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RESEARCH ARTICLE

Autonomic cardiac regulation facilitates acute heat tolerance in rainbow trout: *in situ* and *in vivo* support

Matthew J. H. Gilbert[1,*], Varsha Rani[2], Sean M. McKenzie[1] and Anthony P. Farrell[1,2]

ABSTRACT

Acute warming in fish increases heart rate (f_H) and cardiac output to peak values, after which performance plateaus or declines and arrhythmia may occur. This cardiac response can place a convective limitation on systemic oxygen delivery at high temperatures. To test the hypothesis that autonomic cardiac regulation protects cardiac performance in rainbow trout during acute warming, we investigated adrenergic and cholinergic regulation during the onset and progression of cardiac limitations. We explored the direct effects of adrenergic stimulation by acutely warming an *in situ* working perfused heart until arrhythmia occurred, cooling the heart to restore rhythmicity and rewarming with increasing adrenergic stimulation. Adrenergic stimulation produced a clear, dose-dependent increase in the temperature and peak f_H achieved prior to the onset of arrhythmia. To examine how this adrenergic protection functions in conjunction with cholinergic vagal inhibition *in vivo*, rainbow trout fitted with ECG electrodes were acutely warmed in a respirometer until they lost equilibrium (CT_{max}) with and without muscarinic (atropine) and β-adrenergic (sotalol) antagonists. Trout exhibited roughly equal and opposing cholinergic and adrenergic tone on f_H that persisted up to critical temperatures. β-Adrenergic blockade significantly lowered peak f_H by 14–17%, while muscarinic blockade significantly lowered the temperature for peak f_H by 2.0°C. Moreover, muscarinic and β-adrenergic blockers injected individually or together significantly reduced CT_{max} by up to 3°C, indicating for the first time that cardiac adrenergic stimulation and cholinergic inhibition can enhance acute heat tolerance in rainbow trout at the level of the heart and the whole animal.

KEY WORDS: Upper thermal tolerance, Autonomic regulation, Heart rate, *Oncorhynchus mykiss*, Cardiac arrhythmia

INTRODUCTION

Teleosts are the most abundant class of fishes (>25,000 species) and utilize a variety of strategies in response to warming. Almost universally among these strategies is an increase in heart rate (f_H), a response that has been found in Arctic, Antarctic, tropical and temperate species (Eliason and Anttila, 2017; Farrell and Smith, 2017). Furthermore, where measured, the increase in f_H mediates an increase in cardiac output ($\dot{Q}$) (Brett, 1971; Ekström et al., 2016; Motyka et al., 2017; Penney et al., 2014; Steinhausen et al., 2008), which presumably services the increased tissue oxygen consumption, as inferred from a proportional increase in whole-animal oxygen uptake (Ekström et al., 2014; Eliason and Anttila, 2017; Farrell, 2009; Farrell and Smith, 2017; Sandblom et al., 2016a). However, as fish approach their upper thermal limit, f_H reaches a peak and thereafter may decline and become arrhythmic (Anttila et al., 2014; Badr et al., 2016; Casselman et al., 2012; Ekström et al., 2014; Eliason et al., 2011). While the peak f_H and the temperatures for peak f_H and arrhythmia vary appreciably among species and even strains, with acclimation temperature and with ontogeny (Chen et al., 2015; Drost et al., 2016), in all cases the inability to increase f_H may constrain convective oxygen delivery at temperature extremes (Eliason and Anttila, 2017; Eliason et al., 2011, 2013; Fry, 1947; Steinhausen et al., 2008). Thus, given the central role of f_H in meeting increased oxygen demand, there is considerable interest in why f_H reaches a peak at warm temperatures and why it can become arrhythmic with further warming (Eliason and Anttila, 2017; Vornanen, 2016, 2017).

Extreme warming frequently causes a delay or loss of the QRS complex despite a normal, rhythmic P-wave in the electrocardiogram (ECG) (Badr et al., 2016; Casselman et al., 2012; Vornanen, 2016). As such, Vornanen (2016) proposed a temperature-dependent depression of electrical excitability hypothesis (TDEE), which suggests that the inward Na^+ current (I_{Na}) responsible for ventricular depolarization is depressed relative to the resting outward K^+ current (I_{K1}) at warm temperatures such that the threshold potential needed to initiate an action potential cannot be reached (Vornanen, 2016; Vornanen et al., 2014). Other putative mechanisms also exist to explain cardiac failure with warming. For example, increased proton leakage in cardiac mitochondria at warm extremes decreases mitochondrial efficiency, which may compromise ATP production and cellular pH (Chung et al., 2017; Iftikar et al., 2014; Leo et al., 2017; O'Brien et al., 2018). Potentially compounding compromised ATP production is a reduced myocardial oxygen supply from luminal venous blood, which is essential in all teleosts; at extremely warm temperatures, venous oxygen partial pressure often declines and blood residence time in the heart decreases because of the elevated f_H (Eliason and Anttila, 2017; Eliason et al., 2013; Farrell and Clutterham, 2003; Steinhausen et al., 2008). In addition, the acidemia and hyperkalemia accompanying high temperature also likely impair cardiac contractility (Farrell et al., 1983; Farrell, 2009; Gesser and Jorgensen, 1982; Hanson et al., 2006).

