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RESEARCH ARTICLE

Reduced ventricular excitability causes atrioventricular block and depression of heart rate in fish at critically high temperatures

Jaakko Haverinen* and Matti Vornanen

ABSTRACT

At critically high temperature, cardiac output in fish collapses as a result of depression of heart rate (bradycardia). However, the cause of bradycardia remains unresolved. To investigate this, rainbow trout (*Oncorhynchus mykiss*; acclimated at 12°C) were exposed to acute warming while electrocardiograms were recorded. From 12°C to 25.3°C, electrical excitation between different parts of the heart was coordinated, but above 25.3°C, atrial and ventricular beating rates became partly dissociated because of 2:1 atrioventricular (AV) block. With further warming, atrial rate increased to a peak value of 188±22 beats min^{-1} at 27°C, whereas the ventricle rate peaked at 124±10 beats min^{-1} at 25.3°C and thereafter dropped to 111±15 beats min^{-1} at 27°C. In single ventricular myocytes, warming from 12°C to 25°C attenuated electrical excitability as evidenced by increases in rheobase current and the size of critical depolarization required to trigger action potential. Depression of excitability was caused by temperature-induced decrease in input resistance (sarcolemmal K^+ leak via the outward I_{K1} current) of resting myocytes and decrease in inward charge transfer by the Na^+ current (I_{Na}) of active myocytes. Collectively, these findings show that at critically high temperatures AV block causes ventricular bradycardia owing to the increased excitation threshold of the ventricle, which is due to changes in the passive (resting ion leak) and active (inward charge movement) electrical properties of ventricular myocytes. The sequence of events from the level of ion channels to cardiac function *in vivo* provides a mechanistic explanation for the depression of cardiac output in fish at critically high temperature.

KEY WORDS: Fish heart, Electrocardiogram, Cardiac arrhythmia, Sodium current, Chronaxie, Rheobase

INTRODUCTION

Physical performance of fish is largely dependent on the oxygen supply to the tissues, which is determined by the quantity of blood delivered to them. Cardiac output is the volume of blood circulated through the body in a unit of time. It is the product of heart rate (f_H), or the number of heart beats per minute, and stroke volume, or the volume of blood pumped by the ventricle in one contraction. In fish, acute warming initially increases cardiac output, but at temperatures slightly below the critical thermal maximum of the animal, cardiac output first plateaus and then sharply collapses (Stevens et al., 1972; Mendonca and Gamperl, 2010; Sandblom et al., 2016; Eliason and Anttila, 2017; Ekström et al., 2019). The temperature-induced collapse of cardiac output is caused by depression of f_H (bradycardia), since stroke volume is either maintained or only slightly increased by acute warming (Randall, 1968; Gollock et al., 2006; Sandblom and Axelsson, 2007; Steinhausen et al., 2008; Clark et al., 2008; Mendonca and Gamperl, 2010; Ekström et al., 2014, 2016, 2019; Penney et al., 2014; Motyka et al., 2016). Despite several studies on this topic, the mechanism of the temperature-induced bradycardia remains unknown. Several speculations about the mechanistic basis of the temperature induced depression of f_H have been suggested but thus far no definitive experimentally verified explanation has been provided. Since the mechanism of bradycardia remains unknown, there is also uncertainty about its physiological role: is it a useful (adaptive) or harmful (non-adaptive) trait?

Rate and rhythm of the heart beat is determined in the sinoatrial pacemaker and therefore an obvious candidate for bradycardia is the direct temperature-dependent slowing of the primary pacemaker rate (Haverinen and Vornanen, 2007). On the other hand, temperature-induced bradycardia may in principle be analogous to hypoxic bradycardia, which improves oxygenation of the myocardium (Farrell, 2007). In the latter scenario, high temperature results in hypoxemia and activation of oxygen-sensitive chemoreceptors, which trigger a controlled (adaptive) bradycardia via the cholinergic system (Hughes and Roberts, 1970; Rantin et al., 1998; Gollock et al., 2006; Ekström et al., 2016) or, alternatively, the effect may be mediated by thermal receptors (Hughes and Roberts, 1970). The possibility that temperature-induced elevation of external K^+ concentration (hyperkalemia) suppresses the pacemaker rate has also been raised (Heath and Hughes, 1973). One final putative explanation is that an acute rise in temperature changes the fluidity of the lipid membrane and impairs Na^+, Ca^{2+} and K^+ ion passage through the sarcolemma (Lennard and Huddart, 1991).

The above alternatives, with the exception of the last one, suggest that the bradycardia is regulated at the level of the sinus venosus or the atrium. However, *in vitro* force recordings from fish hearts from almost 90 years ago show that during acute warming the ventricle stops working first, then the atrium arrests, whereas the sinoatrial pacemaker is able to withstand high temperatures better (Markowsky, 1933; Koehnlein, 1933; von Skramlik, 1935). However, it has not been directly tested whether the temperature-induced bradycardia is due to: (1) the suppression of the supraventricular tissues (sinoatrial pacemaker, atrium) or (2) failure of the ventricular excitation. We can resolve this by locating the site of bradycardia among the several specialized compartments of the fish heart. If the hypothesis 1 is true, then the P wave (atrial depolarization) and QRS complex (ventricular depolarization) should always be coupled, and atrial and ventricular beats should slow down in the same rhythm. Conversely, if hypothesis 2 is correct, the P wave and QRS complex should occur partly independently of one another and beating rate of the ventricle should be lower than that of the atrium. Therefore, the first aim of the present study was to test the two alternative hypotheses of the temperature-induced bradycardia by *in vivo* ECG recordings in rainbow trout (*Oncorhynchus mykiss*). A careful examination of

University of Eastern Finland, Department of Environmental and Biological Sciences, 80101 Joensuu, Finland.

*Author for correspondence (jaakko.haverinen@uef.fi)

J.H., 0000-0002-2300-6849; M.V., 0000-0003-0953-1425

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List of symbols and abbreviations

AP	action potential
APD_{50}	action potential duration at 50% of repolarization
CD	critical depolarization
C_m	membrane capacitance
ECG	electrocardiogram
EE	electrical excitability
f_H	heart rate
f_{HA}	beating rate of atrium
f_{HV}	beating rate of ventricle
I_{th}	threshold current
R_{in}	input resistance
T_{BP}	breakpoint temperature
V_{th}	threshold potential
V_{rest}	resting membrane potential

ECGs showed that the P wave and QRS complex became dissociated at critically high temperatures, thus supporting the latter hypothesis. Thereafter, the second aim of the study was to seek a mechanistic explanation for the dissociation of atrial and ventricular depolarizations. The previous findings from brown trout (*Salmo trutta*) and roach (*Rutilus rutilus*) ventricular myocytes have shown that temperature dependencies of the Na^+ inward current (I_{Na}) and the inward rectifier K^+ current (I_{K1}) – two antagonistic ion currents critical for initiation of cardiac AP (Varghese, 2016) – are widely different. I_{Na} is depressed at relatively low temperatures, while I_{K1} is heat resistant and increases almost linearly with rising temperature (Vornanen et al., 2014; Badr et al., 2017). The temperature-induced mismatch between I_{Na} and I_{K1} is considered to result in temperature-dependent depression of electrical excitation (TDEE) (Vornanen, 2016). If the failure of the fish ventricle is due to a temperature-induced mismatch between depolarizing I_{Na} and repolarizing I_{K1}, then the inward charge required to trigger AP should exceed the charge provided by I_{Na}. To test this hypothesis, we measured the temperature dependence of inward charge transfer by I_{Na} and temperature dependence of the charge demand of the quiescent ventricular myocytes.

