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Effects of autonomic blockade on acute thermal tolerance and cardioventilatory performance in rainbow trout, *Oncorhynchus mykiss*

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ABSTRACT

Predicted future increases in global temperature may impose challenges for ectothermic animals like fish, but the physiological mechanisms determining the critical thermal maximum (CT_{max}) are not well understood. One hypothesis suggests that impaired cardiac performance, limited by oxygen supply, is an important underlying mechanism. Since vagal bradycardia is suggested to improve cardiac oxygenation and adrenergic stimulation may improve cardiac contractility and protect cardiac function at high temperatures, we predicted that pharmacological blockade of cardiac autonomic control would lower CT_{max}. Rainbow trout was instrumented with a flow probe and a ventilation catheter for cardioventilatory recordings and exposed to an acute thermal challenge until CT_{max} following selective pharmacological blockade of muscarinic or β-adrenergic receptors.

Contrary to our prediction, CT_{max} (~26 °C) was unchanged between treatments. While β-adrenergic blockade reduced heart rate it did not impair cardiac stroke volume across temperatures suggesting that compensatory increases in cardiac filling pressure may serve to maintain cardiac output. While warming resulted in significant tachycardia and increased cardiac output, a high cholinergic tone on the heart was observed at temperatures approaching CT_{max}. This may represent a mechanism to maintain scope for heart rate and possibly to improve myocardial contractility and oxygen supply at high temperatures. This is the first study evaluating the importance of autonomic cardiac control on thermal tolerance in fish. While no effects on CT_{max} were observed, this study raises important questions about the underlying mechanisms determining thermal tolerance limits in ectothermic animals.

1. Introduction

Current climate change models predict that the average global temperature will increase within the next hundred years (Ficke et al., 2007; Pachauri and Reisinger, 2007), and the occurrence of extreme weather phenomena such as transient heat waves is expected to increase in frequency and magnitude (Doney et al., 2012; Ficke et al., 2007). These changes in temperature may pose significant challenges for aquatic ectothermic animals such as fish, yet many aspects of the thermal biology of fish remain unknown (Beitinger and Lutterschmidt, 2011; Clark et al., 2013). For example, acute thermal tolerance limits have been extensively studied in fishes for more than a century (Carter, 1887; Davenport and Castle, 1895; Davy, 1863), but the physiological mechanisms determining the critical thermal maximum (CT_{max}) are still not understood (Beitinger and Lutterschmidt, 2011; Clark et al., 2013).

While heart rate and cardiac output typically increase with acute temperature elevation due to the stimulatory effects of temperature on cardiac pacemaker cells (Harper et al., 1995), a plateau or reduction in heart rate has often been reported at temperatures approaching CT_{max} (Clark et al., 2008; Gollock et al., 2006; Heath and Hughes, 1973; Mendonca and Gamperl, 2010). This response is often interpreted as impending cardiac failure and the pumping capacity of the heart has therefore been pointed out as a possible limiting factor setting thermal tolerance limits in fish (Casselman et al., 2012; Farrell et al., 2009). This idea is related to the importance of the partial pressure of oxygen in venous blood ($P_{v_{O_2}}$) for adequate diffusion of oxygen to cardiac tissues, as most fishes either lack coronary arteries or only have a coronary supply to the outer compact myocardium (Axelsson, 1995; Davie and Farrell, 1991; Tota, 1983). Thus, as cellular respiration and oxygen extraction of systemic and cardiac tissues increase with

Abbreviations: ; Q, cardiac output; CT_{max}, critical thermal maximum; f_H, heart rate; Hct, hematocrit; Hb, hemoglobin content; Q_{max}, maximum cardiac output; f_{Hmax}, maximum heart rate; f_{Vmax}, maximum ventilation rate; MCHC, mean corpuscular hemoglobin content; P_O, opercular pressure; $(P_{v_{O_2}})$, partial pressure of venous oxygen; RSM, relative spleen mass; RVM, relative ventricle mass; V_S, stroke volume; f_V, ventilation rate

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temperature to meet the elevated metabolic demands, $P_{v_{O_2}}$ gradually decreases and may reach a critical threshold where myocardial oxygenation becomes insufficient (Farrell, 2007; Farrell and Clutterham, 2003). In addition the blood becomes progressively more acidotic and hyperkalemic (Cech et al., 1976; Clark et al., 2008; Currie et al., 2013; Heath and Hughes, 1973; Steinhausen et al., 2008; Vanlandegehm et al., 2010), which may impair cardiac function further (Driedzic and Gesser, 1994; Hanson et al., 2006; Kalinin and Gesser, 2002; Nielsen and Gesser, 2001).