Despite the uncertainty regarding the exact mechanism(s) for the decline in cardiac performance in teleosts at high temperature, autonomic regulation of f_H may protect cardiac function. Autonomic regulation via the sympathetic and parasympathetic control systems involves β-adrenergic stimulation and muscarinic-cholinergic inhibition of the teleost heart (Sandblom and Axelsson, 2011). Together, they regulate the spontaneous depolarization frequency of pacemaker tissue, modifying the excitability and conductivity of atrial, ventricular and nodal tissues. Importantly, acutely warming fish greatly increases circulating catacholamines (adrenaline and

[1]Department of Zoology, University of British Columbia, 6270 University Boulevard, Vancouver, BC, Canada V6T 1Z4. [2]Faculty of Land and Food Systems, University of British Columbia, 2357 Main Mall, Vancouver, BC, Canada V6T 1Z4.

*Author for correspondence (gilbert1@zoology.ubc.ca)

M.J.H.G., 0000-0001-8000-7568

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noradrenaline) (Currie et al., 2013, 2008), which are known to protect the performance of *in situ* perfused hearts, cardiac strips and isolated cardiomyocytes under a range of physiological challenges including hypoxia, acidosis, hyperkalemia and temperature (Aho and Vornanen, 2001; Farrell et al., 1996, 1986; Graham and Farrell, 1989; Hanson et al., 2006; Shiels et al., 2003). An elevated cardiac β-adrenergic receptor density is also associated with improved thermal tolerance in wild sockeye salmon (Eliason et al., 2011). Furthermore, adrenergic agonists have long been recognized in human medicine as an effective first line clinical treatment when atrioventricular (AV) conduction or ventricular excitability is limited (i.e. AV block-type arrhythmia) (Bellet, 1964; Marshall, 1935). Cholinergic regulation of the heart is restricted to direct innervation of the nodal and atrial tissue and, importantly, does not act on the ventricle (Sandblom and Axelsson, 2011; Vornanen, 2017). A cholinergic mediated depression of f_H at high temperatures could increase the time for electrochemical recovery, cardiac filling and oxygen diffusion between heartbeats, as well as decrease cardiac energy and oxygen demands (Ekström et al., 2014; Eliason and Anttila, 2017; Farrell, 2007).

Despite these multiple lines of evidence to suggest an autonomic protection of cardiac performance during extreme warming, only Ekström et al. (2014) have directly tested this hypothesis by blocking β-adrenergic and muscarinic receptors while measuring cardiac output in rainbow trout (*Oncorhynchus mykiss*) as they were acutely warmed until they lost equilibrium (CT_{max}). However, autonomic blockade had no significant effect on peak f_H, peak $\dot{Q}$ or CT_{max} (Ekström et al., 2014). Instead, the reduced β-adrenergic stimulation of f_H observed at high temperatures indicated a possible decline in β-adrenergic sensitivity, as suggested previously (Ask et al., 1981; Eliason and Anttila, 2017; Sandblom and Axelsson, 2011; Shiels et al., 2003). Nonetheless, the sensitivity of the heart to β-adrenergic stimulation has yet to be directly quantified at temperatures that induce cardiac arrhythmia and loss of equilibrium. Also, the effect of dual cholinergic and adrenergic blockade on thermal performance has not been studied. Therefore, our objective was to test the hypothesis that sympathetic and parasympathetic mechanisms protect the rainbow trout heart and enhance warm tolerance. To test this hypothesis, sympathetic cardio-protection was directly studied by acutely warming an *in situ* working, perfused heart to an arrhythmic temperature while adjusting the adrenaline concentration in the perfusate. We predicted that β-adrenergic stimulation can stabilize or improve f_H near the upper thermal tolerance limit of rainbow trout. Also, we investigated whether parasympathetic and sympathetic regulation *in vivo* protects f_H in rainbow trout (as measured with ECG electrodes in a respirometer) during acute warming with and without injections of β-adrenergic and muscarinic antagonists (independently and together). We predicted that sympathetic and parasympathetic control of f_H exists near the upper critical temperature (CT_{max}), which was measured as a humane endpoint for comparison of upper thermal tolerance with previous research.

MATERIALS AND METHODS

Animals

Animal housing and experimental protocols were approved by the University of British Columbia animal care committee (A16-0049 and A15-0035). Rainbow trout, *Oncorhynchus mykiss* (Walbaum 1792), obtained from Sun Valley Trout Farm (Mission, BC, Canada) were used to measure *in vivo* f_H and oxygen uptake (n=49; both sexes; body mass: 357±6 g, mean±s.e.m.). Rainbow trout obtained from Miracle Springs Hatchery (Mission, BC, Canada) were used to measure cardiac performance with an *in situ* working, perfused heart preparation (n=8; all female; body mass: 209±8 g). All fish were housed for at least 3 weeks prior to experimentation at the University of British Columbia Biological Sciences Aquatics Facility (Vancouver, BC) in 200–400 l holding tanks containing dechlorinated municipal tap water with partial recirculation and circular waterflow. The fish were acclimated to 12°C under a 12 h:12 h light:dark photoperiod.