MATERIALS AND METHODS

Animals

Rainbow trout [*Oncorhynchus mykiss* (Walbaum 1792)] 5.6±17.8 g in body mass (mean±s.e.m., n=37), were bought from the local fish farm (Kontiolahti, Finland). In the animal facilities of the university, the fish were maintained in 500 litre metal aquaria for a minimum of 3 weeks before the experiments. Water temperature was regulated at 12±0.5°C (Computec Technologies, Joensuu, Finland) and full oxygen saturation was maintained by aeration with compressed air. Ground water was constantly flowing through the aquaria at the rate of 150–200 litres per day. Trout were fed 5 times per week *ad libitum* with trout feed (EWOS, Turku, Finland). The experiments were authorized by the national animal experimental board in Finland (permission ESAVI/8877/2019).

Recording of electrocardiogram (ECG)

ECG recordings were made using the previously described methods (Vornanen et al., 2014; Badr et al., 2016). Anaesthetized trout (neutralized tricaine methane sulfonate, 0.3 mg l^{-1}, Sigma, St Louis, MO, USA) were placed ventral side up on an operating table and the gills were irrigated with circulating tap water. Two thin stainless-steel electrodes (diameter 0.23 mm; A-M Systems, Carlsborg, WA, USA) were obliquely inserted from the ventral surface close to the pericardium. The electrode wires were fixed on the ventral side of the fish by two sutures. The fish was placed into a cylindrical Perplex chamber (length 29 cm, diameter 9 cm, volume 1.84 litres) with open ends. The chamber and the reference electrode were immersed in a large (250 litre) temperature-regulated (12±0.5°C) stainless steel aquarium with constant aeration of the water. The fish were allowed to recover from the operation for 1–2 days. The recovery was considered complete when a clear and steady f_H variability appeared in the ECG. Temperature dependence of the ECG was studied using a procedure where water temperature was warmed at the constant rate of 3°C h^{-1} (Computec Technologies, Joensuu, Finland) starting from the acclimation temperature of the fish (12°C). The experiment was discontinued when ventricular beating rate began to steadily decline. Immediately after the experiment the fish was killed by a strong blow to the head and pithing. During the experiment ECG and water temperature were continuously recorded via bioamplifiers (ML 136, ADInstruments, Colorado Springs, CO, USA) and the digital recording system (PowerLab, ADInstruments) on the computer. In ECG recordings, the P wave, the QRS complex and T wave correspond to atrial depolarization, ventricular depolarization and ventricular repolarization, respectively. The following parameters were measured from these waveforms: PQ (PR) interval (the time taken by impulse conduction from the beginning of atrial contraction to the beginning (peak) of ventricular contraction), QRS duration (the time taken by impulse transmission through the ventricle) and QT interval (the average duration of ventricular AP) (Vornanen et al., 2014; Badr et al., 2016). f_H was calculated separately for the ventricle (f_{HV}) and the atrium (f_{HA}) from the number of QRS complexes and P waves, respectively. The break point temperature (T_{BP}) is the highest temperature above which there is a sustained decrease in f_{HV}.

Patch–clamp methods

Ventricular myocytes were enzymatically isolated with the established method for fish hearts (Vornanen, 1997; Vornanen et al., 2002). Cell isolation was conducted at room temperature (21–22°C) and the isolated myocytes were stored at 5°C. Fresh cells were prepared on each experimental day.

The whole-cell current-clamp and voltage-clamp measurements were made using the standard methods and equipment of the whole-cell patch–clamp as reported previously in detail (Vornanen, 1997; Badr et al., 2018; Abramochkin et al., 2019). For recording of APs or ion currents, a small aliquot of cell suspension was placed into a chamber which received a constant flow of temperature-controlled external saline solution (see below). After establishing a gigaohm seal and gaining electrical access to the cell, transients due to series resistance (4–8 MΩ) and pipette capacitance were cancelled, and the capacitive size of ventricular myocytes was determined. APs and I_{Na} tracings were digitized at 10 kHz and low pass filtered at 5 kHz. Off-line analysis of the recordings was done using the Clampfit 10.4 (Molecular Devices, Saratoga, CA, USA) software package. The external solution used for AP recordings contained (mmol l^{-1}): 150 NaCl, 3 KCl, 1.2 $MgCl_2$, 1.8 $CaCl_2$, 10 Hepes, 10 glucose, and pH adjusted with NaOH to 7.7 at 20°C. The composition of the internal saline solution was as follows (mmol l^{-1}): 140 KCl, 5 Na_2ATP, 1 $MgCl_2$, 0.03 Tris-GTP, 10 Hepes (pH adjusted with KOH to 7.2 at 20°C) (Badr et al., 2018; Abramochkin et al., 2019). The same solutions were used for recording of the inward rectifier K^+ current, I_{K1}.The external saline solution for I_{Na} recordings was composed of (mmol l^{-1}): 20 NaCl, 120 CsCl, 1 $MgCl_2$, 0.5 $CaCl_2$, 10 glucose and 10 Hepes with pH adjusted to 7.7 with CsOH at 20°C. Nifedipine (10 µmol l^{-1}, Sigma) was included to block I_{Ca}. The pipette solution contained (in mmol l^{-1}) 5 NaCl, 130 CsCl, 1 $MgCl_2$, 5 EGTA, 5 Mg_2ATP and 5 Hepes (pH adjusted to 7.2 with CsOH at 20°C) (Haverinen and Vornanen, 2006).

The following AP parameters were analysed off-line: resting membrane potential (V_{rest}, mV), threshold potential for AP initiation (V_{th}, mV), threshold current (I_{th}, pA), critical depolarization (CD=V_{th}–V_{rest}, mV), AP overshoot (mV), AP amplitude (mV), AP duration at 50% repolarization level (APD_{50}, ms), maximum rate of AP upstroke (+dV/dt, mV ms^{-1}) and the maximum rate of AP repolarization (−dV/dt, mV ms^{-1}).

