Steady-state effects of temperature acclimation on the transcriptome of the rainbow trout heart

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Vornanen, Matti, Minna Hassinen, Heikki Koskinen, and Aleksei Krasnov. Steady-state effects of temperature acclimation on the transcriptome of the rainbow trout heart. Am J Physiol Regul Integr Comp Physiol 289: R1177–R1184, 2005—Cold-acclimated (CA) phenotype of trout heart was induced by 4-wk acclimation at 4°C and was characterized by 32.7% increase in relative heart mass and 49.8% increase in ventricular myocyte size compared with warm-acclimated (WA; 18°C) fish (P < 0.001). Effect of temperature acclimation on transcriptome of the rainbow trout heart was examined using species-specific microarray chips containing 1,380 genes. After 4 wk of temperature acclimation, 8.8% (122) of the genes were differently expressed in CA and WA hearts, and most of them (82%) were upregulated in the cold (P < 0.01). Transcripts of genes engaged in protein synthesis and intermediary metabolism were most strongly upregulated, whereas genes contributing to the connective tissue matrix were clearly repressed. Extensive upregulation of the genes coding for ribosomal proteins and translation elongation and initiation factors suggest that the protein synthesis machinery of the trout heart is enhanced in the cold and is an essential part of the compensatory mechanism causing and maintaining the hypertrophy of cardiac myocytes. The prominent depression of collagen genes may be indicative of a reduced contribution of extracellular matrix to the remodeling of the CA fish heart. Temperature-related changes in transcripts of metabolic enzymes suggest that at mRNA level, glycolytic energy production from carbohydrates is compensated in the heart of CA trout, while metabolic compensation is absent in mitochondria. In addition, the analysis revealed three candidate genes: muscle LIM protein, atrial natriuretic peptide B, and myosin light chain 2, which might be central for induction and maintenance of the hypertrophic phenotype of the CA trout heart. These findings indicate that extensive modification of gene expression is needed to maintain the temperature-specific phenotype of the fish heart.

mechanisms during synthesis and degradation, temperature-dependent changes in transcription of genes are one of the key events in modifying the proteome of the tissues (16, 37).

Circulatory system integrates different body functions through the transport of material and humoral messages and serves the well being of all body cells by providing oxygen and fuels for energy production. As a power supply of the circulatory system, the heart is in the focal point of physiological plasticity and sets limits for the activity level of the animal in different thermal conditions. Although several aspects of heart function have been studied in thermally acclimated fish and a number of structural changes have been noticed on exposure to different temperatures (10, 47), the cellular and molecular mechanisms involved in chronic thermal stress of the heart are only partially elucidated. An interesting and poorly examined feature of the CA phenotype of the trout heart is the enlargement of the cardiac muscle, which attenuates the depressive effect of low temperature on cardiac pump function (19).

The heart is a complex organ composed of multiple tissues, which together provide the system all of the necessary qualifications for cardiac pump function. In addition to the contractile and metabolic machinery of the cardiac myocytes, the amount and quality of the extracellular matrix also contribute to the properties of the heart as a muscular pump (48). Because of the complexity of the heart, an enormous amount of research effort is needed to find out and examine all crucial aspects of cardiac plasticity required for thermal acclimation. In this regard, the screening of gene expression by cDNA microarrays might provide a broader view to genomic basis of cardiac remodeling under changing temperatures (18, 34) and help reveal the candidate genes which are important for thermal acclimation and which might remain unnoticed by traditional biochemical and physiological methods. In the present study, we assess the steady-state effect of temperature acclimation on gene expression of the rainbow trout heart. We hypothesized that under steady-state conditions only relatively few genes, which are vital for the maintenance of proper cardiac function at the new temperature regime, would be differentially expressed. It appeared, however, that of the 1,380 analyzed genes, more than 100 were differentially expressed in trout acclimated to cold and warm temperatures. This indicates that extensive modification of gene expression is needed to maintain the temperature-specific phenotype of the fish heart.