Assessment of the effects of acute warming and adrenergic stimulation on f_H using an *in situ* working perfused heart preparation

The *in situ* working perfused heart preparation isolates the heart from any neural or humoral inputs while retaining an intact pericardium and allowing the heart to beat at its intrinsic, pacemaker rhythm, unless modulatory agents such as adrenaline are added to the perfusate (Farrell et al., 1988). As such, the preparation is ideal for examining the effects of acute warming on intrinsic f_H and the interactive effects of adrenergic regulation. The preparation in rainbow trout has previously been described in detail (Farrell et al., 1988; Hanson et al., 2006). Briefly, individual fish were anesthetized in freshwater at 12°C containing 100 mg l^{-1} tricaine methanesulfonate (TMS) before transfer to an acrylic surgery cradle where their gills were irrigated with 75 mg l^{-1} TMS (TMS solutions were buffered with 110 mg l^{-1} $NaHCO_3$). Inflow and outflow cannula were inserted into the sinus venous via a hepatic vein and the ventral aorta, respectively, and all major remaining veins returning to the heart were ligated. The preparation was transferred to a jacketed bath containing 0.9% NaCl at 12°C, where the heart was perfused at a constant filling pressure with a physiological perfusate (NaCl, 140 mmol l^{-1}; KCl, 2.8 mmol l^{-1}; $CaCl_2{\cdot}2H_2O$, 1.8 mmol l^{-1}; $MgSO_4$, 1.2 mmol l^{-1}; NaH_2PO_4, 1.2 mmol l^{-1}; TES acid, 3.9 mmol l^{-1}; TES sodium salt, 6.1 mmol l^{-1}; D-glucose, 10 mmol l^{-1}; pH 7.85 at 10°C). The perfusate was contained in jacketed reservoirs for temperature regulation and bubbled with 100% oxygen. Precise temperature control was achieved by attaching two recirculating chillers (Isotemp 3016D, Fisher Scientific, Hampton, NH, USA) to the bath and perfusate reservoirs with valves that allowed the temperature control fluid to flow through either chiller. One chiller was used to control the rate of warming and the other was maintained at a cold temperature (>10°C below target temperature) to allow rapid cooling when needed. Temperature was continuously recorded in the bath near to the heart (Microx 3, PreSens, Regensburg, Germany). Also, an ECG was acquired by placing one subdermal 21 gauge stainless steel electrode on the ventral surface over the heart, and another on the body. The inflow and outflow lines were fitted with pressure transducers (DPT 6100, Smiths Medical, Kirchseeon, Germany) to monitor filling pressure and afterload of the heart, respectively. A low afterload (2.94 kPa=30 cmH_2O) was selected for the study to limit the potential for rundown with the knowledge that afterload has minimal effect on f_H in the current preparation (Farrell et al., 1986). A flow transducer (SWF-4, Zepeda Instruments, Seattle, WA, USA) was placed in the outflow line and used to monitor cardiac output ($\dot{Q}$) at the start of the trial and ensure the preparations remained functional during warming. $\dot{Q}$ was initially set on the upper end of the published range for routine values at similar temperatures *in vivo* (~30 ml min^{-1} kg^{-1}; Farrell and Smith, 2017) by adjusting the filling pressure. Data for $\dot{Q}$, stroke volume and pressure measurements during the experiments are presented in Fig. S1 and Table S1, but were not corrected for the potential effects of temperature on measurement equipment. After the preparation had

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stabilized with an adrenaline-free perfusate (~10 min), temperature controllers warmed the bath saline and perfusate reservoirs at a continuous rate of 5°C h^{-1} until a temperature was reached at which the heartbeat became arrhythmic (T_{arr}). f_H did not exhibit marked beat-to-beat variation during warming and so the onset of arrhythmia was always unambiguous and characterized by a decrease in instantaneous f_H of approximately half or more when one or more beats were missed. At the onset of arrhythmia, the preparation was immediately and rapidly (~5–10 min) cooled by 1–2°C to restore a rhythmic heartbeat and stable $\dot{Q}$ before adrenaline was added to the perfusate (final concentration: 5 nmol l^{-1}). The preparation was then rewarmed at the same rate until cardiac arrhythmia was again observed. This protocol of warming to produce arrhythmia, cooling to restore rhythmicity and rewarming to produce arrhythmia was repeated while progressively increasing the adrenaline concentration in the perfusate (10, 100 and 1000 nmol l^{-1}). The expectation was that 1000 nmol l^{-1} would produce maximum adrenergic stimulation (Ask et al., 1981; Farrell et al., 1986; Graham and Farrell, 1989). The ECG signal was amplified and filtered (100×, low pass: 3 Hz, high pass: 100 Hz) with a Grass P55 pre-amplifier (Natus, CA, USA). All electrical signals were digitally displayed and recorded using a PowerLab data acquisition unit and Labchart 8.0 software (ADInstruments, Colorado Springs, CO, USA). The ECG was then digitally filtered (low pass: 4–10 Hz, high pass: 40–45 Hz) to optimize automated heartbeat detection. Accurate beat identification was ensured through visual inspection. A moving 30 s average of f_H was calculated during each experiment and peak f_H at each adrenaline concentration was taken as the maximum 30 s average at that concentration.