The following I_{Na} parameters were determined. The size of I_{Na} is expressed either as current density (pA pF^{-1}) or charge transfer (pA ms^{-1} pF^{-1}). The rate of I_{Na} inactivation is expressed as the time constant (τ) of the monoexponential fit $\{I_{Na}=a\times[1-\exp(-\tau\times t)]\}$ to the recorded current transients, where a is the peak amplitude of I_{Na}, τ is time constant of inactivation and t is time. Voltage dependence of steady-state activation and inactivation were constructed by Boltzmann fits to the experimental data using $V_{SS-Act/SS-Inact}=1/\{1+\exp[(V-V_{0.5})/k]\}$. In the equation $V_{0.5}$ is the half-voltage and the slope factor (k) for the voltage dependence. Recovery from inactivation was measured by the double pulse protocol, where the time distance between the pulses (from −120 mV to −20 mV for 50 ms) was stepwise increased.

Input resistance and strength–duration relationship

Input resistance (R_{in}) of isolated ventricular myocytes was measured at 12°C and 25°C under the current-clamp mode of the patch–clamp using K^+-based external and internal solutions (see above). Small depolarizing current pulses were injected into the cell at the V_{rest} of the cell and the changes of the membrane voltage were recorded. The time course of the voltage change was fitted using a mono exponential equation (see above) to obtain the time constant of the membrane, which is the product of membrane resistance and membrane capacitance ($\tau=R_{in}\times C_m$). Knowing τ (ms) and the cell size (pF) R_{in} (MΩ) could be calculated from the equation.

The classical strength–duration relationship was determined under the same conditions as R_{in}. The current (I) threshold for the initiation of AP was determined for square wave pulses of different durations (d, ms), which were delivered into the cell in random order at the rate of 1 Hz. The response of the cell was presented as a Weiss plot: the impulse strength is the charge ($Q=I\times d$, pA ms^{-1}) of the pulse (y-axis) which was plotted as a function of the pulse duration (x-axis) (Weiss, 1901; Geddes, 2004). This scatter plot was fitted by an equation for the straight-line ($Q=k+b\times d$), where k is the intersection point of the fitted line and the y-axis (the stimulus charge for infinitely short pulse) and b the slope of the line. The slope of the line (b) represents the rheobase, the smallest stimulus current that can induce AP when the pulse duration is infinitely long. The intersection of the fitted straight line and x-axis gives the chronaxie, the stimulus pulse duration required for initiation of AP when the current amplitude is equal to double the rheobase current (Irnich, 1980; Geddes and Bourland, 1985).

Statistics

The results are given as means and the data variation is shown either as ±s.e.m. or 95% confidence limits. Statistical differences between means were tested using t-test or Mann–Whitney U-test. Tests were performed using IBM SPSS software (version 25.0). A P-value <0.05 was considered to indicate a statistically significant difference between means.

RESULTS

Electrocardiogram (ECG)

ECGs were recorded from conscious and slightly restrained fish with intact autonomic nervous regulation. QRS complexes and T waves were well reproduced in the ECG of all fishes (n=8), while P waves were clearly seen in the ECG of 4 fishes. At the acclimation temperature of the fish (12°C) atrial and ventricular excitation were completely coordinated, i.e. each P wave was always associated with QRS complex. However, at temperatures higher than 25°C atrial and ventricular excitations became partly dissociated in all 4 fishes where the P wave was clearly visible (Fig. 1A). Typically, every other P wave occurred without the associated QRS complex, indicating a temperature-induced 2:1 atrioventricular (AV) block. With further warming, this reached 3:1 or higher-level AV block. Because of the AV block, ventricular rate (f_{HV}) increased only to the point where atrial and ventricular beating remained coordinated and then turned into bradycardia. The breakpoint temperature (T_{BP}) for f_{HV} was 25.3±0.4°C (Fig. 1A,B) and the peak value was 124±10 beats min^{-1}. Atrial beating rate (f_{HA}), calculated from the number of P waves, steeply increased above the ventricular T_{BP} (Fig. 1A,B). The peak f_{HA} was significantly higher (188±33 beats min^{-1} at 27.0°C) than the peak f_{HV} (P<0.05). PQ interval (time taken by AP conduction through atrium and atrioventricular canal) and QT interval (mean duration of ventricular AP) became progressively shorter with warming (Fig. 1C,E). The duration of QRS complex (time taken by depolarization to spread through the ventricle) was fairly insensitive to temperature although it tended to prolong at temperatures higher than 25°C (Fig. 1C–E) (P>0.05).

Action potentials (APs)

To understand the cellular effects of high temperature, we measured the responses of ventricular AP to acute warming. APs were elicited by 4 ms current pulses of increasing strength at the rate of 1 Hz at 12°C and 25°C (Fig. 2A,B). Statistically significant differences (P<0.05) were found in 6 out of 9 AP parameters (Fig. 2C–F). V_{rest} and V_{th} became more negative at 25°C relative to 12°C but the change was bigger for V_{rest}. Consequently, CD and I_{th} increased at 25°C. These findings indicate reduced EE of ventricular myocytes at 25°C. In addition, APD_{50} was strongly shortened and the amplitude of AP slightly enhanced at 25°C.

Input resistance (R_{in}) and strength–duration relationship

These experiments were done to reveal the charge requirement of AP initiation and its dependence on sarcolemmal ion leakage. R_{in} is a measure of the ion leak of the plasma membrane of quiescent ventricular myocytes and it was measured from the same cells at 12°C and 25°C. At 12°C the R_{in} was 2.1 times larger (528±59 MΩ) than at 25°C (253±28 MΩ) (P<0.01), indicating a leakier sarcolemma of ventricular myocytes at 25°C (Fig. 3A,B).

The inward charge transfer needed to bring V_{rest} to the V_{th} was examined at 12°C and 25°C by determining the classical stimulus strength–duration relationship in ventricular myocytes. Amplitude and duration of the current pulse were varied in random order and the charge needed to trigger AP at different pulse durations was plotted as a function of the pulse duration (Weiss plot) (Fig. 3C). At all pulse durations, the charge needed to trigger AP was higher at 25°C than at 12°C (P<0.05). The rheobase current was 36.2% higher at 25°C (81.0±8.3 pA) than at 12°C (51.7±3.7 pA) (P<0.05), whereas the chronaxie duration was significantly shorter at 25°C (14.8±1.3 ms) than at 12°C (19.2±1.0 ms) (P<0.05). These data show that a larger absolute threshold current/charge is needed to trigger AP at 25°C than at 12°C, while shorter pulses are more effective at 25°C.