However, another possibility, that has received less attention in the literature, is the possibility that the bradycardia at high temperatures in fish may be beneficial and mediated actively via the autonomic nervous system, i.e. through vagal (cholinergic) inhibition of heart rate. Farrell (2007) suggested that the reduction in heart rate during environmental hypoxia (i.e. hypoxic bradycardia), which represents another environmental challenge that results in reduced $P_{v_{O_2}}$, should improve myocardial oxygen diffusion by increasing the end-diastolic volume and the residence time of blood in the lumen of the heart. In addition, as peak coronary blood flow occurs during ventricular diastole (Axelsson and Farrell, 1993), hypoxic bradycardia and a prolonged diastole would potentially facilitate myocardial oxygenation (Farrell, 2007). Another possible benefit of actively induced bradycardia during heat stress could be to avoid accumulation of deleterious high intracellular Ca^{2+} levels by reducing the influx of Ca^{2+} across the sarcolemma and prevent cardiac arrhythmia and loss of contractility (Rantin et al., 1998). However, few studies have examined the importance of autonomic nervous control of cardiac function during temperature challenges in fishes *in vivo* and available information is surprisingly contrasting. For example, Axelsson et al. (1992) reported a decline in vagal tone with acute temperature increase in the Antarctic fish *Pagothenia bernachii*, while an increase in vagal tone consistent with the ideas presented above was observed with a similar experimental protocol in the closely related Antarctic bald notothen, *Pagothenia borchgrevinki* (Franklin et al., 2001; Lowe et al., 2005).

There are also indications that the sympathetic (adrenergic) component of the autonomic nervous system is important for thermal tolerance and cardiac performance at temperatures approaching the upper lethal limit in fish. Although the stimulatory effect of catecholamines decreases with increasing temperature in *in situ* perfused fish hearts (Keen et al., 1993) and in *in vitro* heart preparations (Aho and Vornanen, 2001; Ask et al., 1981), β-adrenergic stimulation improves cardiac function during hyperkalemic and acidotic conditions (Farrell et al., 1983; Hanson and Farrell, 2007; Hanson et al., 2006; Kalinin and Gesser, 2002), both of which may occur during conditions of increased metabolic demand at high temperature (Cech et al., 1976; Clark et al., 2008; Heath and Hughes, 1973). In fact, adrenergic stimulation was shown to increase the thermal tolerance of *in vitro* heart strip preparations from 4 °C acclimated rainbow trout (Aho and Vornanen, 2001). This is probably because adrenergic stimulation of myocardial β-receptors increases Ca^{2+} re-uptake across the sarcoplasmic reticulum, and maintains cardiac function by preventing accumulation of excessively high intracellular Ca^{2+} levels at high contraction frequencies at high temperature (Aho and Vornanen, 2001; Driedzic and Gesser, 1985). Thus, it may not be surprising that circulating catecholamines typically increase substantially during acute heat challenges in fishes *in vivo* (Currie et al., 2013, 2008; Forster et al., 1998; LeBlanc et al., 2012, 2011). Moreover, Eliason et al. (2011) showed that the most thermally tolerant population of Fraser River sockeye salmon (*Onchorhynchus nerka*) had the highest cardiac β-receptor density indicating that cardiac β-adrenergic stimulation may be important for thermal tolerance in wild salmonids.

In the present study we used pharmacological tools to specifically block cholinergic and β-adrenergic control systems in order to characterize the importance of these systems on cardiac performance and CT_{max} in rainbow trout during an acute temperature challenge. We predicted that if cardiac function and circulatory oxygen transport are important determinants of CT_{max}, and if adrenergic and cholinergic control mechanisms have beneficial influences on cardiac oxygenation and performance at temperatures approaching CT_{max}, selective blockade of these systems would translate into reduced CT_{max}. Furthermore, since positive correlations between CT_{max} and relative ventricular mass and blood oxygen transporting capacity have been found in other fish species (Anttila et al., 2013; Beers and Sidell, 2011), ventricle mass and blood parameters were recorded along with the cardioventilatory variables to further explore the physiological mechanisms underlying acute thermal tolerance.

2. Materials and methods

2.1. Experimental animals

Adult rainbow trout (*Oncorhynchus mykiss*, Walbaum, mass: 683 ± 136 g) was obtained from a commercial fish farm (Antens laxodling AB, Alingsås, Sweden). The fish were kept in tanks supplied with a constant flow of aerated water from a recirculating freshwater system (UV-treated and mechanically filtered) at a temperature of 10 ± 1 °C. The fish were fed to satiation weekly with commercial fish pellets. Newly arrived fish were acclimated to the holding conditions for at least one week before any experimental procedures were initiated. All experiments were approved by the regional ethics committee (ethical permit number: 65-2012).

