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https://doi.org/10.1242/jeb.091272
Acute heat tolerance of cardiac excitation in the brown trout (Salmo trutta fario)

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Short title: Upper thermal tolerance of brown trout heart

Key words: fish heart, heart rate, heat tolerance, electrocardiogram, ion currents

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Abstract
The upper thermal tolerance and mechanisms of heat-induced cardiac failure in the brown trout (Salmo trutta fario) was examined. The point above which ion channel function and sinoatrial contractility in vitro, and electrocardiogram (ECG) in vivo, started to fail (break point temperature, BPT) was determined by acute temperature increases. In general, electrical excitation of the heart was most sensitive to heat in the intact animal (ECG) and least sensitive in isolated cardiac myocytes (ion currents). BPTs of Ca$^{2+}$ and K$^+$ currents of cardiac myocytes were much higher (>28°C) than BPT of in vivo heart rate (23.5±0.6°C) (P<0.05). A striking exception among sarcolemmal ion conductances was the Na$^+$ current (I$_{Na}$), which was the most heat sensitive molecular function with a BPT of 20.9±0.5°C. The low heat tolerance of I$_{Na}$ was reflected as a low BPT for the rate of action potential upstroke in vitro (21.7±1.2°C) and the velocity of impulse transmission in vivo (21.9±2.2°C). These findings from different levels of biological organization strongly suggest that heat-dependent deterioration of Na$^+$ channel function disturbs normal spread of electrical excitation over the heart, leading to progressive variability of cardiac rhythmicity (missed beats, bursts of fast beating), reduction of heart rate and finally cessation of the normal heartbeat. Among the cardiac ion currents I$_{Na}$ is ‘the weakest link’ and possibly a limiting factor for upper thermal tolerance of electrical excitation in the brown trout heart. Heat sensitivity of I$_{Na}$ may result from functional requirements for very high flux rates and fast gating kinetics of the Na$^+$ channels, i.e. a trade-off between high catalytic activity and thermal stability.

Key words: high temperature tolerance, fish heart, action potential, ion currents, electrocardiogram
Introduction

All biological functions have strict thermal limits, making environmental temperature a decisive factor in geographical distribution of animal species (Precht et al., 1955). During >500 million years of evolution fishes have experienced changes in ambient temperature, which have resulted in adaptations to a wide variety of thermal niches. Depending on the extent of temperature specialization fishes can be classified as steno-, meso- and eurythermal having narrow, moderate and wide thermal tolerance range, respectively (Beitinger and Bennett, 2000). Fishes living in stenothermal environments are usually specialists and tolerate only a narrow range of temperatures, the most striking examples being those of the Southern Ocean (Verde et al., 2006). Many freshwater fishes of north-temperate latitudes experience large seasonal temperature changes and are therefore adapted to operate under a wider range of temperatures, which extends from freezing point up to ~40°C (Horoszewicz, 1973; Bennett and Beitinger, 1997; Ford and Beitinger, 2005). Practically all such fishes tolerate freezing winter waters, although the upper thermal tolerance limit varies considerably among species. Salmonid fishes (family Salmonidae) usually prefer cool habitats and show an upper thermal tolerance range of 22-28°C. For example, brown trout (Salmo trutta fario) which manage best in cool waters with high oxygen content, have an upper incipient lethal temperature of 22-25°C, and are therefore classified as mesothermal fish (Elliott and Elliott, 2010).

Although thermal dependences of various molecular and cellular processes are known, factors that set the ultimate thermal tolerance limits of ectotherms are still poorly understood. Evolutionary thermal adaptation is expressed in mitochondrial volume density, membrane lipid composition, metabolic enzyme kinetics, functions of membrane transporters and contractile proteins (Hazel and Williams, 1990; Somero, 1995; Johnston et al., 1998). Interestingly, the limits of thermal tolerance appear first at the level of intact animals, and only later in the function of tissues, cells and molecules (Lagerspetz, 1987). For example, heat tolerance of proteins and lipid membrane structure are often higher than the upper thermal tolerance of the whole organism (Cossins and Prosser, 1978; Hochachka and Somero, 1984; Somero, 1995). Although thermal disturbances first appear in higher level functions, they ultimately reflect temperature-related deterioration or suboptimal function of some cellular and molecular processes or mismatch of linked physiological processes.
Recent findings from both invertebrate and vertebrate ectotherms suggest that heart function could be a limiting factor for upper thermal tolerance of animals (Stillman and Somero, 1996; Seebacher et al., 2005; Farrell, 2009). Similarly, the hypothesis of an oxygen-limited thermal tolerance suggests that the circulatory system is one of the key factors in setting thermal tolerance limits of ectotherms (Frederich and Pörtner, 2000). Therefore, it would be interesting to know which molecular mechanisms might be limiting for the heat tolerance of ectothermic hearts.

Ion channels of fish cardiac myocytes are flexible entities that strongly respond to chronic temperature changes (Haverinen and Vornanen, 2004; Hassinen et al., 2007; Hassinen et al., 2008b; Haverinen and Vornanen, 2009), implying that they are intimately involved in thermal tolerance and temperature acclimation of cardiac function. Species-specific differences in channel composition and subunit assemblies may provide different thermal dependencies to electrical excitability of the fish heart. For example, the inward rectifier potassium current, $I_{K1}$, which is responsible for maintaining the negative resting membrane potential, is formed by different Kir2 channels in different species (Hassinen et al., 2007; Hassinen et al., 2008b). In cardiac myocytes of rainbow trout (Oncorhynchus mykiss) the $I_{K1}$ is produced by Kir2.1 and Kir2.2 channels, while in cardiac myocytes of crucian carp the same current is generated by Kir2.2 and Kir2.5 channels. The absence of Kir2.5 channels in trout is probably the reason why cold-acclimation decreases $I_{K1}$ in rainbow trout heart, while crucian carp myocytes show cold-induced increase of the $I_{K1}$ (Hassinen et al., 2007; Hassinen et al., 2008b).