Sodium current (I_{Na}) and inward rectifier K^+ current (I_{K1})

In these experiments the charge provided by the active ventricular myocytes and the resting leak via I_{K1} were measured. Current–voltage relationship of I_{Na} was determined at 12°C and 25°C. The

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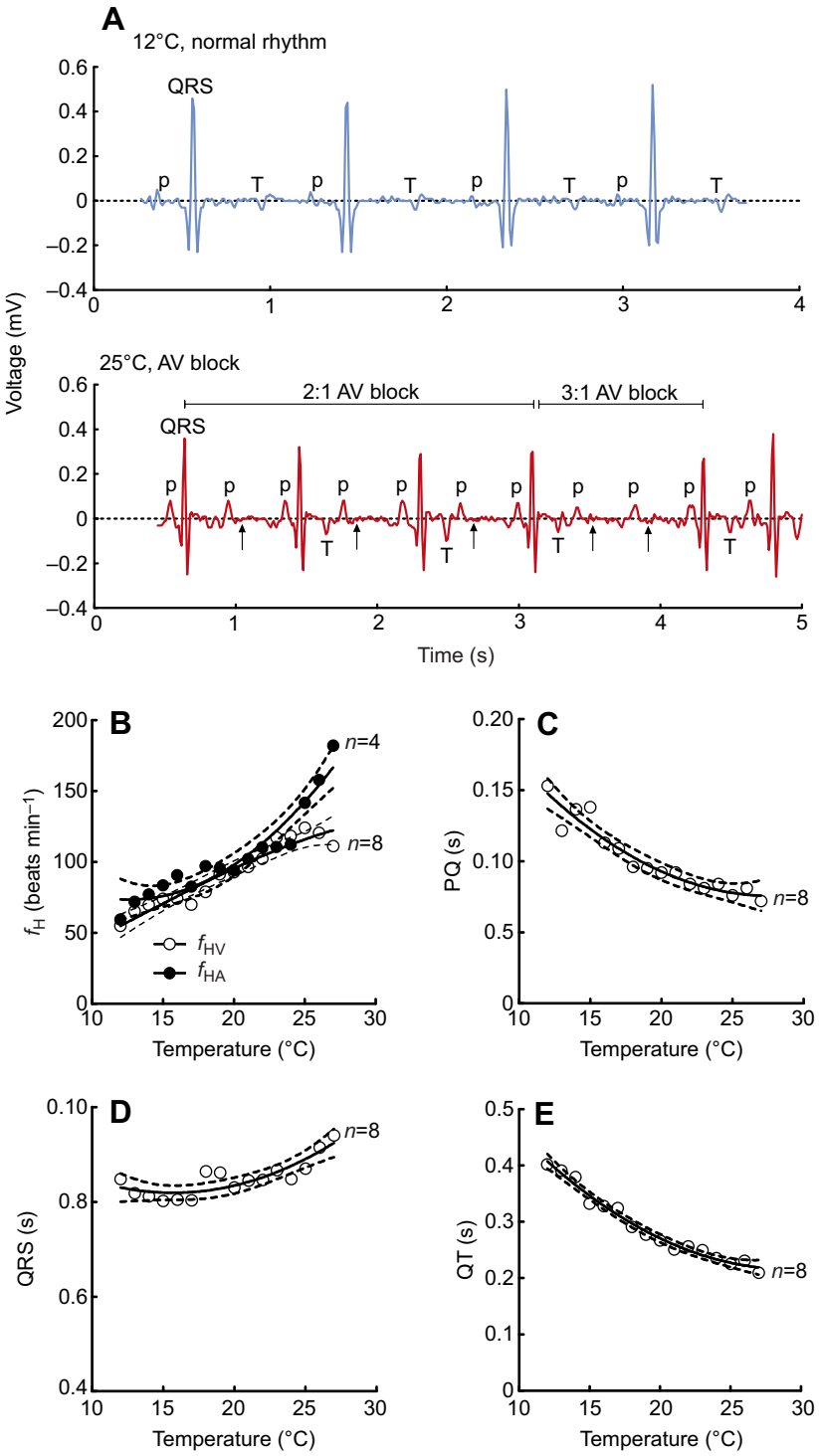

Fig. 1. Effects of acute warming on ECG recordings in rainbow trout. (A) Representative ECG recordings at 12°C and 25°C. The lower panel shows 2:1 atrioventricular block which ends up in 3:1 AV block (successive missing of two QRS complexes, small arrows). P and T waves are highlighted. (B) Effect of temperature on atrial (f_{HA}; n=4) and ventricular (f_{HV}; n=8) beating rate determined from the number of P waves and QRS complexes, respectively. (C–E) Effect of temperature on PQ interval (C), QRS duration (D) and QT interval (E). Results are means of 8 fish. Dotted lines show ±95% confidence limits.

peak density of I_{Na} was 20% larger at 25°C (−70±4 pA pF^{-1}) than at 12°C (−56±3 pA pF^{-1}) (P<0.05) (Fig. 4A,B). In contrast, the charge transfer, i.e. the time integral of I_{Na} was 31% smaller (−40±2 pA ms^{-1} pF^{-1}) at 25°C than at 12°C (−58±3 pA ms^{-1} pF^{-1}) (P<0.001) (Fig. 4C). T_{BP} values of I_{Na} and I_{K1} were sought by gradual increases of the experimental temperature (3°C min^{-1}) while the changes in current amplitudes/charge transfer were recorded (Fig. 4D,F). T_{BP} values were 18.3±0.6°C and 34.0±1.1°C for the density of inward I_{Na} and outward I_{K1}, respectively (P<0.001). Notably, the charge transfer by I_{Na} started to decline immediately upon warming (Fig. 4E). The density of I_{Na} had declined to its starting level at 12°C when temperature was 27°C. At the T_{BP} of f_{HV} the charge transfer by I_{Na} was reduced by 46%. Collectively, this shows that supply of the charge decreases and demand for the charge increases at high temperature.

Effect of warming on biophysical properties of I_{Na} was examined at 12°C and 25°C. Acute warming did not have an effect on the voltage dependence of steady-state inactivation but shifted the steady state activation curve by 4 mV to more positive voltages (P<0.05) (Fig. 5A). The rate of current inactivation became faster at