Assessment of *in vivo* f_H and oxygen uptake

Sub-dermal ECG electrodes represent a fairly minor surgical intervention to monitor f_H *in vivo* (Campbell et al., 2004) and follow f_H during acute warming. To this end, individual fish were anesthetized and transferred to a surgery cradle, as indicated above, to fit two 21 gauge sub-dermal needle electrodes placed diagonally on the ventral surface across the heart. An intraperitoneal (i.p.) cannula was inserted to permit injection of the antagonist drugs, as previously done by Ekström et al. (2014). The electrodes and the cannula were sutured in place at the site of insertion and at the rostral edge of the dorsal fin using 2-0 silk thread. Fish were placed into a 9.9 l respirometer (Loligo Systems, Viborg, Denmark) at 12°C to recover with recirculating flow and an automated 12 min intermittent flush cycle (420 s flush, 120 s wait and 180 s measure; Aquaresp 3.0 software, www.aquaresp.com; Svendsen et al., 2016). Fish were allowed to recover overnight for 15–18 h prior to the start of experimentation and random assignment to one of four treatment groups: saline control (0.9% NaCl), muscarinic blockade with atropine (1.2 mg kg^{-1}), general β-adrenergic blockade with sotalol (2.7 mg kg^{-1}) or dual blockade with both antagonists. All treatments were delivered as a 1 ml kg^{-1} 0.9% saline injection into the i.p. cannula, followed by a second 1 ml kg^{-1} injection of saline to flush the cannula. Following the injection, fish were left for 24 min (two oxygen uptake measurement cycles) to allow the drugs to take effect and observe a change in f_H. This wait period was shorter than in a previous study (Ekström et al., 2014) and the full effect of the drugs may have occurred shortly thereafter. Fish were then warmed in 1°C increments every 12 min (5°C h^{-1}) until they lost equilibrium (CT_{max}), at which point they were removed and killed with an overdose of buffered TMS (200 mg l^{-1}). Warming was achieved by recirculating tank water through a stainless steel coil in a pre-heated bath. During the experiments, oxygen concentration and temperature were continuously recorded every second using a 4-channel Firesting optical sensor (PyroScience GmbH, Aachen, Germany) while ECG signals were displayed and acquired as described above. *In vivo* f_H was calculated as the average over the final ~60 s segment at each temperature unless signal quality did not permit accurate beat detection, in which case a preceding ~60 s segment was used. If a fish did not complete a temperature step, final f_H was measured for ~60 s at that step. Peak f_H was then taken as the maximum average f_H from these ~60 s measurement periods. We also characterized the temperature at which an individual first approached its peak f_H by reporting the temperature at 95% of peak f_H. While this approach helped in dealing with situations where peak f_H exhibited a plateau, it underestimates the temperature when peak f_H was first reached by about 1°C. Because CT_{max} terminated the experiment, we report the percentage of fish that showed a plateau or collapse of peak f_H before CT_{max} was reached.

Data analysis

Repeated measures analysis of variance (ANOVA) was used to assess the effect of increasing adrenaline concentration on peak f_H and T_{arr} for the *in situ* perfused heart, with Holm's adjusted pairwise comparisons to evaluate differences among adrenaline concentrations. Linear mixed effects models were constructed with backwards elimination of non-significant interaction terms to assess differences in $\dot{M}_{O_2}$ and f_H between treatment groups during warming from 12 to 20°C. In both cases, body mass was included in the initial model as a covariate and fish ID was included as a random factor to account for repeated measures. The 12–20°C temperature range was chosen to avoid peak f_H being reached in some fish above 20°C. We tested the *a priori* predictions that f_H at a given temperature would be lower than control with sotalol treatment and higher with atropine treatment using one-tailed *t*-tests at each temperature whenever >3 individuals remained in the dataset. *P*-values were adjusted using the Benjamini–Hochberg procedure to control the false discovery rate. An ANOVA followed by Holm's adjusted pairwise comparisons was also used to assess *in vivo* differences in peak $\dot{M}_{O_2}$, temperature at peak $\dot{M}_{O_2}$, peak f_H, and temperature at 95% peak f_H and CT_{max} among treatment groups. As above, we used directional *a priori* predictions for the response of peak f_H with sotalol and atropine treatments and applied a one-tailed test for comparison with control fish. Again, body mass was included as a covariate in all ANOVA, but it was removed if it was not statistically significant. Differences in temperature at 95% peak f_H and CT_{max} among treatment groups were also evaluated as cumulative frequency or survival plots using Cox proportional hazards survival analysis. Data analysis and presentation were completed in R studio (R Development Core Team, 2014; http://www.R-project.org) and Sigmaplot 11.0 (Systat Software, San Jose, CA, USA) and α was set at 0.05 for all analyses.

RESULTS

In situ perfused heart

Adrenaline-free perfusion produced an intrinsic f_H at 12°C of 69.0±2.6 beats min^{-1}, which gradually increased during acute warming to 91.6±3.9 beats min^{-1} at 20°C (Q_{10}: 1.4±0.1; Fig. S1). Peak intrinsic f_H (96.8±6.2 beats min^{-1}) was reached at 22.0±1.0°C (Fig. 1), a value similar to the intrinsic f_H measured *in vivo* at 22°C after dual pharmacological blockade (101.0±6.4 beats min^{-1}; see below). Further warming resulted in an arrhythmic intrinsic heartbeat at 23.1±1.0°C.