MATERIALS AND METHODS

Animals. Rainbow trout (Oncorhynchus mykiss) were obtained from the local fish farm (Kontiolahti, Finland). In the lab, the fish were

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randomly divided in two groups and gradually brought to the acclimation temperatures of 4°C or 18°C, at which the animals were maintained for at least 4 wk before sampling. The fish were fed commercial trout fodder (Biomar, Brande, Denmark) once a day.

Design of the microarray. We used a salmonid fish microarray containing 1,380 nonredundant clones from normalized and subtracted cDNA libraries targeted at characterizing transcriptome responses to environmental stressors. Each clone was printed in six replicates on the chip, and each sample was hybridized to two microarrays so that each gene was measured in 12 replicates. Multiple replications combined with the dye-swap design of hybridization (see Microarray analyses) allow for accurate detection of relatively small alterations in expression levels, which is important for the functional interpretation of results. Design and construction of this platform have been recently described in detail elsewhere (29). Functional annotation of genes was made by the categories of gene ontology (5).

Microarray analyses. For each sample, atria and ventricles were pooled from 3–4 individuals, and the total RNA of atrial and ventricular tissue was separately extracted with Trizol reagent (Invitrogen, San Diego, CA). Quality of the RNA was verified with gel electrophoresis. Labeling with Cy3- and Cy5-deoxycytidinetriphosphate (dCTP) (Amersham Pharmacia, Piscataway, NJ) was made using SuperScript III reverse transcriptase (Invitrogen) and oligo(dT) primer; cDNA was purified with Microcon YM30 (Millipore, Bedford, MA). We used a dye-swap experimental design (28, 52). For the first slide, test and control cDNA were labeled with Cy5 and Cy3, respectively, and for the second array, dye assignment was reversed. The slides were pretreated with 1% BSA (fraction V), 5 × saline-sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) (30 min at 50°C) and washed with 2 × SSC (3 min) and 0.2 × SSC (3 min), and hybridized overnight in a cocktail containing 1.3 × Denhardt’s, 3 × SSC, 0.3% SDS, 0.67 μg/μl polyadenylate and 1.4 μg/μl yeast tRNA. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO). Slides were washed with 0.5 × SSC, 0.1% SDS (15 min), 0.5 × SSC, 0.01% SDS (15 min), and twice with 0.06 × SSC (2 min). Scanning was performed with ScanArray 5000 and images were processed with QuantArray (GSI Luminonics, Munich, Germany).

Data analyses. The measurements in spots were filtered by criteria I – B ≥ 3 and (I – B)/(S + B) ≥ 0.6, where I and B are the mean signal and background intensities and S is the standard deviation. After subtraction of mean background, lowess normalization (8) was performed. To assess differential expression of genes, the normalized logarithm base 2 of the ratio of the background-subtracted fluorescent signals was analyzed for every pair of dye-swap slides, and P values were determined with Student’s t-test (P < 0.01). In more than 90% of cases the expression of genes was changed similarly in atrial and ventricular muscle, and therefore, the data were combined for functional annotation.

The annotated genes were then categorized into functional groups and analyzed based on the gene expression levels. The relative contributions (W, weight) of different functional gene classes to transcriptome responses were calculated as

\[ W = \frac{n \sum \log_2(\text{FoldChange})}{N} \]

where n is number of differentially expressed genes and N is total number of genes on a slide in each functional Gene Ontology Consortium category.

Assessment of myocyte size. The lipid plasma membrane behaves as a capacitor when surrounded by ionic solution on both sides, and therefore allows cell size measurement by electrophysiological techniques. Cell capacitance is a direct measure of the surface membrane area and can be used as an indirect index of cell volume if surface-to-volume ratio of the cell type is known (38). Because surface area:volume ratio of fish cardiac myocytes is close to one (1.15) (45), capacitance measurements of cell surface area will, in practice, give a good estimate of cell volume changes without any transformations. The capacitance of cardiac myocytes was determined from the same population of trout used for cDNA microarray analyses. Cardiac myocytes were enzymatically isolated with established protocols, and cell capacitance was determined from the time-constant of voltage decay after small current injection into the cell under the whole cell patch-clamp recording mode. Protein concentration of ventricular muscle was determined with the Lowry method (31). Statistical comparisons between acclimation groups were made by unpaired t-test.