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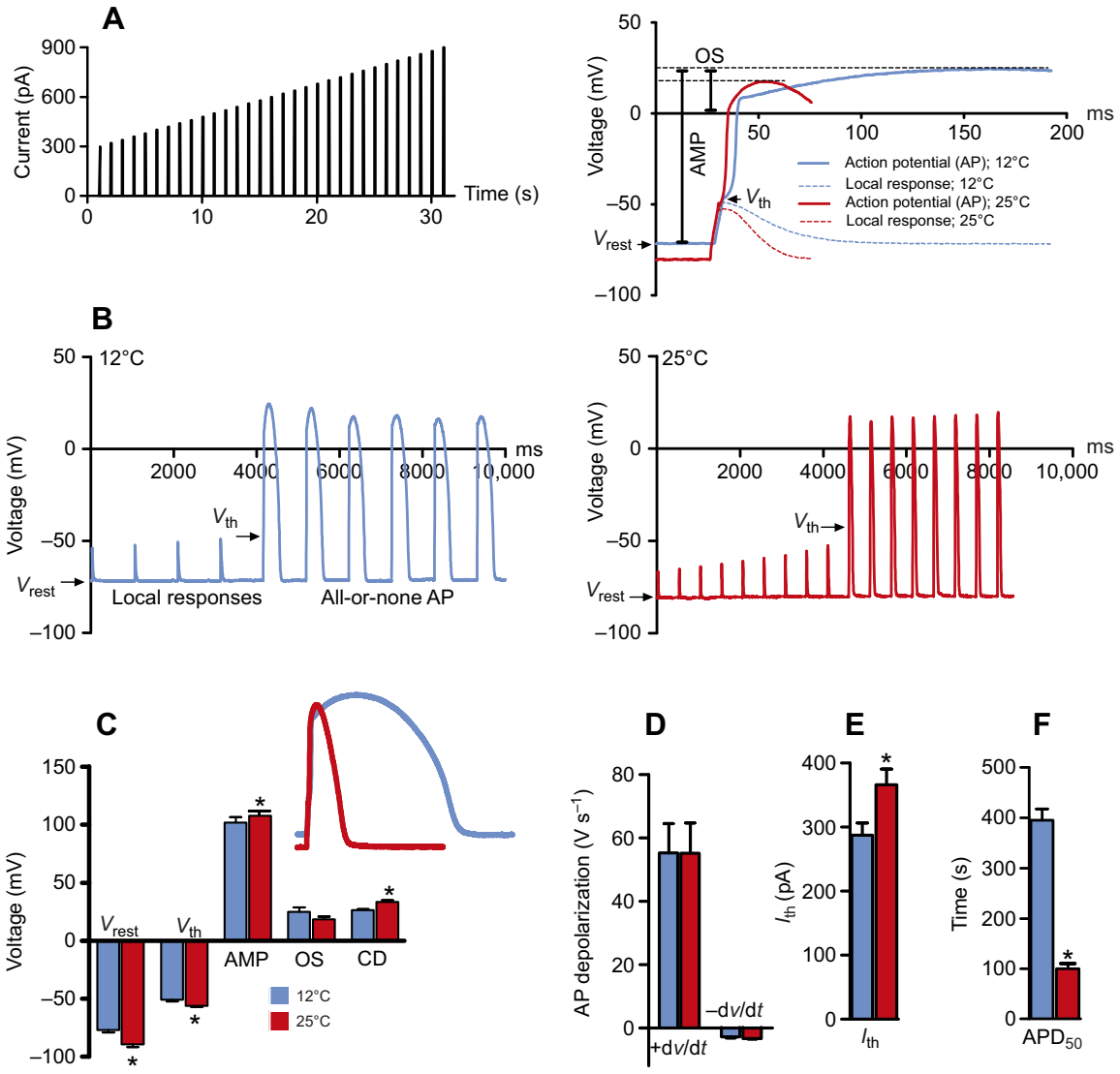

Fig. 2. Effect of temperature on AP initiation of rainbow trout ventricular myocytes at 12°C and 25°C. (A) Stimulus current protocol (left) and representative fast sweep recordings of APs at 12°C and 25°C. The measured AP parameters are shown for the AP at 12°C (right). (B) Slow sweep recordings of ventricular APs 12°C (left) and 25°C (right) showing smaller voltage responses to stimulus current and a more negative V_{th} at 25°C compared with 12°C (left). (C) Effects of temperature on resting membrane potential (V_{rest}), threshold potential (V_{th}), AP amplitude (AMP), AP overshoot (OS) and critical depolarization (CD). (D) Effects of temperature on the maximum rates of AP depolarization (+ dV/dt) and repolarization (–dV/dt). Effects of temperature on threshold current (I_{th}) (E) and AP duration at 50% of repolarization (APD_{50}) (F). Asterisks show statistically significant differences (P<0.05) between 12°C and 25°C.

25°C (Fig. 5B). Surprisingly, the rate of I_{Na} recovery from inactivation was not affected by acute temperature change (P>0.05) (Fig. 5C).

DISCUSSION

The dissociation of atrial and ventricular beating rates at the T_{BP} of f_{HV} indicates that our hypothesis 2 was correct, i.e. that the temperature-induced bradycardia was due to a failure of ventricular excitation. The sharp increase of f_{HA} above the T_{BP} of f_{HV} suggests that the beating rate of the primary pacemaker and the ability of the atrium to follow it are not impaired at temperatures above the upper critical temperature of the rainbow trout (25.6–26.9°C) (Hokanson et al., 1977; Kaya, 1978; Ekström et al., 2014; Gilbert et al., 2019). In contrast, the ventricle cannot keep pace with the pacemaker rate and therefore the heart has two separate beating rates at critically high temperatures, one for the atrium and another for the ventricle. The nearly exponential increase and the high peak value of f_{HA} in rainbow trout (188 beats min^{-1}at 27°C) matches well with the thermal response of AP rate in the isolated pacemaker cells of the brown trout (193 beats min^{-1} at 26°C) (Haverinen et al., 2017). Collectively, these findings indicate that the temperature-induced bradycardia is specific for the ventricle, and strongly suggest that the high-temperature-induced depression of cardiac output in fish (Gollock et al., 2006; Steinhausen et al., 2008; Clark et al., 2008; Mendonca and Gamperl, 2010; Ekström et al., 2016) is due to the inability of the ventricle to follow the sinoatrial rate. Our results are consistent with the early *in vitro* findings of both teleost and elasmobranch hearts, in that the ventricle is the most heat-sensitive cardiac compartment of the fish heart, which loses the ability to follow the increasing beating rate of the upper cardiac compartments (Koehnlein, 1933; Markowsky, 1933).

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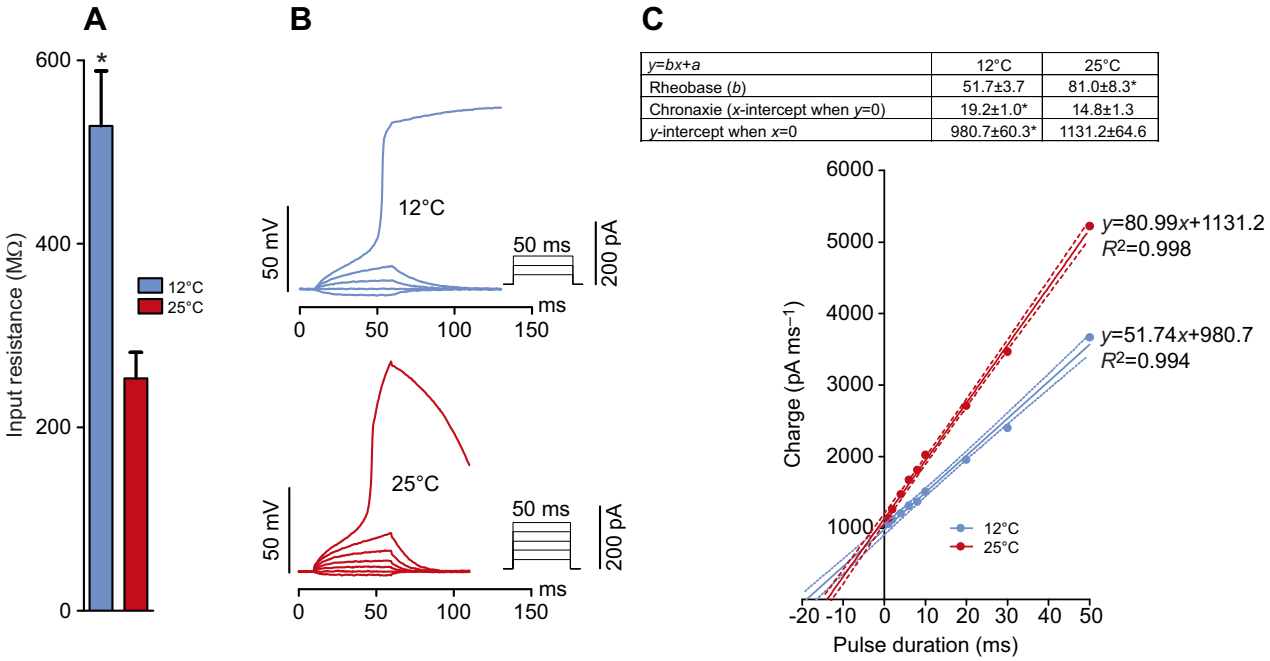