2.2. Instrumentation and experimental setup

Fish were anesthetized in water containing tricaine methanesulfonate (MS-222, 120 mg l^{-1}), buffered with sodium bicarbonate ($NaHCO_3$, 240 mg l^{-1}), and was placed right side up on wet foam on a surgical table. The gills were constantly irrigated with aerated water (10 °C) containing MS-222 (120 mg l^{-1}) buffered with sodium bicarbonate ($NaHCO_3$, 240 mg l^{-1}). A small incision was made in the opercular cavity to expose the ventral aorta without damaging the pericardium. A custom made Perspex cuff with a 20 MHz Doppler flow probe (Iowa Doppler products, Iowa City, IA, USA) was then fitted around the vessel. The wire from the probe was secured with silk sutures to the skin along the opercular cavity and anterior to the dorsal fin. A heat flared polyethylene catheter (PE-90), inserted through a small hole in the operculum, was used to record ventilatory pressure changes in the opercular cavity (Holeton and Randall, 1967). The catheter was locked in place from the outside using a short piece of tight-fitting PE-160 catheter and sutured to the skin anterior to the dorsal fin. A PE-50 catheter for drug administration was inserted into the abdominal cavity, ventrally, and approximately 5 cm posterior to the pectoral fin and sutured in place close to the point of insertion (Sandblom et al., 2010).

Following instrumentation, the fish were placed individually in opaque experimental chambers (width: 131 mm, length: 340 mm and height: 170 mm) with flow through water or in holding tubes (Ø: 105 mm, length: 450 mm) floating in the holding tanks. Fish were always given at least 20 h of post-surgical recovery before any experiments started. In those cases where the fish was first placed in a holding tube, it was transferred to the experimental chamber in a water filled plastic bag at least 15 h before the experiments started. A constant flow of well-oxygenated water

from the recirculating water system supplied the test chambers throughout the experiments. To avoid any external visual stimuli, the test chambers were shielded with black plastic drapes and the fish were monitored using a camera mounted on the experimental setup. During the acute thermal challenge (see below) the water to the test chamber was heated using a partly recirculating and aerated water system containing a thermostatically controlled heater. Water oxygen saturation was always above 80% during the thermal challenge.

2.3. Experimental protocol

Resting cardioventilatory variables were first recorded for at least one hour. Fish were then randomly injected with one of the following drugs via the abdominal catheter: atropine sulfate (1.2 mg kg^{-1}) to block muscarinic receptors; sotalol hydrochloride (2.7 mg kg^{-1}) to block β-adrenergic receptors or saline (0.9% NaCl) in the control. All pharmacological substances were dissolved in saline and injected at a total volume of 1 ml kg^{-1} followed by 0.5 ml of saline to flush the catheter. Chemicals and pharmacological substances were purchased from Sigma-Aldrich (St. Louis, MO, USA). The drugs were allowed to take effect for approximately one hour while recordings of cardioventilatory variables were continuously performed to confirm when a new steady state had been reached.

The temperature was subsequently elevated in 1 °C increments (3 °C h^{-1}) and maintained at each temperature for 15 min to allow the body temperature of the fish to thermally equilibrate with the surrounding water (Crawshaw, 1976; Stevens and Sutterlin, 1976). The critical thermal maximum (CT_{max}) was determined as the temperature where the righting reflex was lost (Beitinger et al., 2000). Thus, the total duration of the thermal challenge from the drug injection to CT_{max} ranged from 311 to 392 min. At this point, the fish was immediately removed from the experimental chamber and euthanized with sharp blow to the head and a blood sample was quickly taken from the caudal vessels using a heparinized syringe. Blood hemoglobin content (Hb) was determined using a handheld Hb 201^{+} meter (Hemocue® AB, Ängelholm, Sweden). Hematocrit (Hct) was determined using heparinized microhematocrit tubes, spun at 10.000 g for 5 min and mean corpuscular hemoglobin content (MCHC) was determined as MCHC= Hb/(Hct/100). The ventricle and spleen were removed, blotted dry and weighed and the relative wet masses were determined.

2.4. Data acquisition

The ventilation catheter was connected to a pressure transducer (model DPT-6100, Pvb Medizintechnik, Kirchseeon, Germany) calibrated against the water level in the experimental chamber and a static water column. The signal from the pressure transducer was amplified using a Senselab 4ChAmp amplifier (Somedic sales, Hörby, Sweden). The Doppler probe was connected to a directional-pulsed Doppler flowmeter (545C-4, Bioengineering, University of Iowa, Iowa City, USA) and the signal range was adjusted to obtain the maximum signal intensity in the untreated fish. The water temperature in the experimental setup close to the fish was recorded continuously using a custom-built thermostat and temperature logger (EW 7221, Crn Tecnopart, Barcelona, Spain). The analog outputs from the recording equipment were fed into a PowerLab system (ADinstruments, Castel Hill, Australia) connected to a computer running LabChart Pro (version 7.03; ADInstruments, Bella Vista, Australia) for continous on line data acquisition and subsequent off line data analysis.