RESULTS

Cold-acclimated phenotype of trout heart. Acclimation of rainbow trout to low temperature induced an increase in relative heart mass, which resulted in 32.7% larger hearts in animals acclimated to 4°C (0.154 ± 0.012) compared with animals acclimated to 18°C (0.116 ± 0.006) (P < 0.02). To test the contribution of cellular hypertrophy to the cardiac enlargement, capacitive membrane area of individual myocytes was measured with the patch-clamp technique. In equal-sized fish, mean cell capacitances indicate a 49.8% increase (P < 0.001) in surface area of trout ventricular myocytes as a consequence of acclimation to the cold (Fig. 1A). Cell capacitance increase in atrial myocytes was 30.3% (Fig. 1B). Thus cold acclimation is associated with significant hypertrophic growth of cardiac myocytes, which is able to account for the cardiac enlargement. Protein concentration (125.8 ± 11.3 mg/g and 132.3 ± 3.58 mg/g for CA and WA fish, respectively) (P > 0.05) and water content (79.5 ± 0.61% and 78.0 ± 1.11% for CA and WA fish, respectively) (P > 0.05) were not different between acclimation groups.

![Image](https://example.com/image.png)

**Fig. 1.** Acclimation to cold increases capacitive membrane area of ventricular (A) and atrial (B) myocytes of the rainbow trout heart. Surface area of sarcolemma was determined electrophysiologically from equal-sized fish acclimated at +4°C or +18°C. ***Significantly different (P < 0.001) from the value WA trout.
Expression of gene transcripts. We grouped the gene expression data by the functional categories, which can help to see an overall picture of temperature-dependent changes in the expression pattern. Of the 20 functional gene groups, 16 were in different degrees upregulated and only 4 were repressed (Fig. 2). The most strongly upregulated gene groups were those of protein biosynthesis and metabolism, whereas the contribution of other functional groups to differential gene expression was substantially smaller.

With respect to protein biosynthesis, the majority of the upregulated genes encode ribosomal proteins, although a number genes involved in the initiation and elongation of protein translation were also upregulated (Fig. 3). Downregulated genes represent various molecular chaperones, even though individual chaperones reacted differently to the chronic temperature change, for example, Hsp70 was downregulated but Hsp90 was upregulated (Fig. 4).

In regard to energy metabolism, genes involved in carbohydrate breakdown, e.g., glycogen phosphorylase, glyceraldehyde 3-phosphate dehydrogenase, and malate dehydrogenase were upregulated, whereas transcripts for mitochondrial ATP/ADP translocases, cytochrome c, and mitochondrial creatine kinase were suppressed (Fig. 4).

Among structural genes, strong suppression of collagen genes was the most drastic change. A slight upregulation of genes involved in the formation of cytoskeleton and endoplasmic reticulum was evident, whereas cell adhesion molecules and microtubule proteins were repressed. Transcripts of myosin light chain 2, atrial natriuretic peptide B, and muscle LIM-protein—all established markers of cardiac hypertrophy—were markedly upregulated (Fig. 4).

DISCUSSION

Rainbow trout are relatively eurythermal (0–25°C) and active throughout their thermal tolerance range. Maintenance of proper cardiac function at different thermal conditions requires a profound change of the cardiac phenotype, which appears as compensatory changes in relative heart mass, energy metabolism, nervous and humoral control of cardiac contractility, and in electrical and mechanical properties of the trout heart (2, 10, 26, 27, 46). Such an extensive structural and functional remodeling of the heart probably necessitates both qualitative and quantitative changes among thousands of macromolecules, which constitute the cardiac phenotype and cannot, therefore, be solely based on posttranslational modification of proteins but is expected to require differential gene expression. The present study indicates that even the maintenance of the temperature-induced cardiac phenotype is dependent on differential transcription of almost 10% of the genes. This is in line with other studies in thermally acclimated fish (18, 25, 34).

Selected aspects of steady-state changes in trout cardiac transcriptome are discussed below.