Fig. 3. Effect of temperature on input resistance (R_{in}) and strength–duration relationship of rainbow trout ventricular myocytes. (A) R_{in} of resting ventricular myocytes at 12°C and 25°C. (B) Effect of temperature on strength–duration relationship of ventricular myocytes. Representative current-clamp recordings showing the requirement of increased stimulus current/charge for AP initiation of the same myocyte at 25°C (bottom) relative to that at 12°C (top). (C) Mean results of strength–duration relationship at 12°C and 25°C presented in the form of Weiss plots (right) (n=13 myocytes from 4 fishes). The dotted lines indicate ±95% confidence limits. The mean values (±s.e.m., n=13) for rheobase, chronaxie and the charge for infinitely short pulse (y intercepts of the lines) are shown in the box above the plot. Asterisks show statistically significant differences (P<0.05) between 12°C and 25°C.

In the normally functioning vertebrate heart, the P wave always precedes the QRS complex. In the 12°C-acclimated rainbow trout, the atrial P wave and ventricular QRS complex became partially dissociated at ∼25°C. This is a functional AV block and the cause for the temperature-induced ventricular bradycardia. In all 4 trout where P was clearly visible, at some point of warming every other ventricular beat was missed, causing a 2:1 AV block. With further warming, this progressed to a 3:1 AV block, which explains the deepening ventricular bradycardia above T_{BP} of f_{HV}. In the human heart, AV block is classified in three categories (Vogler et al., 2012). In the first-degree AV block, each QRS complex is preceded by a P wave. The only deviation from the normal heart function in this benign condition is the slowed AP conduction (prolongation of PR interval) in the AV node. Characteristic for the second-degree AV block is the intermittent block of AP propagation between the atria and the ventricles, i.e. occasional dissociation of the P wave and QRS complex. Based on ECG morphology, two types of the second-degree AV block, Mobitz type 1 and Mobitz type 2, can be identified. The type 1 block is caused by the malfunction of the AV node which results in non-conducted P waves. ECG recordings of the type 1 block are characterized by a prolonged PR interval, i.e. there is a gradual slowing of AP conduction with successive beats in the AV node which then results in a skipped ventricular beat. The type 2 block also appears as dissociation of the P wave and QRS complex but without the conduction delay in the AV node. In the type 2 block, the failure occurs in the ventricular conducting system and is often associated with an increase in QRS duration. The AV block of the trout heart resembles the human second-degree type 2 block in that the P wave and QRS complex are dissociated but without prolongation of the AV conduction time. In fact, PQ interval was gradually shortened and there was a slight increase in QRS duration when the fish was acutely warmed. Similarly, in tench (*Tinca vulgaris*), eel (*Anguilla vulgaris*) and torpedo (*Torpedo ocellatta* and *Torpedo marmorata*) conduction time between atrium and ventricle shortened at temperatures between 5°C and 25°C (for tench and eel) and 2°C and 28°C (for torpedo). However, at temperatures between 25°C and 30°C, conduction started to slow down, and this was thought to cause AV block and dissociation of atrial and ventricular contractions (Markowsky, 1933). Our present findings *in vivo* (ECG) and those of Markowsky and Kohnlein *in vitro* (contractile studies) are closely similar, but the explanations of the phenomenon differ. While Markowsky and Kohnlein favour the failure of impulse conduction in the AV canal as a causal factor, we are suggesting that it is the increased excitation threshold of ventricular myocytes which precludes the ventricular contraction. In fact, both factors could be involved. It has been noticed in the sinoatrial pacemaker cells of the brown trout that high temperature does not depress AP frequency, but it shortens the duration and reduces the amplitude of the nodal AP (Hassinen et al., 2017). If the same holds for the nodal cells of the AV canal, the ventricular failure could be partly related to the small APs of the AV nodal cells and partly caused by increased excitation threshold of ventricular myocytes. APs are conducted through the AV canal, but they are too small and short to trigger APs in ventricular cells whose V_{th} is elevated (note that the strength–duration relationship shows that short pulses require a larger current to trigger AP). However, little is known about the temperature-induced changes in excitability of the fish AV canal. Clearly, the role of the AV canal in thermal responses of the fish heart needs to be examined.

It has been suggested that the warming-induced bradycardia in fish could be an adaptive reaction to high temperature by improving myocardial oxygenation (Farrell, 2007; Ekström et al., 2014, 2019; Gilbert et al., 2019). Mechanistically, this could happen via the release of acetylcholine from the vagal nerve endings and subsequent