Electrical excitation of the heart involves a range of molecular mechanisms that potentially could be responsible for thermal limitation of fish cardiac function. Therefore, the aim of the current study was to examine whether ion channel function of the brown trout heart is sensitive to temperatures close to the upper thermal tolerance limit of the fish, and therefore represent a limiting factor for electrical excitability of the heart. To this end acute thermal tolerances of ion currents of cardiac myocytes, contractility of spontaneously beating sinoatrial preparations and in vivo electrocardiogram were compared. Based on thermal sensitivity of cardiac function in other ectotherms, we hypothesized that electrical excitability of brown trout heart is vulnerable to temperatures that lead to heat death of the fish, and that this can be attributed to thermal deterioration or suboptimal function of one or more ion currents.
Materials and Methods

Animals

Experiments were conducted on cultivated brown trout (Salmo trutta fario) (113.3 ± 10.8 g, n=37) that were obtained from a local fish farm (Kontiolahti, Finland). In the animal house of the university the fish were maintained in 500 L metal aquaria at water temperature of 12 ± 1°C, and fed commercial food pellets (Ewos; Turku, Finland) 5 times per week. Photoperiod was a 12:12h light-dark cycle. All experiments were authorized by the national Animal Experimental Board in Finland (permissions STH998A and PH472A).

Recording of electrocardiograms

ECG recordings were made according as previously described (Campbell et al., 2004; Campbell et al., 2006). Trout were anaesthetized in tricaine methanesulfonate (MS-222, 0.3 mg L⁻¹, Sigma) and placed dorsal side up on an operating table and the gills irrigated with a tap water. Recording electrodes (7-strand Teflon coated wire, length 40 cm, diameter 0.23 mm; A-M Systems, Carlsborg, WA, USA) were hooked into the end of a 24 G hypodermic needle and obliquely inserted from the ventral surface at the level of pectoral fins forward close to the pericardium. The trailing wires were attached by a suture to the belly of the fish and by a second suture in the front of the dorsal fin. Whilst still docile the fish was placed into a respiratory chamber (1 L, initial O₂ content ~9 mg L⁻¹). Bipolar ECG signals were recorded using a bioamplifier (ML 136) interfaced with a digital recording system (PowerLab, ADInstruments, Oxford, UK). After implantation of electrodes the fish were allowed to recover from the operation for about 2 days before the thermal challenge. ECG recordings were started at the temperature of about 10°C and temperature was raised at the rate of ~1.5°C·h⁻¹ until disturbances appeared in the ECG, with uninterrupted recordings of ECG and temperature made throughout (LabChart, ADInstruments). The amplified signal was plotted in real time, and inter-beat intervals extracted from the raw trace, defined as the period between the R waves of successive heart beats.

Calculation of heart rate variability

An index of short term heart rate (HR) variability in the time domain was calculated as standard deviation of successive interbeat intervals, with normalized variability given as coefficient of variation, using manual identification of component from 20-
30 consecutive beats. The underlying physiological control of heart rate was examined using power spectral analysis to reveal the frequency of oscillatory components due to autonomic modulation of intrinsic cardiac pacemaker activity, after converting a selected ECG trace into a tachogram of 256 consecutive interbeat (R-R) intervals (Supplementary Fig. 2) (Campbell et al., 2004). A fast Fourier transformation was then applied using a Hanning window to minimize spectral leakage, and the resulting output plotted graphically. The ratio of low frequency to high frequency components of the spectra was calculated as an index of sympathovagal balance. Four fish gave sufficiently stable recordings across the whole temperature range.

Velocity of impulse conduction over the ventricle was determined from the width of the QRS complex at the zero voltage level. Q-T interval was used as a measure for the average duration of the ventricular action potential (AP).

Patch-clamp recordings

Atrial and ventricular myocytes were isolated with enzymatic digestion using the same solutions and enzymes as in our original method for fish hearts (Vornanen, 1997). In brief, 7 minute perfusion of the heart with Ca\(^{2+}\)-free saline was followed by a 15-minute perfusion with solution containing collagenase (Sigma Type IA 0.75 mg mL\(^{-1}\)), trypsin (Sigma Type III, 0.5 mg mL\(^{-1}\)) and fatty acid-free bovine serum albumin (Sigma, 1 mg mL\(^{-1}\)). A small aliquot of dissociated cells were placed in a 150 \(\mu\)L chamber (RC-26, Warner Instrument, USA) mounted on the stage of an inverted microscope (Nikon Eclipse 200). Cells were allowed to adhere to the bottom of the chamber and then superfused continuously with the external solution precooled to 4 ± 1°C. Voltage (ion currents, I) and current (action potentials, AP) clamp recordings were made using an Axopatch 1D amplifier (Axon Instruments) equipped with a CV-4 1/100 head-stage.

The external solution for AP and K\(^+\) current recordings contained (in mmol L\(^{-1}\)) 150 NaCl, 5.4 KCl, 1.5 MgSO\(_4\), 0.4 NaH\(_2\)PO\(_4\), 2.0 CaCl\(_2\), 10 glucose, and 10 HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) at pH 7.82 at 4°C. This solution, without any blockers, was used for AP recordings. For measurement of K\(^+\) currents 1 \(\mu\)M tetrodotoxin, 10 \(\mu\)M nifedipine and 30 \(\mu\)M glibeclamide were added to block Na\(^+\), Ca\(^{2+}\) and ATP-sensitive K\(^+\) currents, respectively. The pipette solution for AP recordings contained (in mmol L\(^{-1}\)) 140 KCl, 5 Na\(_2\)ATP, 1 EGTA (ethylene glycol
tetraacetic acid), and 10 HEPES at pH 7.2, and for K$^+$ currents 140 KCl, 4 MgATP, 1
MgCl$_2$, 5 EGTA, and 10 HEPES at pH 7.2. $I_{K1}$ was measured as a Ba$^{2+}$ (0.2 mM)
sensitive current and $I_{Kr}$ as an E-4031 (N-[4-[1-[2-(6-Methylpyridin-2-
yl)ethyl]piperidine-4-carbonyl]phenyl]) (2 µM) sensitive current. Temperature
sensitivity of the peak outward $I_{K1}$ was measured from currents elicited by 1-s voltage
ramp pulses. Temperature-dependence of $I_{Kr}$ was measured for the peak tail current at
-20 mV.