Cellular hypertrophy and structural proteins. Acclimation of rainbow trout to cold is associated with 20–40% enlargement of the ventricular mass (3, 19). Whether this represents growth of existing myocytes (hypertrophy) or is contributed by increase in the number of myocytes (hyperplasia), has not been resolved. Electrophysiological measurement of membrane capacitance indicates that the surface area of the cardiac myocytes is increased as much as 49.6% by acclimation to the cold. Provided that the shape of the myocytes and the proportion of surface membrane invaginations (caveolae) are not affected by temperature acclimation (45), the present findings indicate that the cold-induced enlargement of the trout heart represents true cellular hypertrophy, which is sufficient to explain the 32.7% increase in relative heart mass (myocytes comprise about 68% of the fish heart; Ref. 12). Cold-induced hypertrophy of cardiac myocytes has been also suggested for the striped bass (Morone saxatilis) (36).

If the enlargement of the heart is accounted by myocyte hypertrophy, it means that the relative portion of the connective tissue compartment is decreased. In accordance with this,
cDNA microarray analysis indicates that the expression of genes involved in the synthesis of intercellular collagen matrix is repressed 5- to 6-fold in the cold. The specific increase in myocyte size might point to the significance of increasing the proportion of force producing contractile tissue at the cost of more passive extracellular component. Connective tissue matrix is not, however, a mere supporting substance but directly affects diastolic and systolic function of the heart (48). Unfortunately, temperature-dependent changes in the composition of cardiac connective tissue have not been examined in trout, and thus the putative effects of transcriptional changes on collagen matrix and elastic properties of the trout heart remain an interesting topic for future research.

**Hypertrophy markers.** The cold-induced phenotype of the trout heart is also a hypertrophic cardiac phenotype (see above). Besides its contribution to thermal compensation of heart function, the hypertrophic trout heart may have more general interest, as it presumably represents purely physiologic overgrowth without those pathological features that impair the function of the hypertrophied mammalian heart. In this regard, it is especially interesting to look at how gene transcripts of the established hypertrophy markers behave under thermal acclimation.

In mammalian cardiac myocytes, cardiac overload, and endocrine factors cause hypertrophic growth together with transcriptional upregulation of the several hypertrophy marker genes, for example, atrial natriuretic peptides, myosin light chains, β-myosin heavy chain, α-skeletal actin, and muscle LIM protein (MLP) (30, 41). Interestingly, in the ventricle of CA trout, the transcripts of atrial natriuretic peptide B (ANB), myosin light chain 2 and MLP were elevated by about 3-, 3-, and 5-fold, respectively, showing that cold-induced hypertrophy of the fish heart shares several markers with the hypertrophy of the mammalian heart. The increase in mRNA of ANB and its possible association with temperature-dependent increase in the relative heart mass has been recently documented in another salmonid fish (*Salmo salar*) (43). ANB is produced by hypertrophic ventricular myocytes, and it exerts stabilizing or moderating effect on cardiac hypertrophy. ANB slightly attenuates hypertrophic growth of cardiac myocytes, and more importantly, it restrains the development of cardiac fibrosis by antagonizing the growth factor-induced proliferation of cardiac...
fibroblasts and their collagen production (14, 23, 42). In light of these data, the repression of collagen genes can be seen as a downstream effect of the enhanced ANB expression.

Upregulation of the muscle LIM protein gene was especially striking among the different cytoskeletal or myofibrillar proteins. MLP is a member of the LIM domain (double zinc finger) protein family, which is highly expressed in striated muscle cells during myogenic differentiation (4). In adult cardiac myocytes, MLP is localized at the Z disk, where it anchors the sarcomere to the sarcolemma by interacting with alpha-actinin and beta-spectrin (13). MLP has also been localized in the nucleus, and it seems to be intimately involved in hypertrophic growth of cardiac myocytes. In cultured cardiac myocytes, MLP alone can induce cellular hypertrophy and the production of atrial natriuretic peptide B (22, 53).