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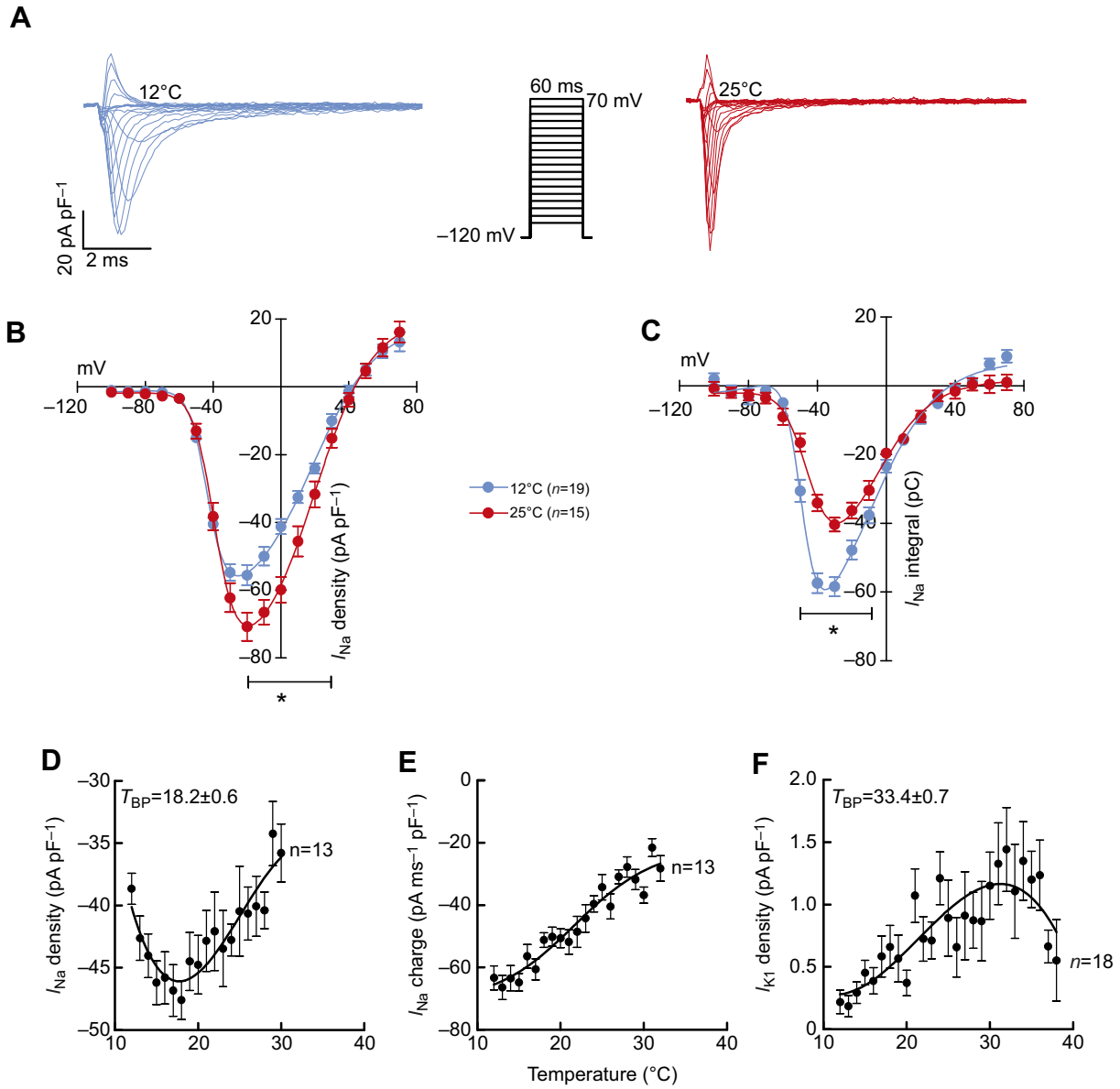

Fig. 4. Effects of temperature on I_{Na} and I_{K1} of rainbow trout ventricular myocytes. (A) Representative tracings of the voltage-dependence of I_{Na} at 12°C and 25°C. (B) Voltage dependence of I_{Na} density at 12°C (n=19 cells from 5 fish) and 25°C (n=15 cells from 5 fish). (C) Voltage dependence of I_{Na} charge transfer at 12°C (n=19 cells from 5 fish) and 25°C (n=15 cells from 5 fish). (D,E) Effects of acute warming on I_{Na} density (D) and charge transfer (E). The results are means±s.e.m. of 13 cells from 3 fishes. (F) Effect of acute warming on the density of the outward I_{K1} in trout ventricular myocytes (n=18 cells from 4 fishes). Asterisks show statistically significant differences (P<0.05) between 12°C and 25°C.

hyperpolarization of the maximum diastolic potential of the pacemaker cells, if the cholinergic tone was increased by acute warming (Campbell et al., 1989). However, the findings on the cholinergic regulation of the thermal tolerance of f_H in rainbow trout are contradictory. Some studies suggest that blocking of the cholinergic nervous transmission has no effect on the temperature tolerance of f_H (Ekström et al., 2014, 2019), while others suggest that temperature tolerance is decreased by the cholinergic block (Gilbert et al., 2019). Both conclusions are based on the beating rate of the ventricle. The present findings show that f_{HA} and f_{HV} become separated when f_H is about 120 beats min^{-1}. In the light of the present study, the 2°C depression in T_{BP} of f_H in the presence of cholinergic block (Gilbert et al., 2019) is explained by the slightly higher f_H of the atropine-treated fish, i.e. the 'cut-off' frequency of the AV block (about 120 beats min^{-1}) is achieved at slightly lower temperature. Thus, the cholinergic tone *in vivo* can indirectly increase T_{BP} of f_{HV} by shifting the cut-off frequency of the AV block to slightly higher temperatures. However, the strong increase of f_{HA} above the T_{BP} of f_{HV} indicates that the sinoatrial rate remains intact and therefore excludes the cholinergic 'brake' as an explanation for the temperature-induced bradycardia. To conclude, the present findings show that the temperature induced ventricular bradycardia is a non-adaptive trait because it is caused by malfunction of the cardiac excitation system.

At the cellular level, the reduced ventricular excitability was evident as temperature-induced increase in CD and I_{th}. With acute warming, both V_{rest} and V_{th} become more negative but the effect of temperature on V_{rest} was stronger than on V_{th}, and therefore the voltage distance between the two (CD) increased: more current (I_{th}) was needed to trigger AP. According to the TDEE hypothesis, the failure of ventricular excitation is due to the discordant temperature-

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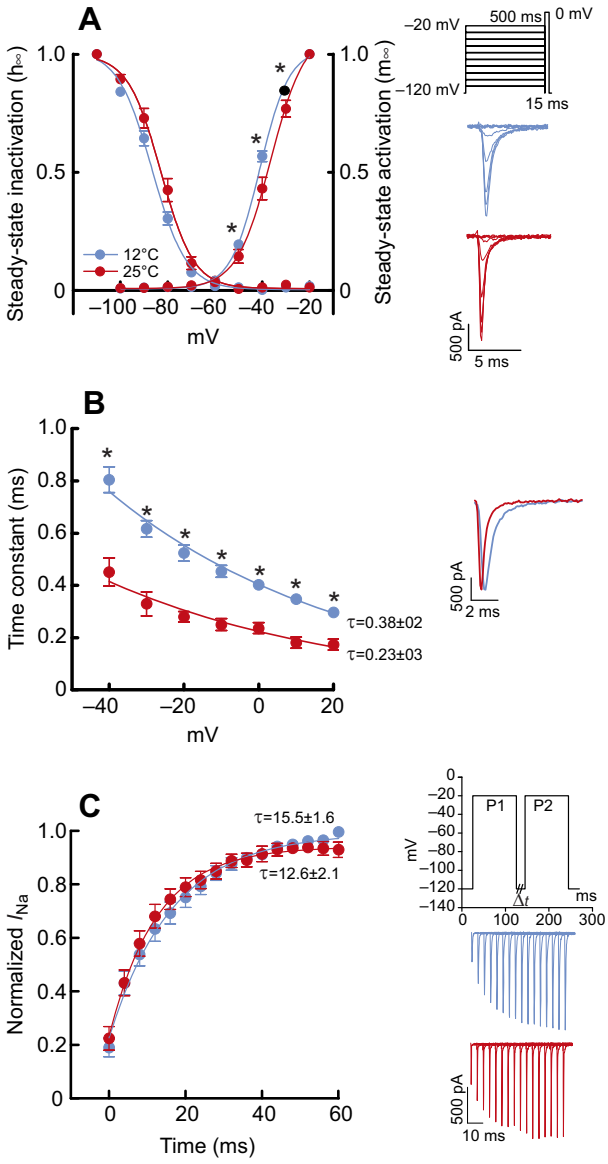