Na$^+$ current ($I_{Na}$) was measured in Cs$^+$-based, low-Na$^+$ saline solution which
contained (in mmol L$^{-1}$): 20 NaCl, 120 CsCl, 1 MgCl$_2$, 0.5 CaCl$_2$, 10 glucose and 10
HEPES (pH adjusted to 7.82 with CsOH). In addition, 10 mM nifedipine (Sigma) was
added to both solutions to block L-type Ca$^{2+}$ currents. The pipette solution contained
(mmol L$^{-1}$): 5 NaCl, 130 CsCl, 1 MgCl$_2$, 5 EGTA, 5 MgATP and 5 HEPES (pH
adjusted to 7.2 with CsOH). First, the myocytes were perfused with normal K$^+$-based
saline so that gigaseal and whole-cell patch clamp recording of the myocyte were
established. Internal perfusion of the myocytes with pipette solution was continued in
this solution for at least 3 min in order to allow buffering of intracellular Ca$^{2+}$ with
5 mmol L$^{-1}$ EGTA. Then, solution flow could be switched to a low-Na$^+$ external
solution without inducing contracture of the patched myocyte. To ensure adequate
voltage control a minimum of 80% series resistance compensation was applied. $I_{Na}$
was elicited from the holding potential of -120 mV (Haverinen and Vornanen, 2004).
Effect of temperature on $I_{Na}$ was measured at the pulse potential of -30 mV.

The composition of the physiological solution used for recording of Ca$^{2+}$ current
($I_{Ca}$) was as follows (in mmol L$^{-1}$): 130 NaCl, 5.4 CsCl, 1.5 MgSO$_4$, 0.4 NaH$_2$PO$_4$, 1.8
CaCl$_2$, 10 glucose and 10 HEPES (adjusted to pH 7.82 with CsOH). The pipette
solution contained (in mmol L$^{-1}$): 130 CsCl, 5 MgATP, 15 TEACl, 1 MgCl$_2$, 5
oxaloacetate, 5 EGTA, 0.03 Na$_2$GTP, and 10 HEPES (adjusted to pH 7.2 with CsOH).
The difference between the peak current and the current at the end of 300 ms
depolarizing pulse from the holding potential of -60 mV to +10 mV was taken as $I_{Ca}$ at
different temperatures.

All current and voltage recordings were started at 4°C and then temperature was
gradually raised at the rate of ~3°C min$^{-1}$ until ion current or membrane voltage
showed evident decline. The temperature after which they started to decline is termed
the break point temperature (BPT) and is regarded as the upper thermal tolerance limit
of the corresponding membrane function. Effects of temperature on ionic currents are
shown relative to the initial current density at 4°C, which was set as 1 (outward current) or -1 (inward current). Electrophysiological experiments were mainly performed on ventricular myocytes with the exception of the $I_{Kr}$ current that was measured from atrial cells. $I_{Kr}$ is much bigger in atrial than ventricular myocytes, while the opposite is true for $I_{K1}$ current. The dichotomy in the densities of $I_{Kr}$ and $I_{K1}$ currents between the two cell types enables easy and clean measurement of $I_{Kr}$ and $I_{K1}$ from atrial and ventricular myocytes, respectively.

Sinoatrial contractility

For measuring intrinsic HR, force and time-course of atrial contraction, sinoatrial preparations consisting of the sinus venosus and the whole atrium was dissected free and gently fixed from one atrial corner with insect pins on a Sylgard-coated 10 mL recording chamber filled with continuously oxygenated (100% $O_2$) physiological saline (in mmol L$^{-1}$): 150 NaCl, 3 KCl, 1.2 MgSO$_4$, 1.2 NaH$_2$PO$_4$, 1.8 CaCl$_2$, 10 HEPES and 10 glucose adjusted to pH 7.82 with NaOH at 4°C. From another corner the atrium was fixed via a small hook and a braided silk thread to the force transducer (Grass FT03) (Vormanen, 1979). The muscle was slightly stretched and allowed to spontaneously beat at the intrinsic HR. Force signals were digitized (Digidata-1340 AD/DA board Axon Instruments, Saratoga, CA, USA) with a sampling rate of 2 kHz before storing on the computer with the aid of Axotape (Axon) acquisition software. HR and contractile variables were analyzed with Clampfit software (Axon) and graphs were constructed in SigmaPlot. The preparation was allowed to equilibrate at 3°C for about 1 h to reach a stable beating rate before responses to rising temperature were determined. Temperature was gradually raised at the rate of ~1°C min$^{-1}$ until increasing HR clearly reversed direction or when the heartbeat ceased. BPT of HR contractile parameters was determined as in patch clamp experiments.

Statistics

Results are given as mean ± SEM. Statistically significant differences (P<0.05) among different variables, obtained by each research method (in vivo ECG, in vitro sinoatrial contractility, current clamp of single myocytes, voltage clamp of single myocytes) were assessed using one-way ANOVA after checking normality of distribution and making necessary transformation of variables. Paired comparisons between two means were done by Tukey’s HSD post hoc test.
Results

Temperature dependence of ECG

Acute temperature changes had a profound effect on brown trout’s ECG. From time-domain analysis, in vivo HR showed a curvilinear increase with temperature, and the R-R interval a linear decrease \((Q_{10}=2.17; -87.0 \pm 3.0 \text{ ms} \cdot ^\circ\text{C}^{-1}; \text{Fig. } 1)\). We used the R-T interval (instead of the Q-T) as an index of ventricular AP duration, due to the waveform of fish ECG preventing a consistent identification of Q wave position, which also showed a linear change with temperature \((Q_{10}=2.17; -29.2 \pm 0.9 \text{ ms} \cdot ^\circ\text{C}^{-1}; \text{Fig. } 1)\). Similar temperature dependences of R-R and R-T intervals suggest that systolic/diastolic duration remains fairly constant under acute temperature changes.