Although changes in myosin light chain expression are regularly noted in hypertrophic mammalian hearts, its significance in cardiac remodeling is not completely clear. Thermal plasticity of myosin ATPase activity in fish skeletal muscle is associated with changes in myosin heavy chain composition and/or with a change in the ratio of myosin light chain isoforms (24). In the trout heart, acclimation to cold increases the activity of myosin-ATPase (2), but its molecular basis is unknown. In analogy with fish skeletal muscle, the change in expression of the regulatory myosin light chain 2 might contribute to thermal compensation of myosin-ATPase activity and shortening velocity also in the trout cardiac muscle.

Taken together, the cold-induced hypertrophy of the rainbow trout heart shows some similarities with the hypertrophic growth of the mammalian heart. In the CA trout heart, the gene transcripts of the hypertrophy-inducing factor (MLP) and antifibrotic factor (ANB) are enhanced, while fibrosis promoting collagen genes are repressed. These findings suggest that transcripts that code for adaptive cardiac hypertrophy are activated, while transcripts that are known to promote cardiac fibrosis and can lead to cardiac failure in mammals (30) are repressed in the CA trout heart. Clearly, the hypertrophic trout heart might be a useful model to separate those factors that are responsible for compensatory hypertrophy and pathologic overgrowth of the vertebrate heart, respectively.

Protein synthesis. The most dramatic effect of thermal acclimation was directed on transcripts of cardiac protein synthesis. The remarkable overall upregulation of genes coding for ribosomal proteins, translation initiation, and translation elongation factors suggests that protein synthesis machinery of the fish heart is enhanced in the cold. Taking into account the cold-induced enlargement of the rainbow trout heart and the depression of protein synthesis rate by acute temperature drop (49), an increase in gene transcripts involved in protein synthesis is not surprising.

In mammalian heart, compensatory hypertrophy occurs first as a result of increased translational rates on existing ribosomes, that is, by increase in translational efficiency, and later by production of additional ribosomes for protein synthesis (i.e., by increase in translational capacity) (20). In the CA trout heart, gene transcripts for both translation initiation and elongation factors and for ribosome biosynthesis are upregulated, suggesting that both translation efficiency and translation ca-
Expression of genes coding for different chaperones suggests that the aerobic ATP production is not enhanced in CA trout heart by increasing the amount of enzyme transcripts. During translation, polypeptides are bound by different kinds of chaperones, which assist in correct folding, oligomerization, and compartmentalization of proteins and prevent the formation of nonspecific protein aggregates (11). Although clearly an exception within the general transcriptional pattern of this functional gene group, this is in accordance with the finding that acclimation to cold (5°C) does not induce positive thermal compensation in the rate of protein synthesis in mitochondria of the rainbow trout heart (49). Because all 13 peptide subunits synthesized in mitochondria are involved in the oxidative phosphorylation, the absence of thermal compensation suggest that the aerobic ATP production is not enhanced in CA trout heart by increasing the amount of enzyme transcripts. Mitochondrial proteins account for approximately one-third of the total protein content of the heart, and most of them are synthesized in cytoplasm. Only 13 polypeptides are encoded by mitochondrial genome and synthesized on ribosomes within the organelle itself (35). In the present study, only 1 of the 40 genes coding for different ribosomal proteins was downregulated by cold acclimation and that one was the mitochondrial 28S ribosomal protein, S34mt. Although clearly an exception within the general transcriptional pattern of this functional gene group, this is in accordance with the finding that acclimation to cold (5°C) does not induce positive thermal compensation in the rate of protein synthesis in mitochondria of the rainbow trout heart (49). Because all 13 peptide subunits synthesized in mitochondria are involved in the oxidative phosphorylation, the absence of thermal compensation suggest that the aerobic ATP production is not enhanced in CA trout heart by increasing the amount of enzyme transcripts.

Metabolism. Fish cardiac myocytes use both carbohydrates and fats for aerobic energy production, and increased activities of glycolytic and mitochondrial enzymes are found in response to cold acclimation in fish hearts (9). Activities of nonequilibrium enzymes like hexokinase, carnitine palmitoyltransferase, citrate synthase, and cytochrome oxidase are increased by cold acclimation in some fish species (9), while concentrations of near-equilibrium enzymes like lactate dehydrogenase, glyceraldehydes-3-phosphate dehydrogenase, pyruvate kinase correlate positively with decreasing habitat temperature of 15 different Fundulus species (32–34). Because both nonequilibrium and near-equilibrium reactions can be rate-limiting for ATP production in muscle cells (40), thermal compensation of ATP production can, in principle, occur at almost any step of the metabolic pathway.