Progressive increases in adrenaline concentration in the perfusate from 0 to 1000 nmol l^{-1} significantly increased, in a dose-dependent manner, both peak f_H (by 34%: 129.3±6.4 beats min^{-1}) and T_{arr} (by

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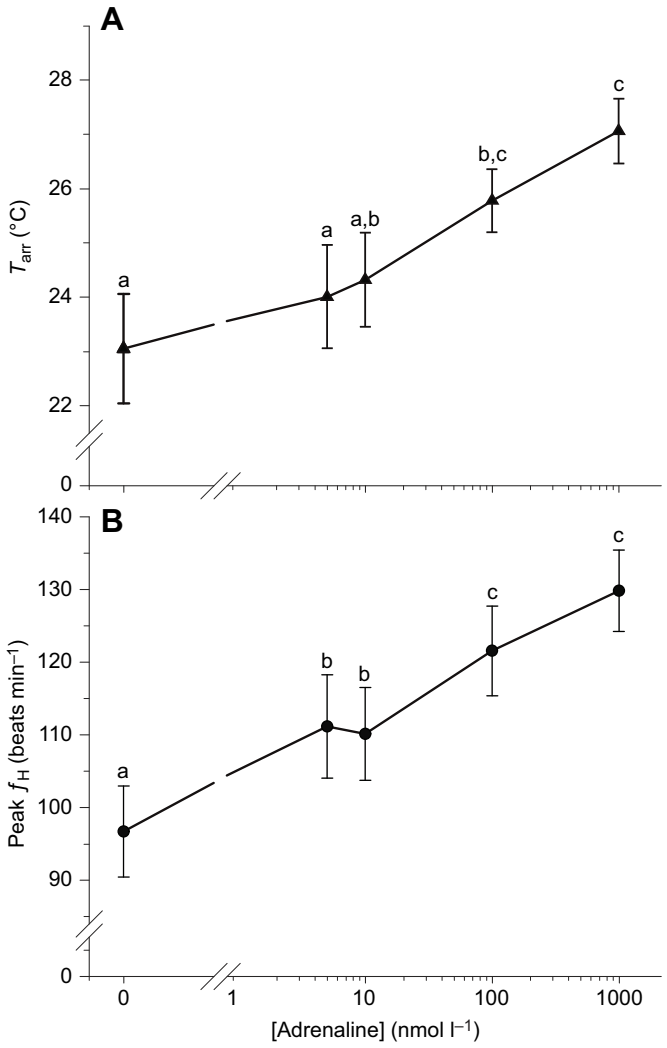

Fig. 1. Effect of increasing adrenergic stimulation and acute warming on temperature at arrhythmia and peak heart rate in an *in situ* perfused heart preparation. (A) Temperature at arrhythmia (T_{arr}). (B) Peak heart rate (f_H). Dissimilar letters indicate significant differences between adrenaline concentrations (α=0.05).

17%: 27.1±0.6°C) (peak f_H: $F_{4,38}$=19.2, P<0.001; T_{arr}: $F_{4,38}$=12.287, P<0.001; Fig. 1). Thus, the perfused heart performed significantly better during acute warming with β-adrenergic stimulation.

On average, peak f_H occurred 0.4±0.1°C below T_{arr} ($F_{1,77}$=3.76, P=0.06) and peak f_H was 4.5±0.9 beats min^{-1} higher than the average f_H at arrhythmia ($F_{1,77}$=8.55, P=0.005).

In vivo cardiorespiratory responses to acute warming with and without autonomic antagonists

As expected, $\dot{M}_{O_2}$ increased with temperature between 12 and 20°C (Q_{10}=2.1), a response that was not significantly different among treatment groups [treatment: $F_{1,40}$=1.27, P=0.298, temperature: $F_{1,349}$=508.82, P<0.001, log(mass): $F_{1,40}$=18.19, P<0.001; Figs 2 and 3]. Nevertheless, peak $\dot{M}_{O_2}$ during warming for the saline control group was significantly greater than for the dual antagonist treatment group, but not for either the atropine- or sotalol-treated group (treatment: $F_{3,40}$=4.19, P=0.01, mass: $F_{1,40}$=11.34, P=0.002; Table 1). The temperature at peak $\dot{M}_{O_2}$ was also higher in the saline group than in the atropine group (treatment: $F_{3,40}$=3.00, P=0.04, mass: $F_{1,40}$=13.73, P<0.001; Table 1).

Warming between 12 and 20°C increased f_H by a similar amount for all four treatment groups (saline Q_{10}=1.9; atropine Q_{10}=1.8; sotalol Q_{10}=1.8; both antagonists Q_{10}=2.0) (treatment: $F_{3,46}$=16.62, P<0.001, temperature: $F_{1,399}$=2303.62, P<0.001; Figs 2 and 3). As expected, atropine treatment significantly elevated f_H more than all the other treatments, while the sotalol-treated fish had a lower f_H than that of fish in all other treatments (Figs 2 and 3). In addition, f_H in fish treated with both antagonists was similar to f_H of the saline control fish (Fig. 3), indicating approximately equal and opposing adrenergic and cholinergic tone on the heart.

Peak f_H (and its plateau) occurred at a temperature lower than CT_{max} in all control and atropinized fish, but in only 62% of sotalol-treated fish and in only 83% of fish that received both antagonists (Figs 2 and 3). While peak f_H was not significantly different in the atropine and saline treatment groups, the temperature at which a fish reached 95% peak f_H was significantly lower (−9%) in atropinized fish than in control (treatment: $F_{3,46}$=6.76, P<0.001; Fig. 2, Table 1). Peak f_H in sotalol-treated fish was significantly lower than following the atropine (−17%) and control (−14%) treatments (treatment: $F_{1,40}$=3.65, P=0.02; Table 1). Peak f_H in fish that received both antagonists was intermediate to that of the sotalol and atropine treatment groups (Table 1).

Relative to the saline control group, CT_{max} was significantly reduced by a similar amount in all treatment groups (atropine −9.0%; sotalol −5.6%; both antagonists −6.8%) (treatment $F_{3,46}$=10.34, P<0.001, mass: $F_{1,46}$=11.57, P=0.001; Table 1, Fig. 4) such that CT_{max} was not significantly different among antagonist treatments.