When approaching the upper thermal tolerance limit of the animal, irregularities appeared in cardiac rhythmicity and HR started to decline at BPT of 23.5 ± 0.6°C. Arrhythmicity first appeared as increasing heterogeneity of interbeat intervals and later on as bursts of repetitive activity (Supplementary Fig. 1). The break-up of normal cardiac rhythm was obvious in the parameters of HR power spectra (BPT=21.6 ± 5 °C) (Fig. 2). Power spectral analysis of a broader selection replicated the HR vs. temperature relationship, and showed a consistent thermal sensitivity among animals \((NN=-82.7 \pm 3.0 \text{ ms} \cdot ^\circ\text{C}^{-1}; \text{Supplementary Fig. } 2)\). HR variability followed a similar trend to that of NN vs. temperature \((-8.68 \pm 0.92 \text{ ms} \cdot ^\circ\text{C}^{-1})\) (Fig. 2A).

Coefficient of variation, a normalized measure for variability of interbeat intervals, showed a transient increase as animals approached their BPT (Fig. 2B, circled data), but thereafter declined (overall thermal sensitivity of -0.14 ± 0.09 ms·°C⁻¹). Although total spectral power varied among individual fish, there was a clear trend of an inverse relationship with temperature (Fig. 2C). The relative sympathovagal balance also varied among fish, with two showing a relative thermal insensitivity of low frequency/high frequency ratio and two showing a gradual rise until the BPT, after which all fishes showed a clear upward shift (Fig. 2D, the circled data points). With further increases in temperature spectral analysis became difficult due to electromyograph interference from respiratory muscles and unreliable due to heterogeneity in interbeat intervals. This was evident around the BPT, clearly seen as deviations in the Poincare plots and period histograms (Supplementary Fig. 1).

One obvious change in ECG was an abrupt increase in QRS complex duration at high temperatures. First, the duration of the QRS slightly reduced with rising
temperature up to the BPT of 21.9 ± 2.2 ºC, after which it strongly increased (Fig. 3). Duration of QRS complex is a measure for the velocity of impulse transmission over the ventricle and therefore broadening of QRS complex indicates depression in the rate of AP spread over the heart.

Atrial contractility in vitro

Spontaneously beating sinoatrial preparations were used to determine temperature tolerance of cardiac contractility (Fig. 4). The intrinsic HR increased linearly from 25 ± 5 beats min⁻¹ at 3°C to a maximum of 124 ± 6 beats min⁻¹ at 25°C (overall $Q_{10}=2.15$), then declined at higher temperatures and often completely ceased if temperature was not immediately lowered. The BPT for intrinsic HR was 25.8 ± 0.6°C (Table 1). The force of atrial contraction decreased linearly with rising temperature up to a similar BPT (25.6 ± 0.7°C) and then increased at higher temperatures, i.e. the temperature-related decline in force of contraction was partly due to the negative force-frequency relationship. Atrial pumping capacity (product of rate and force of contraction) doubled between 3°C and 25.7°C, and declined above the BPT (25.4 ± 0.4°C). Kinetics of contraction accelerated in curvilinear manner as a function of rising temperature up to the BPT, with little change or slight decline at higher temperatures.

Thus, for an acute temperature rise contractility of the sinoatrial tissue improves up to the BPT of ~25°C and declines at higher temperatures.

Membrane potentials

Temperature sensitivities of resting membrane potential (RMP) and action potential (AP) were measured from enzymatically isolated ventricular myocytes. RMP increased linearly upon warming from -60.6 ± 1.5 mV at 4°C to -89.1 ± 3.4 mV at ~33°C (Fig. 5), being then essentially equal to the theoretical equilibrium potential of K⁺ ions (-86.9 mV). The BPT of RMP was 29.6 ± 1.2°C. Amplitude of AP first increased with increasing temperature between 4°C and 20°C, and then leveled off between 20°C and the BPT of 26.4 ± 1.3°C. The duration of ventricular AP ($APD_{50}$) decreased with temperature in curvilinear manner from 776 ± 124 ms at 4°C to 36 ± 6 ms at 36°C. The shortening of AP was much stronger at low temperatures ($Q_{10}=3.20$ between 4°C and 19°C) in comparison to high temperatures ($Q_{10}=2.16$ between 19°C and 36°C).
BPTs for AP amplitude, RMP and APD$_{50}$ of the ventricular myocytes were somewhat higher (26.4 – 29.6°C) than BPTs for atrial contractile parameters suggesting that none of the three membrane potential parameters, are directly causative for malfunction of sinoatrial contractility. A clear exception was the upstroke velocity of ventricular AP, which showed a much lower BPT (21.7 ± 1.2°C) in comparison to any other parameter of ventricular AP or sino-atrial contractility (Fig. 5F). The thermal response curve of AP upstroke velocity had a shape of an inverted V with a peak at the BPT and minimum values at 4°C and 35°C.

Potassium currents

Outward K$^+$ currents are repolarizing, i.e. they promote shortening of APD. Similar to other teleosts the major cardiac K$^+$ currents of brown trout cardiac myocytes are the inward rectifier current (I$\text{K}_1$) and the rapid component of the delayed rectifier current (I$\text{Kr}$) (Vornanen et al., 2002) (Fig. 6). Temperature dependence of I$\text{Kr}$ was measured from atrial myocytes, where this current is larger in comparison to ventricular myocytes. Respectively, I$\text{K}_1$ was measured from ventricular myocytes where the I$\text{K}_1$ is much larger than in atrial myocytes. The tail current density of I$\text{Kr}$ increased 6.3-fold between 4°C and BPT of 27.3 ± 0.6°C ($Q_{10}$=2.22), while the outward I$\text{K}_1$ increased 2.22-fold between 4°C and 32.0°C ($Q_{10}$=1.33) (Fig. 6). Thermal tolerance of I$\text{K}_1$ was better than that of I$\text{Kr}$, without a clear BPT at temperatures below 32°C. Collectively, these results show that heat tolerances of both I$\text{Kr}$ and I$\text{K}_1$ are much higher than the upper thermal tolerances of sinoatrial contractile parameters or ECG in vivo.