In the present study, the two most strongly induced metabolic genes were glyceraldehyde-3-phosphate dehydrogenase and glycogen phosphorylase, suggesting cold-induced enhancement of aerobic glycolysis and improved use of glycogen stores in the CA trout heart. Increased transcript levels of cytosolic malate dehydrogenase are in line with this conclusion, because this enzyme is crucial for continuous glycolytic flux by regenerating NAD$^+$ from NADH. It has been recently shown that mammalian heart responds to an acute increase in energy demand by selective oxidation of glycogen (17). Preferential oxidation of glycogen in response to the acute increase in cardiac contractile activity, for example, under β-adrenergic stimulation, suggests that cardiac glycogen serves as a buffer for rapid changes in substrate demand. Glycolysis is also the preferential energy source for sarcolemmal Na$^+$-pump (15) and SR Ca$^{2+}$-pump (51). Because β-adrenergic responsiveness (26) and SR Ca$^{2+}$-uptake activity are enhanced in the heart of CA trout (1), the upregulation of transcripts of glycolytic and glycogenolytic enzymes might be needed to satisfy these demands.

Unlike aerobic skeletal muscle, increases in mitochondrial volume density are not evident in CA fish cardiac myocytes, even though the rate of oxygen consumption is increased (7, 36). This suggests that in fish heart, the mitochondrial population is quite adequate to satisfy ATP demand and does not require further expansion in cold acclimation. In the present study, genes for several mitochondrial markers including cytochrome c and two isoforms of the adenine nucleotide translocator (ANT) were downregulated. Cytochrome c is part of the respiratory chain, and ANT is located in the inner mitochondrial membrane, where it exchanges intramitochondrial ATP for cytosolic ADP. ANT is functionally coupled to mitochondrial creatine kinase, whereby the newly formed phosphoryl group of ATP is transferred to creatine and further to the myofibrils (44). This type of energy and metabolite shuttle is much more effective than diffusive transport of ATP, and ADP and is also present in the trout heart (6). Interestingly, also, the transcripts of mitochondrial creatine kinase were repressed in the CA trout. Reconciliation of these findings suggests that the whole sequence of events from ATP production in mitochondrial respiratory chain down to ANT and creatine kinase-dependent transport of ATP/ADP might be downregulated at the transcriptional level.

Taken together, temperature-related changes in gene transcripts of metabolic enzymes suggest that capacity for glycolytic energy production from carbohydrates might be increased, while mitochondrial function is not compensated for in the heart of CA rainbow trout. The apparently imbalanced effect of thermal acclimation on gene transcripts of cytosolic and mitochondrial metabolic machinery might be simply due to the possibility that the activities of membrane-bound mitochondrial enzymes are improved by homeoviscous adaptation without increases in enzyme concentration.

Limitations of the study. Although cDNA microarray analysis showed that a large number of genes are differentially regulated in CA and WA fish, it does not necessarily mean that exactly the same changes or changes to the same extent would appear at the proteome level. Furthermore, the increases in mRNA expression does not necessarily mean increased gene activity but might be contributed by increased mRNA stability in the cold (50). Second, this study did not examine transient changes in transcriptome, and the results represent only one time point in the myocardial adaptation to temperature changes. It is likely that at transition from one cardiac phenotype to another is associated with, at least partly, different transcriptome than the steady-state situation. Third, physiologically important changes in transcripts of excitation-contraction coupling sequence might have remained unnoticed, as genes for different ion channels, ion exchangers, and ion pumps were not sufficiently represented on the chip. Regardless of these...
limitations, the present study provides some interesting clues for future research, for example, on the relative significance of interstitial and myocyte components in the CA cardiac phenotype or the activation of hypertrophy markers genes and their significance to thermal acclimation of ectothermic hearts.

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