DISCUSSION

It has long been recognized for teleosts that both f_H and $\dot{Q}$ increase with $\dot{M}_{O_2}$ during acute warming, but they plateau or collapse at critically warm temperatures (Brett, 1971; Eliason and Anttila, 2017; Farrell, 2009; Fry, 1947). Past research suggests that autonomic regulation and specifically β-adrenergic stimulation of

Table 1. Morphometrics, peak f_H and $\dot{M}_{O_2}$, and corresponding critical temperatures for 12°C-acclimated rainbow trout during acute warming with and without autonomic blockade

	Saline	+Atropine	+Sotalol	+Atropine+sotalol
n	11	14	13	12
Mass (g)	377±14	344±12	363±14	346±13
Length (cm)	31.4±0.8	30.9±0.4	31.3±1.4	30.9±1.3
Peak f_H (beats min^{-1})	125.0±6.0^{a}	129.7±3.9^{a}	107.5±4.2^{b}	116.4±6.5^{a,b}
Temperature at 95% peak f_H (°C)	24.3±0.3^{a}	22.3±0.3^{b}	23.8±0.3^{a}	23.1±0.4^{a,b}
Peak $\dot{M}_{O_2}$ (mg O_2 kg^{-1} min^{-1})	8.1±0.5^{a} (10)	7.3±0.4^{a,b} (13)	6.8±0.3^{a,b} (11)	6.6±0.5^{b} (11)
Temperature at peak $\dot{M}_{O_2}$ (°C)	24.8±0.7^{a} (10)	23.0±0.5^{b} (13)	24.1±0.3^{a,b} (11)	24.1±0.5^{a,b} (11)
CT_{max} (°C)	26.6±0.4^{a}	24.2±0.3^{b}	25.1±0.4^{b}	24.8±0.4^{b}

Data are presented as mean±s.e.m. Dissimilar letters indicate significant differences between treatments within a row. Sample size (*n*) is shown in parentheses if different from the total number of fish used per group.

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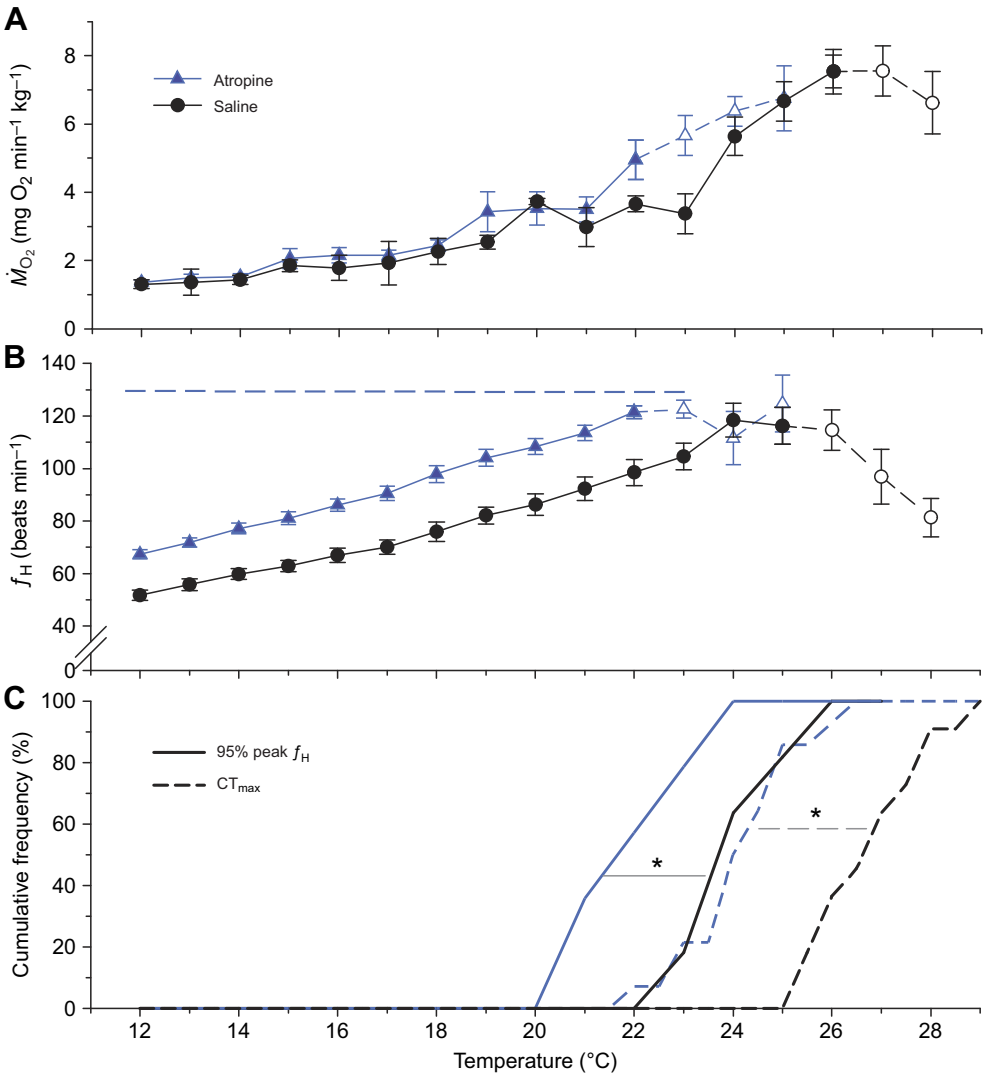