Calcium current

Density and current-voltage relation of the nifedipine sensitive L-type I$\text{Ca}$ is similar as in rainbow trout (Oncorhynchus mykiss) (Fig. 7) (Vornanen, 1998; Shiels et al., 2000). The density of I$\text{Ca}$ increased with an overall $Q_{10}$ of 1.7 between 10°C and a BPT of 30.1 ± 0.5°C, above which I$\text{Ca}$ steeply declined. These findings indicate that I$\text{Ca}$ of the brown trout heart is fairly resistant to high temperatures.

Sodium current

I$_{Na}$ of the brown trout was similar to that of the rainbow trout in regard to voltage-dependence and current density (Fig. 7) (Haverinen and Vornanen, 2004). I$_{Na}$
increased with an average $Q_{10}$ of 2.3 between 4°C and a BPT of 20.9 ± 0.5°C, above which the current strongly decreased. The V-shaped temperature dependence curve of $I_{\text{Na}}$ is a mirror image to the inverted V-shape curve of AP upstroke velocity (Fig. 5F). $I_{\text{Na}}$ density at 35°C is only slightly higher than at 5°C. The BPT of $I_{\text{Na}}$ was significantly lower than the BPTs of $I_{\text{Kr}}$, $I_{\text{K1}}$ and $I_{\text{Ca}}$ ($P<0.05$), and was the most sensitive molecular function to high temperatures among the measured electrical or contractile parameters of the brown trout heart.

**Discussion**

To explain the thermal sensitivity of a particular organ function or an intact organism, temperature dependences of the underlying molecular processes need to be described. Ideally, it would be illustrative to compare the BPT of an organ function with BPTs of all those molecular entities that generate it. Electrical excitability of the heart (ECG, AP, HR) is a thoroughly characterized higher level function and its underlying molecular entities (ion channels/currents) are also well-known for fish cardiac myocytes (Vornanen, 1998; Haverinen and Vornanen, 2004; Hassinen et al., 2008b; Haverinen and Vornanen, 2009). Therefore, the fish heart offers a well-defined model system to test heat tolerance mechanisms of organ function in ectotherms.

**In vivo ECG**

In the present study, cardiac function was measured at different levels of biological organization ranging from the intact fish down to the molecular function of ion channels. Heat resistance of heart function was weakest in the intact animal; electrical activity of the brown trout heart in vivo started to deteriorate at a temperature around 21.6°C (HR variability) resulting in depression of HR above the BPT of 23.5°C. This is consistent with the findings from rainbow trout (Oncorhynchus mykiss) and Atlantic cod (Gadus morhua), where HR started to fall 2-4°C below the incipient lethal temperature (Heath and Hughes, 1973; Gollock et al., 2006). The first signs of disturbed cardiac function in the ECG of brown trout appeared as a progressive increase in HR variability slightly below the actual BPT for HR, indicating that the heart was losing control over the interbeat interval. Beat-by-beat variations in the ECG usually reflect efferent vagal (parasympathetic) activity. The lower in vivo HR in comparison to intrinsic in vitro HR suggests that the parasympathetic tone restrains HR in trout in vivo (Priede, 1974). On the other hand, the increase in low
frequency/high frequency ratio, and reduced total power with a increasing
temperature, are consistent with a relative increase in sympathetic drive contributing
to temperature-induced tachycardia (Wood et al., 1979). However, the relative
increase in sympathetic tone is at odds with suggestions that heat-related reductions in
HR represent vagal bradycardia, i.e. a parasympathetically controlled adaptive HR
response against hypoxia or cellular Ca^{2+} overload (Heath and Hughes, 1973; Rantin
et al., 1998). The increase in low frequency/high frequency ratio could be sooner seen
as a protective mechanism to maintain high HR and cardiac output, since isoprenaline,
a beta-adrenergic agonist, not only increases HR but also provides protection against
heat-dependent depression of HR in rainbow trout (Aho and Vornanen, 2001). It is
unlikely that the abrupt increase in sympathetic drive at elevated temperature is the
immediate cause of large HR variability, arrhythmicity of the heartbeat or depression
of HR.

Another large temperature-related change in the ECG of brown trout was the
widening of the QRS complex at high temperatures. This is indicative for heat-
dependent depression of the velocity of impulse conduction over the heart, which
could result in increases in HR variability and depression of HR. Experiments on
isolated cardiac myocytes provide support for this hypothesis.

Contractility of sinoatrial tissue

In vitro cardiac function, measured from spontaneously beating sinoatrial preparations
was slightly more resistant to high temperatures than in vivo ECG. BPTs for the
contractile parameters of the trout sinoatrial preparation centered around 25°C (range
24.3-25.8°C), probably due to interdependence of the measured contractile parameters.
Changes in beating frequency will affect force and time course of contraction
(?twitch? rise time, decay time, half-width) (Shiels et al., 2002), and therefore heat
sensitivity of HR is reflected in all parameters of atrial contractility. This indicates the
prime importance of HR in the temperature sensitivity of fish heart function. A
temperature-dependent increase in HR is able to compensate for the simultaneous
decrease in force development, but pumping capacity of the heart starts to decline
when heat depresses HR.

Temperature-dependence of cardiac action potential
As expected, increases in temperature were associated with decreases in AP duration (Talo and Tirri, 1991; Haverinen and Vornanen, 2009). This was evident both in vivo (R-T interval of ECG) and in vitro in isolated ventricular myocytes. There was no apparent limit to the shortening of AP in vitro, although reduction in APD got slower at higher temperatures. Because the duration of the AP plateau is determined by the balance between outward (K⁺) currents and inward (Ca²⁺ and Na⁺) currents, shortening of APD with temperature indicates that at high temperatures the ion current balance changes in favor of repolarizing K⁺ currents. This is necessary for cardiac function, because shortening of AP plateau makes room for diastolic filling of the heart when cardiac cycles are abbreviated. Indeed, in vivo ECG suggests that the duration of ventricular AP occupies a similar fraction of the total cardiac cycle at all temperatures.