2.5. Data analysis and statistics

All data are presented as mean ± S.E.M. Cardioventilatory values were typically taken from the last three minutes prior to the pharmacological treatment or temperature change during periods when variables were stable. Heart rate (f_H) was determined from the pulsating blood flow traces and ventilation rate (f_V) from the opercular pressure (P_O) trace. Relative changes in cardiac output (Q) were determined from the changes in blood flow velocity with the initial value taken before the injection of drugs set to 100%. Relative stroke volume (V_S) was calculated as $V_S = Q/f_H$. Statistical analyses were performed using SPSS for Windows (SPSS Inc., Chicago, IL, USA). A linear mixed model followed by Holm–Sidak post-hoc tests, with temperature set as repeated measures, was used for determination of differences in cardioventilatory variables between and within treatment groups. Determination of temperature effects was performed by comparing the 10 °C value after drug injection with values at subsequent temperature steps. A one-way ANOVA, followed by a Tukey post-hoc test, was also performed to specifically determine statistical differences among pharmacological treatment groups at 10 °C, as well as for hematological and morphometric variables. Statistical significance was accepted at $p \leq 0.05$. Pearson's correlation analysis was performed using values from individual control fish to evaluate possible relationships between CT_{max} and cardioventilatory, hematological and morphometric variables.

3. Results

3.1. Basal cardioventilatory variables and effects of pharmacological treatments at 10 °C

Cardioventilatory responses to pharmacological treatment and temperature increase are summarized in Fig. 1. In untreated fish at 10 °C, routine f_H ranged between 42 and 48 beats min^{-1} and routine f_V ranged between 52 and 56 breaths min^{-1}. Atropine treatment significantly elevated f_H by 22% and sotalol treatment significantly reduced f_H by 33% at 10 °C. Nonetheless, cardiac output was maintained unchanged following the drug treatments by a significant compensatory 22% decrease in V_S in atropine-treated fish and a significant 28% increase in sotalol-treated fish. Pharmacological treatments had no significant effects on ventilatory variables (Fig. 1D and E). Body mass and length were not significantly different among treatment groups (Table 1).

3.2. CT_{max} and cardioventilatory responses to temperature after autonomic blockade

CT_{max} of control fish was 25.9 ± 0.2 °C and there were no significant effects of the drug treatments on this variable (Table 1). Cardiac activity at CT_{max} exhibited considerable inter-individual variability, but most individuals maintained regular cardiac rhythmicity even when equilibrium was lost, although a few fish showed clear signs of cardiac arrhythmia prior to CT_{max} (data not shown). No significant differences among treatments were detected when comparing cardioventilatory variables at CT_{max} (data not shown).

In both the control and atropine-treated fish cardiac output increased approximately threefold with the acute temperature increase, but no significant differences were found between the treatment groups. (Fig. 1A and Table 1). The increase in blood flow was mainly due to tachycardia in both the control and atropine-treated groups because f_H increased significantly with temperature until reaching a plateau at 23 °C, which was followed by a subsequent decline in f_H until reaching CT_{max}. However, the