Fig. 2. *In vivo* oxygen uptake, f_H, and temperature at 95% peak f_H and loss of equilibrium in rainbow trout during acute warming with and without autonomic blockade. Data for (A) oxygen uptake ($\dot{M}_{O_2}$), (B) f_H and (C) 95% peak f_H and loss of equilibrium (CT_{max}) with (n=11) and without (n=14) atropine (a muscarinic receptor antagonist) are presented (means±s.e.m.). A dashed data line with open symbols indicates that individuals have been removed following loss of equilibrium, as shown in C. The horizontal dashed line indicates temperatures where f_H is significantly different between atropine and saline groups (Holm-adjusted pairwise comparisons; α=0.05). The asterisks and gray lines in C indicate a significant difference in distributions between treatments (Cox regression; P<0.001).

the heart may be cardio-protective during extreme warming (Aho and Vornanen, 2001; Eliason and Anttila, 2017; Eliason et al., 2011; Hanson and Farrell, 2007; Wood et al., 1979). However, Ekström et al. (2014) found no negative effects of autonomic blockade on cardiac or whole-organism thermal tolerance. In contrast, we found positive chronotropic and antiarrhythmic effects of β-adrenergic stimulation during acute warming of the *in situ* working heart preparation, and negative effects of *in vivo* autonomic blockade on both peak f_H and CT_{max}. As such, we argue below that autonomic regulation of f_H in rainbow trout can facilitate both cardiac and whole-organism thermal tolerance.

f_H, $\dot{M}_{O_2}$ and autonomic regulation over sub-critical temperatures

For commonly studied temperatures, our findings regarding f_H, $\dot{M}_{O_2}$ and autonomic tone align well with current literature. For example, the increase in routine f_H and $\dot{M}_{O_2}$ that we observed with acute warming is well documented for salmonids, typically increasing with Q_{10} coefficients of between 1.5 and 2.5 for f_H and 2 and 4 for $\dot{M}_{O_2}$, as found here (Eliason and Anttila, 2017; Motyka et al., 2017; Penney et al., 2014; Rodnick et al., 2004; Steinhausen et al., 2008). While substantial variation in absolute $\dot{M}_{O_2}$ and f_H values can exist among studies given variable genetic (e.g. between species and strains), environmental (e.g. thermal history) and methodological considerations, notable similarities still exist between our results and previous ones. For instance, desert redband rainbow trout not only increased $\dot{M}_{O_2}$ during acute warming from 14 to 26°C (1.96 to 6.86 mg O_2 kg^{-1} min^{-1}; Rodnick et al., 2004) with a quantitatively similar range to that seen here (1.63 to 7.24 mg O_2 kg^{-1} min^{-1}) but also exhibited a sharp transition at 24°C, as observed in the present study (Fig. 2A). Whether this sharp rise is related to an innate escape behavioral response to thermal stress, which has been documented previously (Peterson and Anderson, 1969), or a biochemical response is unknown. We also observed cholinergic and adrenergic cardiac tone and our estimates suggest they remained stable and approximately equal (~20–30% of intrinsic f_H) up to near-critical temperatures, a suggestion supported by the fact that intrinsic and control f_H were nearly identical. Autonomic cardiac tone is normally calculated based on sequential injections of cholinergic and β-adrenergic antagonists (Altimiras et al., 1997), something that our study design precluded us from doing. Nevertheless, our estimates are within reported ranges for salmonids (i.e. 20–70% of intrinsic f_H; Axelsson and Farrell, 1993; Ekström et al., 2016; Gamperl et al., 1995; Sandblom and Axelsson, 2011).

Beside the present study, only Ekström et al. (2014) have specifically investigated the role of autonomic regulation of f_H during acute warming to CT_{max}. At temperatures below CT_{max}, the quantitative similarities of the two studies are remarkable. For