A temperature dependent increase in K⁺ currents is also seen in hyperpolarization of RMP, which reaches its theoretical maximum at 29.6°C. Hyperpolarization of RMP has a stabilizing effect on electrical excitability, which may protect the heart against ectopic beats. However, at high temperatures the very negative RMP could make myocytes electrically unexcitable and completely prevent the heartbeat, in particular, if the inward I_{Na} current is simultaneously reduced (see Ion currents of cardiac myocytes).

In general, electrical activity of enzymatically isolated cardiac myocytes was more resistant to high temperatures than either in vivo ECG or in vitro contractility of sinoatrial preparations. Since ECG is produced by composite activity of all ion channels of cardiac myocytes, heat-related disturbances in ECG must be due to temperature-dependent failure of one or more ion channels or imbalance between depolarizing or repolarizing ion currents due to their different temperature dependencies. The failure could occur either in the pacemaker cells that initiate the heartbeat or alternatively in the working atrial and ventricular myocytes or in the conducting pathway between the atrium and the ventricle.

Major ion current systems of the vertebrate heart are largely the same in ectothermic and endothermic vertebrates, although isoforms of channel proteins and molecular assemblies of multi-protein channel complexes may differ significantly. I_{Na} and I_{Ca} are the major inward currents, while I_{K1}, I_{Kr} and I_{Ks} are the main outward
currents of fish cardiac myocytes (Vornanen, 1998; Haverinen and Vornanen, 2004; Hassinen et al., 2007; Hassinen et al., 2008a; Hassinen et al., 2011). In mammals these currents operate optimally at the body temperature of 36-39°C. In most fishes body temperature is less than in endotherms, and importantly, it is often highly variable. Consistent with this fact, ion currents of the brown trout cardiac myocytes (perhaps with the exception of $I_{K1}$) started to decline much below the body temperature of endothermic vertebrates. The lower thermal tolerance of brown trout ion currents/channels in comparison to those of endotherms is probably a trade-off between catalytic activity and thermal stability as a consequence of molecular adaptation to optimise function at the lower body temperatures of ectothermic fishes. Catalytic activity and thermal stability are mutually exclusive properties of a protein molecule, in that high catalytic activity at low temperature means low thermal stability at higher temperatures (Zavodszky et al., 1998; Fields, 2001). Whether the relatively low heat tolerance is purely a property of ion channel proteins or is also dependent on the lipid environment of channels remains to be shown. Large variability in heat tolerance between different ion currents (see below) suggests that thermal properties of the bulk lipid membrane are not decisive. However, the contribution of the immediate lipid environment of the ion channel, the lipid annulus, cannot be excluded (Zheng et al., 2011).

The four ion currents of the brown trout heart measured in this study showed different heat resistances. The most resilient to high temperatures was $I_{K1}$ followed by $I_{Ca}$ and $I_{Kr}$. $I_{Na}$ had clearly the lowest threshold for temperature-dependent deterioration with a 6.4°C lower BPT than the next heat sensitive current, the $I_{Kr}$. Opening and closing of ion channels requires conformational changes in the ion channel proteins known as ‘gating’ between conducting and non-conducting states (Bezanilla, 2005). Similar to enzyme reactions, gating of ion channels is strongly dependent on temperature (Collins and Rojas, 1982). Considering the implicit kinetic compromise, the high sensitivity of $I_{Na}$ for thermal inactivation may reside in its high catalytic activity. The kinetics of sodium channel gating is very fast, resulting in an almost instantaneous opening of the channels upon small membrane depolarization, followed by large Na$^+$ influx and rapid inactivation during a maintained depolarization (Patlak, 1991). The high catalytic activity of Na$^+$ channels probably requires high molecular flexibility, which may come with the trade-off of low thermal stability.
The BPTs of $I_{Na}$ density and upstroke velocity of AP are in excellent agreement, differing only 0.8°C. Furthermore, the BPT of the velocity of impulse conduction in vivo also shows low heat tolerance. Since the density of $I_{Na}$ is the main determinant for the velocity of impulse conduction, heat inactivation of $I_{Na}$ is expected to compromise the rate of AP propagation over the heart. This is keeping with the mammalian models, where the loss of cardiac Na$^+$ channel function is associated with slowing of sinoatrial conduction and frequent sinoatrial or atrioventricular conduction blocks (Derangeon et al., 2012). Therefore, the large increases in HR variability, missed beats and depression of HR in brown trout at high temperatures could be due to slowed or impaired AP conduction between cardiac compartments rather than caused by heat inactivation of the impulse generation in the pacemaker center. Indeed, the slightly better heat tolerance of the sinoatrial tissue in vitro suggests that conductive pathways and the ventricle are more sensitive to heat than the pacemaker tissue. Examination of temperature modulation of isolated pacemaker cells is needed to exclude putative contribution of direct heat inactivation of the pacemaker mechanism to thermal deterioration of cardiac contractility.

The threshold potential is the critical level to which the membrane potential must be depolarized in order to initiate an AP, i.e. at that voltage the density of the inward $I_{Na}$ exceeds the total density of the outward K$^+$ currents (mainly the $I_{K1}$). Therefore, large increases in repolarizing K$^+$ currents or decrease in $I_{Na}$ can result in AP failure (Huxley, 1959; Guttmann, 1962; Golod et al., 1998; Rosenthal and Bezanilla, 2002). Under a rising temperature regime, a cardiac myocyte is expected to become electrically unexcitable, if the Q10 value of the repolarizing currents is greater than that of the depolarizing currents, i.e. if the total density of K$^+$ currents increases faster than the density of $I_{Na}$. In this respect it is notable that in brown trout myocytes $I_{Na}$ started to decline above 20.9°C, while $I_{Kr}$ and $I_{K1}$ still continue to increase with temperature. With increasing temperature RMP becomes increasingly negative, while depolarizing power of $I_{Na}$ decreases; the resultant imbalance of repolarizing and depolarizing currents prevents reaching the threshold potential, i.e. AP fails (Fig. 8). This may be the reason why heartbeat in brown trout completely ceases at temperatures slightly above 25°C.

