure 3c). The decreased expression of the RyR2 activator FKBP12 not only reduces CICR but also prevents SR $Ca^{2+}$ leakage, which could cause ectopic beats and therefore cardiac arrhythmias.

High rate of SR $Ca^{2+}$ uptake is observed in the heart of hibernating ground squirrels (Belke et al., 1987). Similarly, in the cold-active teleost species, enhanced CICR caused by increasing RyR2 activity and SR $Ca^{2+}$ uptake seem to be crucial elements in the thermal compensation of cardiac function at cold temperatures. However, in the burbot heart, thermal

compensation is achieved without acclimatory changes in FKBP12 expression, possibly because the sensitivity of CICR is already up-regulated by evolutionary adaptation to cold. Even though burbot are able to survive for some time warm temperatures, long-term increases in water temperature as a consequence of global warming may threaten the survival of the cold-adapted burbot due to the limited ability of this species to

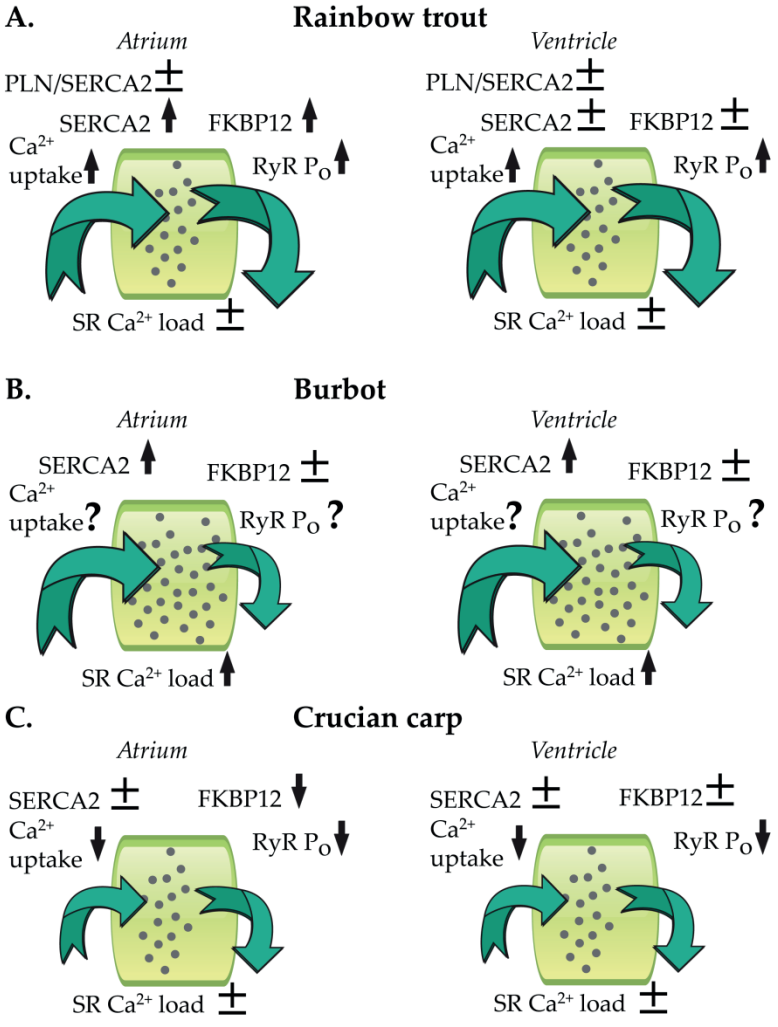


***Figure 3.*** *Hypothetical mechanisms behind the CA-induced changes in SR $Ca^{2+}$ recycling in the myocytes of three studied fish species.*

acclimate to changing temperature. Decreases in SERCA2 expression and SR $Ca^{2+}$ load at high temperatures are expected to weaken CICR, which may reduce cardiac contractility and therefore the aerobic performance of the fish at elevated temperatures. In the light of the hypothesis of oxygen-limited thermal tolerance (Pörtner, 2002), it is expected that studies on the molecular mechanisms of thermal adaptation/acclimation in cardiac contractility will increase our understanding of the heat tolerance of fishes and other ectothermic animals (Somero, 2010).

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**Hanna Korajoki**

# *Effects of temperature acclimation on the molecular machinery of the cardiac sarcoplasmic reticulum in fishes*

The role of intracellular $Ca^{2+}$ stores in the contraction initiation of a fish cardiac myocyte is dependent on the acclimation temperature of the fish. This thesis offers an insight to the effect of thermal acclimation on the expression of four important components of the molecular machinery responsible for the contraction initiation, FKBP12, SERCA2, PLN and CASQ2. This knowledge helps to understand the temperature-induced changes in the cardiac contractility of a fish.

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