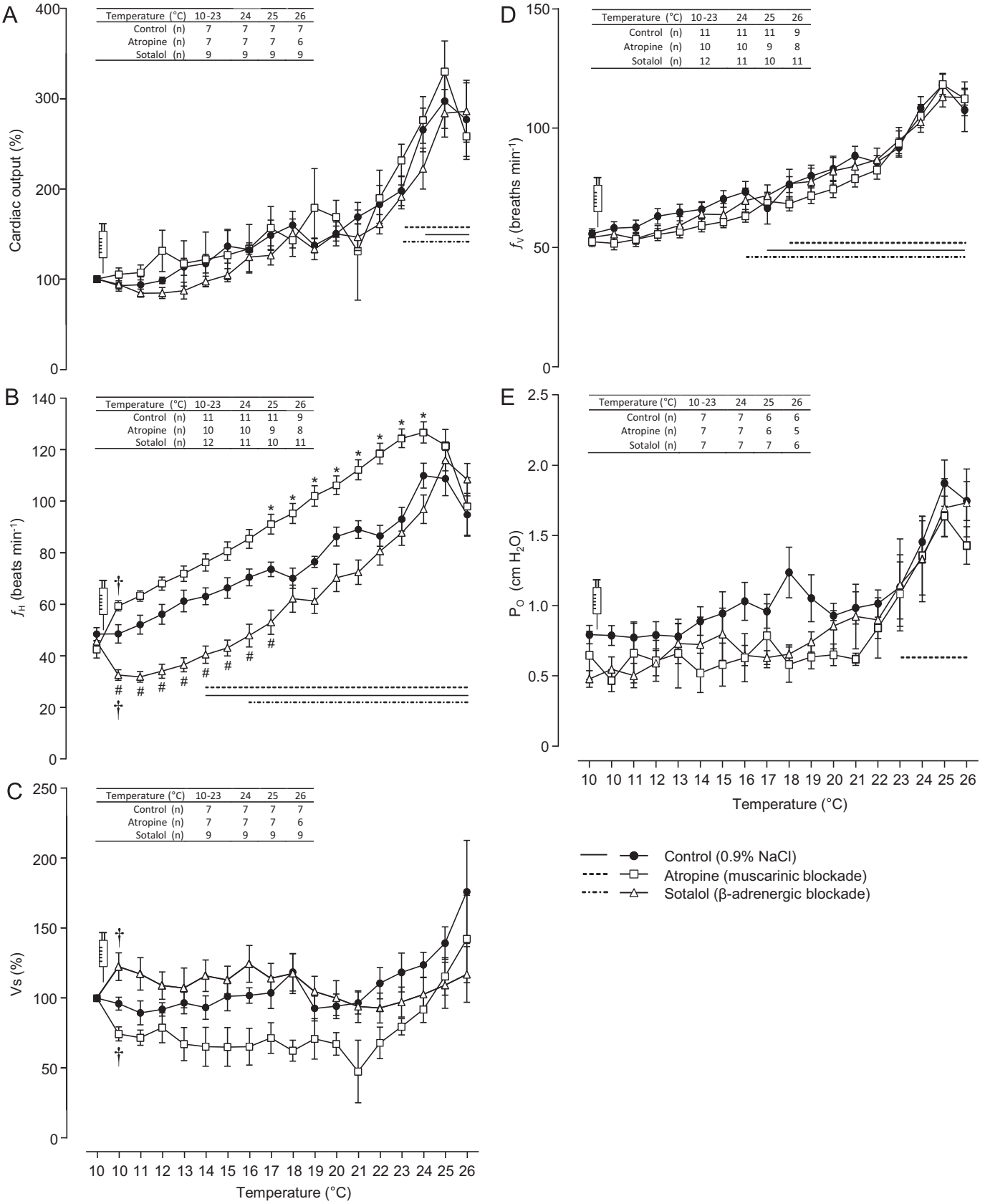

Fig. 1. Cardioventilatory responses in instrumented rainbow trout (*Oncorhynchus mykiss*) during an acute temperature challenge (3 °C h^{-1}). (A) Cardiac output, (B) heart rate and (C) stroke volume (D) ventilation rate and (E) opercular pressure in fish treated with saline, atropine sulfate or sotalol hydrochloride. Daggers indicate significant effects of drug treatment at 10 °C and the horizontal lines indicate statistically significant effects of temperature from the initial post-treatment value at 10 °C (drug injection indicated by syringe symbol). * denotes significant differences between control and atropine treatment across temperatures and # denotes significant differences between control and sotalol treatment across temperatures. The tables show the number of animals (*n*) used in the analysis at each temperature. Data are presented as mean ± S.E.M.

Table 1
Comparison of $CT_{max,}$ cardiovascular, hematological and morphometric variables in rainbow trout (*Oncorhynchus mykiss*) during acute thermal challenge under control conditions and after muscarinic (atropine) or β-adrenergic (sotalol) blockade.

	Control	Atropine	Sotalol
CT_{max} (°C)	25.9 ± 0.2	25.9 ± 0.3	26.0 ± 0.3
Q_{max} (%)	320.8 ± 28.8	365.5 ± 24.3	316.3 ± 32.6
at temp. (°C)	25.0 ± 0.3	25.0 ± 0.5	25.2 ± 0.4
f_{Hmax} (beats min^{-1})	118.4 ± 3.8	127.9 ± 4.0	118.7 ± 3.0
at temp. (°C)	24.3 ± 0.3	23.9 ± 0.2	25.2 ± 0.2*
f_{Vmax} (breaths min^{-1})	120.1 ± 4.7	119.5 ± 3.8	116.6 ± 3.5
at temp. (°C)	25.2 ± 0.3	24.9 ± 0.3	24.6 ± 0.3
Hct (%)	39.1 ± 2.8	39.1 ± 2.2	39.6 ± 1.6
Hb ($g\ l^{-1}$)	114.9 ± 5.6	115.1 ± 7.2	122.5 ± 4.7
MCHC ($g\ l^{-1}$)	301 ± 14.8	295 ± 9	310 ± 22
RVM (%)	0.105 ± 0.006	0.095 ± 0.005	0.094 ± 0.004
RSM (%)	0.101 ± 0.013	0.103 ± 0.008	0.115 ± 0.021