This study shows that function of the cardiac $I_{Na}$ is compromised at high temperatures, which might be a contributing factor to heat-dependent depression of cardiac contractility in the brown trout. However, Na$^+$ channels are not restricted to
cardiac myocytes, but occur in many other cell types, in particular in neurons and muscle cells. At least 8 alpha subunits of voltage-gated Na\textsuperscript{+} channels exist in teleost fishes with different tissue distribution (Widmark et al., 2011). Therefore, it is possible that similar thermal sensitivity of I\textsubscript{Na} as shown here for the trout cardiac myocytes, might also compromise Na\textsuperscript{+} channel function in nervous system and skeletal muscles with possibly dangerous consequences to behavior, locomotion and other vital functions. The trout cardiac I\textsubscript{Na} is mainly produced by the Na\textsubscript{V}1.4a subunits (Haverinen et al., 2007), and it remains to be shown whether other Na\textsuperscript{+} channel isoforms show similar thermal sensitivity as the cardiac isoform. An interesting topic for further research is the molecular mechanism, which makes the fish cardiac Na\textsuperscript{+} channels sensitive to heat inactivation, i.e. which amino acid sequences or ion channels domains are involved in heat-sensitivity of the ectothermic Na\textsuperscript{+} channels. The significance of lipid annulus around the channel protein needs to be also clarified.

Conclusions

The present findings show that ECG in vivo and contractility of sino-atrial preparation in vitro are in general more sensitive to temperature-dependent deterioration than ion channel function in single isolated myocytes. These findings are in agreement with the generalization that body functions at higher level of biological organization (physiology) are more thermally sensitive than are most cellular and molecular mechanisms upon which the higher level functions are based on (Lagerspetz, 1987). In the brown trout heart, a clear exception to this rule is the upper thermal tolerance of the I\textsubscript{Na} which shows a lower BPT than the higher level functions, HR in vivo and HR in vitro.

Cardiac output in fishes increases with increasing temperature mainly via elevations in HR and with little or no changes in stroke volume (Gollock et al., 2006; Steinhausen et al., 2008; Mendonca and Gamperl, 2009). The current study suggests that the upper thermal tolerance of cardiac function in the brown trout might be set by heat sensitivity of the cardiac Na\textsuperscript{+} channels, because I\textsubscript{Na} is thermally the weakest link in electrical excitation of the brown trout cardiac myocyte. Above 20.9°C impaired Na\textsuperscript{+} channel function results in depression of I\textsubscript{Na} density, AP upstroke velocity and conduction spread over the heart with consequent disturbance in cardiac rhythmicity and depression of HR. With further increases in temperature electrical excitability may be completely lost, because increasing amplitude of the I\textsubscript{K1}
overwhelms the decreasing $I_{Na}$ so that the threshold voltage for the regenerative opening of Na$^+$ channels is not achieved with an outcome of cardiac standstill.

**Funding** This study was supported by a grant from The Academy of Finland (No. 14795) to M.V.

**Acknowledgements.** Anita Kervinen is acknowledged for skillful technical assistance.
Table 1. Break point temperatures (BPT) for different variables of in vivo ECG, sinoatrial contractility and electrical excitability of atrial or ventricular myocytes of the brown trout heart.

<table>
<thead>
<tr>
<th>Variable</th>
<th>BPT (°C)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate in vivo</td>
<td>23.5 ± 0.6</td>
<td>5</td>
</tr>
<tr>
<td>QRS duration</td>
<td>21.9 ± 2.2</td>
<td>4</td>
</tr>
<tr>
<td>Heart rate in vitro</td>
<td>25.8 ± 0.6</td>
<td>7</td>
</tr>
<tr>
<td>Force of atrial contraction</td>
<td>25.6 ± 0.7</td>
<td>7</td>
</tr>
<tr>
<td>Atrial pumping capacity</td>
<td>25.4 ± 0.4</td>
<td>7</td>
</tr>
<tr>
<td>Half-width of atrial contraction</td>
<td>24.9 ± 0.4</td>
<td>7</td>
</tr>
<tr>
<td>Rise time of atrial contraction</td>
<td>24.6 ± 0.6</td>
<td>7</td>
</tr>
<tr>
<td>Decay time of atrial contraction</td>
<td>24.3 ± 0.8</td>
<td>7</td>
</tr>
<tr>
<td>Resting membrane potential (ventricle)</td>
<td>29.6 ± 1.2^a</td>
<td>10</td>
</tr>
<tr>
<td>Action potential amplitude (ventricle)</td>
<td>26.4 ± 1.3^b</td>
<td>10</td>
</tr>
<tr>
<td>Action potential duration (ventricle)</td>
<td>31.5 ± 1.3^a</td>
<td>10</td>
</tr>
<tr>
<td>Action potential upstroke velocity (ventricle)</td>
<td>21.7 ± 1.2^c</td>
<td>10</td>
</tr>
<tr>
<td>Current density of I_{Kr} (atrium)</td>
<td>27.3 ± 0.6^b</td>
<td>11</td>
</tr>
<tr>
<td>Current density of I_{K1} (ventricle)</td>
<td>&gt;32^c</td>
<td>11</td>
</tr>
<tr>
<td>Current density of I_{Ca} (ventricle)</td>
<td>30.1 ± 0.5^c</td>
<td>11</td>
</tr>
<tr>
<td>Current density of I_{Na} (ventricle)</td>
<td>20.9 ± 0.5^a</td>
<td>15</td>
</tr>
</tbody>
</table>

The results are means ± SEM of 4-5 fishes, 7 sinoatrial preparations or 10-15 cardiac myocytes as indicated. The lines separate variables that were statistically evaluated as a single group by ANOVA. Different letters show statistically significant differences (P<0.05) between two variables.
Figure captions

Figure 1. Time domain analysis of the in vivo ECG. Individual cardiac cycle elements are plotted against defined temperature records (n=4, least squares regression, ± 95% confidence intervals). (A) Heart rate changed in curvilinear manner with temperature. (B) Thermal sensitivity of R-R interval (middle) and (C) R-T interval (bottom) changed linearly with temperature, both with a similar Q_{10} value of 2.17.