Fig. 5. Temperature dependence of I_{Na} in rainbow trout ventricular myocytes. (A) Voltage dependence of steady-state activation shifted more positive voltages (P<0.05) but steady-state inactivation was not affected (P>0.05) by acute increase in temperature from 12°C to 25°C. Mean results± s.e.m. of 19 cells from 5 animals (left). Representative recordings of I_{Na} at 12°C and 25°C are shown on the right. (B) Acute increase in temperature accelerated the rate of I_{Na} inactivation. Mean values±s.e.m. for the voltage-dependence of I_{Na} inactivation (left) and representative I_{Na} recordings at −20 mV (right) at 12°C and 25°C. (C) Recovery of I_{Na} from inactivation. Acute increase of temperature from 12°C to 25°C did not have any effect on the rate of recovery from inactivation. Mean results±s.e.m. of 13 cells from 4 animals (left). Representative recordings of I_{Na} at 12°C and 25°C are shown on the right. P1 and P2 are voltage pulses 1 and 2. Asterisks show statistically significant differences (P<0.05) between 12°C and 25°C.

dependencies of I_{Na} and I_{K1}, the two opposing currents decisive for the initiation of ventricular AP (Varghese, 2016; Vornanen, 2016). Consistent with the TDEE hypothesis the depression of EE was associated with an increase in the outward I_{K1} of the resting ventricular myocyte and a decrease in the charge transfer by I_{Na} of the active myocytes. Collectively, these findings indicate that a mismatch between the inward I_{Na} and the outward I_{K1} develops at high temperatures, which results in depressed excitability of ventricular myocytes (Vornanen, 2016). This was further tested using the classical method to measure EE of cells and tissues, i.e. to determine the strength–duration relationship (Weiss, 1901; Lapicque, 1909; Geddes, 2004). This analysis gives two variables: (1) rheobase, which is the minimum stimulus strength of an indefinite long depolarizing pulse needed to trigger AP; and (2) chronaxie, which is the minimum duration of stimulus pulse with twice the rheobase strength sufficient for initiation of AP (Irnich, 1980). In the space-clamped ventricular myocytes, the rheobase current was 56.6% larger at 25°C than at 12°C, indicating an increased demand for depolarizing current/charge. This is due to the increased outward leakage of K^+ ions as indicated by the lower R_{in} and the higher I_{K1} at 25°C relative to 12°C. K^+ efflux of the quiescent myocyte shunts the inward current inflow from the upstream cell with the consequence that the diminished inward charge transfer by I_{Na} cannot depolarize the membrane to the V_{th}. Basically, the present results are consistent with the earlier findings on another teleost species, the brown trout (*Salmo trutta fario*) (Vornanen et al., 2014).

In multicellular cardiac tissue, the increased outward K^+ leakage of the quiescent myocytes forms the current sink of the downstream myocyte, which must be exceeded by the inward I_{Na} from the activated upstream myocyte or the current source (Varghese, 2016). Robust excitability of the ventricle requires that there is some excess of the inward I_{Na} relative to the outward I_{K1}. Owing to the surplus of I_{Na}, small changes in physiological or environmental conditions are unlikely to cause failure of ventricular excitation. The excess of the inward current relative to the outward current is called a safety factor or safety margin, which can be defined as the ratio between charge provided by the upstream source cell and charge demanded by the downstream sink cell for excitation (Shaw and Rudy, 1997). When the safety factor is ≥1.0, excitation is ensured and if the safety factor is <1.0 excitation will fail. At high temperatures, the increase of I_{K1} with the simultaneous decrease of I_{Na} charge transfer 'consumes' the surplus of the source current resulting in source–sink mismatch and failure of the ventricular excitation. Notably, in rainbow trout ventricular myocytes, the charge transfer via I_{Na} started to decline immediately when temperature exceeded 12°C. At the T_{BP} of the f_{HV}, the charge transfer by I_{Na} was reduced by about 46% and the membrane leakage was approximately doubled. From these changes it can be estimated that the safety factor at the acclimation temperature of the fish (12°C) was more than 2.0. At 25°C, the safety margin is probably consumed (<1.0) resulting in bradycardia. The main reason for the decline of the charge transfer by I_{Na} is the temperature-induced increase in the rate of current inactivation. Also, the rate of current activation is slightly increased by high temperatures but its effect on charge transfer is less than that of inactivation. The activation of I_{Na} is almost instantaneous even at 12°C, and therefore, not affected much in absolute terms by warming.

Conclusions

AV block caused by the mismatch between I_{Na} source current and I_{K1} sink current (membrane leak) of ventricular myocytes provides a mechanistic explanation for high-temperature-induced ventricular bradycardia. While the source–sink mismatch concept is consistent with the oxygen and capacity limited thermal tolerance (OCLTT) – a hypothesis that oxygen delivery by circulation and ventilation sets the thermal tolerance limits of ectotherms (Pörtner, 2001) – its scope of application might not be limited to cardiac function. Therefore, it may be premature to assume that the temperature-dependent weakening of physiological performance of the fish is due solely to the impairment of cardiac function caused by

bradycardia. Indeed, the OCLTT hypothesis is highly controversial and heavily disputed (Jutfelt et al., 2014), and there are alternative explanations for thermal failure of fish physiology (Clark et al., 2013; Ern et al., 2016; Lefevre, 2016; Motyka et al., 2016). Because electrical excitability of nerve and muscle cells is based on the same principles as that of cardiac myocytes (Koester and Siegelbaum, 2000), it is equally possible that concomitantly with bradycardia (or even at lower temperatures) transmission of nervous system messages and muscle function are also impaired. Future studies should address the possibility that sensory and motor functions, and coordination of body homeostasis in fish are compromised at high temperature as a result of the source–sink mismatch of current flow in various types of excitable cells.

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Competing interests
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Author contributions
Conceptualization: M.V.; Methodology: J.H.; Investigation: J.H.; Writing - original draft: J.H.; Writing - review & editing: M.V.; Visualization: J.H.; Project administration: M.V.; Funding acquisition: M.V.

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