Data are presented as mean ± S.E.M. Hematological and morphometrical variables were collected following the thermal challenge, i.e. at CT_{max}. * denotes significant difference ($p \leq 0.05$) from the control group. Abbreviations are CT_{max} (critical thermal maximum); Q_{max} (maximum cardiac output); f_{Hmax} (maximum heart rate); f_{Vmax} (maximum ventilation rate); Hct (hematocrit); Hb (hemoglobin content); MCHC (mean corpuscular hemoglobin content); RVM (relative ventricle mass) and RSM (relative spleen mass).

atropine treatment revealed that heart rate in control fish was continuously suppressed by an inhibitory cholinergic tone up to temperatures approaching CT_{max}, because f_H after atropine remained significantly elevated relative to the control at temperatures between 17 and 24 °C (Fig. 1B). No significant changes in V_S were observed with increasing temperature in the control and atropine-treated fish. At temperatures above 21 °C, however, there appeared to be a trend for an increase in stroke volume in both treatment groups, but this was not statistically significant.

In the sotalol-treated fish, cardiac output and f_H also increased approximately threefold with temperature and the effects of the temperature increase were statistically significant from 16 to 23 °C, respectively (Fig. 1B). Nonetheless, sotalol treatment resulted in a significantly depressed f_H relative to the control at temperatures between 10 and 17 °C (reduced by 27–40%, Fig. 1B), suggesting that a stimulatory β-adrenergic tonus on the heart was present at the lower end of the temperature range. However, from 18 °C and upwards this difference was absent. If anything, V_S appeared to decline somewhat at the highest temperatures in the sotalol-treated group, but similar to the control and atropine-treated fish this was not statistically significant (Fig. 1C).

An approximately twofold increase in f_V was observed with all treatments, with the temperature effect being significant from 16 to 18 °C (Fig. 1D). A significant increase in ventilation pressure was only observed in the sotalol-treated fish between 23 and 26 °C, although all three treatment groups exhibited apparently similar trends in response to the acute temperature challenge (Fig. 1E).

There were no differences in maximum responses for any of the measured cardioventilatory variables (Table 1). However, when comparing the temperatures where maximum cardioventilatory responses occurred, the temperatures for f_{Hmax} differed significantly among treatments, with the sotalol-treated fish reaching f_{Hmax} at a significantly higher temperature compared to the control (Table 1). No differences in the temperatures for Q_{max} or f_{Vmax} were observed among treatment groups.

3.3. Blood variables and physiological correlates of CT_{max}

No significant differences were found among treatment groups for any of the measured blood variables, relative ventricle mass (RVM) or relative spleen mass (RSM) (Table 1). However, linear correlation analysis in the control group found a significant positive correlation between CT_{max} and RSM ($r^2 = 0.473$, $p \leq 0.05$). No other significant correlations were found between CT_{max} and the tested variables (Table 2).

Table 2
Summary of correlation analysis between CT_{max} and hematological and morphometric variables measured at CT_{max} in the control group.

	R^2	p
Hct	0.003	0.88
Hb	0.001	0.91
MCHC	0.000	0.97
RVM	0.078	0.40
RSM	0.473*	0.02

* indicates statistically significant correlation ($p \leq 0.05$). Abbreviations are Hct (hematocrit); Hb (hemoglobin content); MCHC (mean corpuscular hemoglobin content); RVM (relative ventricle mass) and RSM (relative spleen mass).

4. Discussion

4.1. Thermal tolerance and effects of cardiac autonomic blockade

To our knowledge the linkages between autonomic cardiac control and thermal tolerance have not previously been examined in fish. This is somewhat surprising given that numerous studies suggest a relationship between cardiovascular oxygen transporting capacity and thermal tolerance in fishes (e.g. Casselman et al., 2012; Farrell et al., 2009), and a well-known role of the autonomic nervous system for cardiovascular performance in all vertebrates including fish (Sandblom and Axelsson, 2011). Even so, selective blockade of autonomic function did not significantly affect thermal tolerance in rainbow trout in the present study as CT_{max} was approximately 26 °C in all treatments (Table 1). The CT_{max} values reported here are within the range of previously reported thermal maxima for instrumented and uninstrumented rainbow trout (Beitinger and Bennett, 2000; Heath and Hughes, 1973; Rodnick et al., 2004). This suggests that the surgical procedures and the instrumentation of the fish in the present study did not have a major deleterious effect on thermal tolerance. However, it should be kept in mind that both inter- and intra-specific comparisons of CT_{max} are complicated due to differences among studies in e.g. heating rates in relation to body mass, as well as previous acclimation temperature that may greatly affect thermal responses and tolerance limits (Beitinger and Bennett, 2000; Beitinger and Lutterschmidt, 2011).