Figure 2. Power spectral analysis of the in vivo ECG. In power spectral analysis, it is usual to define cycle durations with respect to normal values, hence R-R is typically denoted NN. (A) Standard deviation of interbeat intervals (SDNN) predictably varies in a similar manner to NN, i.e. with shortening of interbeat interval also the variability of interbeat interval decreases. (B) Coefficient of variation (CVNN), i.e. normalized variability of interbeat interval, shows a much more gradual decline with increasing temperature with the exception of the period prior to substantial changes in the spectral data (circled). (C) Total power is quite heterogeneous among individual fish, reflected in differences in low frequency/high frequency (LF/HF) ratio, but (D) consistently showed an upward shift between 21-23°C (circled). Solid lines show linear regression to the data and dashed lines show 95% confidence limits.

Figure 3. Effect of acute temperature increase on the duration of QRS complex in brown trout ECG. (A) Mean values (± s.e.m.) from 4 fishes. (C) and (D) representative recordings of QRS complex at 10°C and 24°C, respectively.

Figure 4. Effects of acute temperature increases on the contractility of sinoatrial preparations from brown trout in vitro. (A) A slow time-base recording of atrial contraction showing disruption due to rising temperature (26.6°C at the time of recording) on beating rhythm and consequent effects on force of contraction (right). (B) The contractile parameters that were measured at each temperature; F, force of contraction; HW, half-width of contraction; RT, rise time; DT, decay time. Temperature dependences of those variables are shown in (C) heart rate, (D) force of contraction, (E) pumping capacity of atrial muscle, (F) half-width, (G) rise time and (H) decay time of atrial contraction. The results are mean ± s.e.m. of 7 preparations.
Figure 5. Effects of acute temperature increases on ventricular action potential of the brown trout in vitro. (A) A representative recording showing the effects of acute increase in temperature on the shape of ventricular action potential. (B) Three APs at selected temperatures from the same myocyte. Mean values for temperature dependence of (C) resting membrane potential, (D) action potential amplitude, (E) action potential duration at 50% repolarization level and (F) upstroke velocity of the action potential. The results are mean ± s.e.m. from 10 myocytes.

Figure 6. Effects of acute temperature increases on the repolarizing $K^+$ currents, the rapid component of the delayed rectifier $K^+$ current ($I_{Kr}$) and the inward rectifier $K^+$ current ($I_{K1}$) in brown trout atrial and ventricular myocytes, respectively. (A and B) Voltage protocols and representative recordings of $I_{Kr}$ and $I_{K1}$ at 12°C, respectively. An arrow indicates the time point of measurement. (C and D) Effects of temperature on the peak tail current of $I_{Kr}$ at -20 mV and the peak inward current of $I_{K1}$ at -120 mV (bottom) normalized to the current densities at 4°C, respectively. The results are mean ± s.e.m from 11-15 myocytes.

Figure 7. Effects of acute temperature increases on inward $Na^+$ and $Ca^{2+}$ currents of the brown trout ventricular myocytes. (A and B) Current-voltage dependencies of $I_{Ca}$ and $I_{Na}$ at 12°C, respectively. Voltage protocols and representative current recordings are also shown. (C and D) Temperature dependencies of the peak $I_{Ca}$ and $I_{Na}$ normalized to the current densities at 4°C, respectively. The results are mean ± s.e.m. from 11-15 myocytes.

Figure 8. A scheme showing two ionic mechanisms, which are assumed to be closely involved in heat-dependent deterioration of brown trout cardiac excitability. With increasing temperature: (1) the inward rectifier $K^+$ current, $I_{K1}$, increases making the resting membrane potential more negative and the voltage threshold for action potential initiation larger, (2) the $N^+$ current, $I_{Na}$, declines (break point temperature = 20.9°C). These changes have two consequences, first the velocity of impulse conduction slows down and with further increases in temperature $I_{Na}$ becomes too small to cross the threshold voltage, i.e. action potential fails (this does not happen under the current clamp conditions in isolated myocytes, when stimulus strength is
high enough). Red and blue lines show current and voltage at high and low temperature, respectively.
Figure 1

- Heart Rate vs. Temperature
  - $R^2 = 0.949$

- R-R Interval vs. Temperature
  - $R^2 = 0.980$

- R-R Interval vs. Temperature
  - $R^2 = 0.982$
Figure 2
Figure 3
Figure 4

A

Temperature 26.6°C

0.5 mN mg⁻¹

2 s

B

C

Heart rate (beats min⁻¹)

Force (mN mg⁻¹)

Pumping capacity (mN mg⁻¹ min⁻¹)

Leaf width (mm)

Rise time (ms)

Decay time (ms)

Temperature

Temperature

Temperature

Temperature

Temperature

Temperature
Figure 5
Figure 8
References


Rosenthal, J. J. C. and Bezanilla, F. (2002). A comparison of propagated action potentials from tropical and temperate squid axons: different durations and


**Supplementary material**

**Supplementary Figure 1.** Graphical representation of HR variability, representative views at three different temperatures. First column, Poincare plots. The central point represents the mean NN interval, plotting each interbeat interval against the subsequent interbeat interval. The ellipse represents the standard deviation of interbeat intervals perpendicular (SD1, short term HR variability) and parallel (SD2, long term HR variability) to the line of identity. Boundaries are set to identify ectopic beats and data outliers (artefacts). As the BPT is approached, the dispersion widens and eventually separates into clusters representing repetitive rhythm sequences. Middle column, this can be seen in the period histogram, where a gradual rightward expansion leads to a bimodal distribution. Right column, representative ECG traces showing: top, regular rhythmicity of rested trout at low temperature (in this case with a prominent T-wave); middle, appearance of a range of interbeat interval durations; bottom, establishment of a different form of rhythmicity, in this case a long beat followed by three shorter beats.
**Supplementary Figure 2.** Frequency domain analysis. Individual cardiac cycle elements plotted against defined temperature records (n=4, least squares regression, ±95% confidence intervals) from tachograms of 256 consecutive cycles.