4.2. Cardioventilatory responses to temperature and importance of vagal mechanisms

Our data in the control fish are in agreement with previous studies demonstrating that rising temperature elevated $\dot{Q}$ mainly by increased f_H and not V_S (Cech et al., 1976; Clark et al., 2008; Farrell, 2009; Gamperl et al., 2011; Gollock et al., 2006; Mendonca and Gamperl, 2010; Sandblom and Axelsson, 2007b; Steinhausen et al., 2008). The temperature-mediated tachycardia in the present study did not significantly reduce V_S, which might otherwise be expected considering the negative force-frequency relationship (Shiels et al., 2002), as well as from the reduced diastolic filling time with increasing heart rates at elevated temperature (Sandblom and Axelsson, 2007a). In fact, V_S remained relatively constant until approaching the upper critical temperature indicating that cardiac filling pressure may have increased to compensate

for these effects (Clark et al., 2008). As temperatures approached CT_{max}, a slight increase in V_S was observed in the control fish. This response may have resulted from the decrease in f_H close to CT_{max}, which increased cardiac filling pressure and filling time (Altimiras and Axelsson, 2004).

While routine cardiac performance in fishes is influenced by tonic adrenergic and cholinergic input (Altimiras et al., 1997; Sandblom and Axelsson, 2011), few studies have explored how this autonomic control changes with acute temperature change (Axelsson et al., 1992; Franklin et al., 2001; Lowe et al., 2005). An estimate of the autonomic influence on the heart in the present study can be obtained by comparing the difference in f_H between the control group and the respective treatment groups. In the atropine-treated group, the numerical difference in f_H was the greatest between 17 and 23 °C, which likely reflected an increased vagal tone on the heart with increasing temperature (Fig. 1B). These results are similar to previous studies in the Antarctic bald notothen *P. borchgrevinki*, where the cholinergic tone increased by 39–57% (Franklin et al., 2001; Lowe et al., 2005) during rapid heating from −1 °C up to 6 °C. Whether this response is beneficial, detrimental or simply a side-effect of the elevated temperature *per se* is presently not clear (see Section 4). Even so, our study clearly shows that the increase in heart rate and cardiac output with increasing temperature does not involve a withdrawal of vagal tone.

There are several possible reasons why an increased cholinergic tone on the heart may be beneficial at high temperatures in fish. First, an active vagal suppression of heart rate may be an important mechanism to preserve cardiac scope. Salmonids can regulate cardiac output through changes in heart rate (Altimiras and Larsen, 2000), and so the maintained cholinergic tone on the heart even at temperatures close to CT_{max} ensures that cardiac output can be rapidly increased by withdrawal of vagal tone during situations of additionally elevated metabolic demand such as exercise. Second, vagally induced bradycardia during hypoxia has been proposed as a strategy to improve cardiac oxygenation by facilitating myocardial oxygen diffusion (Farrell, 2007). Similar to the situation in hypoxia, $P_{v_{O_2}}$ decreases with increasing temperature and when $P_{v_{O_2}}$ drops below a species-specific critical threshold cardiac failure may result from myocardial oxygen shortage (Farrell, 2007; Farrell and Clutterham, 2003). Based on this reasoning, we hypothesized that blockade of the vagal inhibition of the heart with atropine during an acute temperature challenge would result in an earlier onset of cardiac failure reducing CT_{max}. However, cardiac output was maintained to the same degree in the atropine-treated fish as in the control fish. This suggests that either a compensatory increase in anaerobic energy production maintained cardiac function or that myocardial oxygenation was sufficient for aerobic energy production (Fig. 1B). In the latter case it is possible that if luminal oxygen supply to the heart was reduced with the higher heart rate after atropine, a compensatory increase in coronary blood flow occurred that was either caused by abolished cholinergic coronary vasoconstriction from the atropine treatment, or by some other vasodilatory mechanisms intrinsic to the coronary vasculature (Farrell and Johansen, 1995; Gamperl et al., 1995).

4.3. Adrenergic control of cardiac function and thermal tolerance

Cardiac stimulation by catecholamines binding to β-adrenergic receptors modulates cardiac function by increasing both cardiac chronotrophy and inotropy (Nilsson, 1983; Sandblom and Axelsson, 2011). While elevated temperature can have detrimental effects on cardiac contractility (Aho and Vornanen, 2001; Driedzic and Gesser, 1994; Hanson et al., 2006), this effect can be alleviated by adrenergic stimulation of myocardial strips *in vitro* (Aho and Vornanen, 2001) and in *in situ* perfused hearts (Graham and Farrell, 1989; Hanson et al., 2006). Thus, we hypothesized that pharmacological removal of this cardioprotective adrenergic influence would lead to a reduction in contractility and V_S at high temperatures. However, sympathetic blockade did not affect thermal tolerance or cardiac output, and V_S was maintained or even appeared to increase slightly at the highest temperatures. The maintained V_S may have been attributed to a compensatory increase in cardiac filling pressure (Clark et al., 2008), but whether the venous filling pressure increased even more in the sotalol-treated fish to stimulate cardiac contractility via the Frank–Starling mechanism is not known, but could be interesting to explore in future studies.

While the β-adrenergic inhibition with sotalol caused a significant reduction in f_H relative to the untreated fish up to 17 °C, it was not significantly different from control values at higher temperatures. However, it does not appear that this response was due to a reduced blocking effect of the β-adrenergic antagonist with increasing temperature, but rather by an increased vagal inhibition of f_H in control fish at temperatures above 17 °C that was not present in the sotalol-treated fish. The mechanisms for this is not clear, but it is possibly due to an unspecific effect of the β-blocker as found by Agnisola et al. (2003) where the effect of electrical vagal stimulation in an *in situ* perfused dogfish heart was significantly reduced following β-adrenergic blockade. Thus, it is possible that the adrenergic blockade by sotalol elicited a similar inhibitory action on the vagal tonus in rainbow trout *in vivo* in the present study. The reduced difference in f_H between control and sotalol treated fish may also be explained by an overall reduced sensitivity of the myocardium to adrenergic stimulation at high temperatures, which has been previously reported in rainbow trout (Graham and Farrell, 1989; Shiels et al., 2003).

4.4. What sets thermal limits in fish?

The underlying determinants of the upper thermal limits in fish have been studied for over a century but are still not fully understood (Beitinger and Lutterschmidt, 2011; Clark et al., 2013). One of the current predominating hypotheses suggests that cardiac failure caused by myocardial oxygen limitation sets the upper thermal limit (Farrell et al., 2009; Portner and Farrell, 2008). However, little information exists on how myocardial oxygen supply and cardiac function are affected at critically high temperatures in fish. Further emphasis on the importance of oxygen transport on thermal tolerance is provided by Anttila et al. (2013) who found positive correlations between CT_{max} and relative ventricle mass and myoglobin levels in Atlantic salmon, *Salmo salar*. While we found no support for a linkage between CT_{max} and relative ventricle size, we did find a positive correlation between CT_{max} and relative spleen mass. However, the functional significance of this correlation remains speculative as we cannot determine whether a large relative spleen mass reflects a heightened capacity to release erythrocytes during the temperature challenge (Sandblom and Axelsson, 2007b), or if the larger spleens simply had emptied less completely. Even so, we did not find any significant correlations between CT_{max} and any of the recorded blood variables.

While cardiac oxygen limitation likely plays a role in cardiac failure at temperatures close to CT_{max} (Casselman et al., 2012; Farrell et al., 2009), it remains uncertain whether this is the direct causal factor determinig CT_{max}. In fact, other explanations for the underlying mechanisms behind thermal tolerance limits and why the heart fails at high temperature in ectotherms have been proposed. For example, Iftikar and Hickey (2013) suggested that cardiac failure with acute heating in *Notolabrus celidotus* does not result from lack of oxygen, but from impaired mitochondrial

function as well as from deterioration of enzymatic function and ATP production in myocardial cells. Lennard and Huddart (1991) demonstrated a cessation of action potential generation in rainbow trout hearts exposed to high temperatures and attributed this to changes in membrane fluidity causing disturbances in ion flux across the cardiomyocyte cell membrane. Along these lines, it was recently demonstrated that Na^+ channel function deteriorates at temperatures approaching CT_{max}, which compromises action potential transduction in cardiomyocytes from brown trout (*Salmo trutta*) (Vornanen et al., 2014). Interestingly, Friedlander et al. (1976) found similarities in the occurrence and loss of particular behavioral responses (e.g. coordination of body movement, loss of equilibrium, spams, etc.) when exposing whole fish to an acute temperature challenge and when heating only the cerebellum to critical temperatures. The loss of equilibrium occurred at similar temperatures in both treatments indicating that these responses were caused by central nervous failure. Again, it is possible that failing ion channels was the underlying mechanistic cause (Vornanen et al., 2014). Collectively these studies point to failure of electrical conduction systems as a possible underlying cause for organismal failure at high temperature, further emphasizing that studies on multiple physiological levels and functions are required to resolve what defines an animal's upper thermal limit.

5. Conclusions

In the present study we demonstrate for the first time that whole animal thermal tolerance is unaffected when autonomic control of cardiac function is manipulated in rainbow trout *in vivo*. Pharmacological blockade of the adrenergic component of the autonomic nervous system did not impair cardiac output and stroke volume, which may have been compensated for by an increased cardiac filling pressure at high temperatures. Furthermore, this study confirms that cholinergic tone on the heart in rainbow trout is maintained, and even increases, at temperatures approaching CT_{max}. While actively induced bradycardia did not enhance thermal tolerance in rainbow trout, the possible benefits of this control to maintain the scope for heart rate and possibly to facilitate myocardial oxygenation and cardiac contractility at high temperatures are interesting avenues for further research.

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