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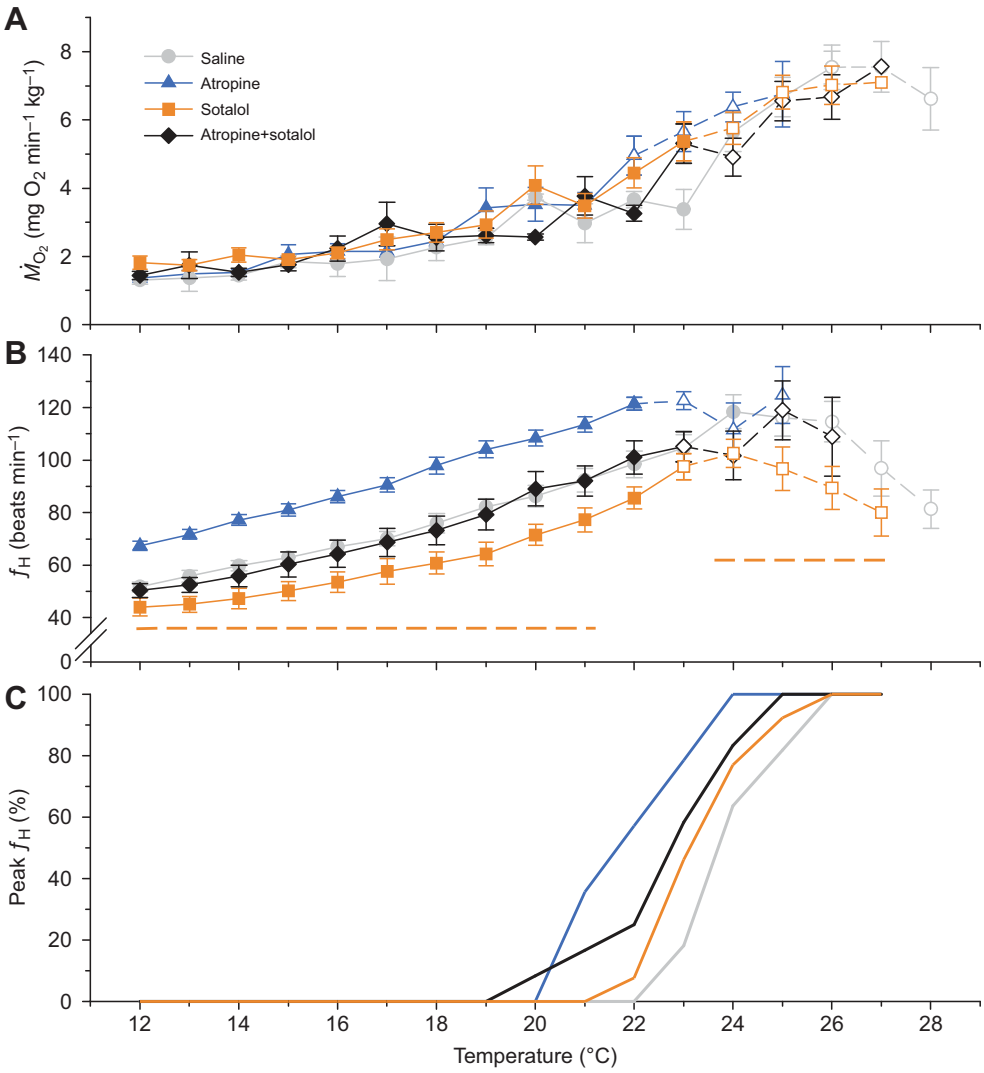

Fig. 3. *In vivo* $\dot{M}_{O_2}$, f_H, and temperature at 95% peak f_H and loss of equilibrium in rainbow trout during acute warming with β-adrenergic or dual blockade. Data for (A) $\dot{M}_{O_2}$, (B) f_H and (C) 95% peak f_H in the presence of sotalol (β-adrenergic receptor antagonist, n=13) or both atropine and sotalol (dual blockade, n=12) are presented (means±s.e.m.). Data for trout treated with saline or atropine (Fig. 2) are included for reference. A dashed data line with open symbols indicates that individuals have been removed following loss of equilibrium (Fig. 4A). The horizontal dashed line indicates temperatures where f_H is significantly different between atropine and saline groups (Holm-adjusted pairwise comparisons; α=0.05).

instance, atropine and sotalol treatment had a similar magnitude of effect on f_H, and f_H in all common treatments (saline, atropine and sotalol) increased with temperature in a nearly identical manner [e.g. atropine 12–20°C: 68 to 106 beats min^{-1} for Ekström et al. (2014) versus 67 to 108 beats min^{-1} in the present study]. A subtle difference between the two studies is that cholinergic tone appeared to increase at warm temperatures in Ekström et al. (2014) but was unchanged in the present study. Intrinsic f_H in the present study was similar to routine f_H and intermediate to that of atropine- and sotalol-treated fish in both studies, suggesting similar autonomic tone in the two studies even though Ekström et al. (2014) did not assess intrinsic f_H. Ekström et al. (2014) also did not measure routine $\dot{M}_{O_2}$, but instead measured ventilation rate and opercular pressure, both of which increased exponentially with temperature over sub-critical temperatures and were not affected by autonomic blockade, as was the case for $\dot{M}_{O_2}$ in the present study. These similarities over moderate temperatures are highlighted to contrast the novelty of our findings at extreme warm temperatures regarding the cardio-protective role of autonomic regulation of f_H.

Cardio-protective effects of autonomic regulation at high temperatures

Previous *in situ* and *ex vivo* studies with rainbow trout have demonstrated the positive inotropic (Farrell et al., 1996; Shiels and Farrell, 1997; Shiels et al., 2003) and chronotropic (Aho and Vornanen, 2001) effects of β-adrenergic stimulation (up to 22°C). β-Adrenergic stimulation is also known to be protective under adverse cellular conditions such as acidemia, hypoxemia and hyperkalemia (Hanson et al., 2006; Roberts and Syme, 2018), which are likely to occur *in vivo* at high temperatures. Furthermore, elevated β-adrenergic receptor density in sockeye salmon has been associated with improved aerobic performance at high temperatures (Eliason et al., 2011). However, the potential for direct β-adrenergic stimulation of the heart to protect against cardiac collapse with acute warming to critically warm temperatures has not previously been investigated. Here, we show that progressive β-adrenergic stimulation repeatedly rescued an *in situ* perfused working heart from warm-induced cardiac arrhythmia and increased peak f_H (+23%). Correspondingly, β-adrenergic blockade *in vivo* reduced the peak f_H attained during extreme acute warming (by 14–17%). Peak f_H of the perfused heart with maximal adrenergic stimulation was also similar to that observed *in vivo* in atropinized trout, suggesting that a similar stimulation may occur *in vivo* in the absence of cholinergic inhibition. Together, these results definitively show for the first time that β-adrenergic stimulation can facilitate achievement of peak f_H at critically warm temperatures, and is thus cardio-protective during extreme warming.

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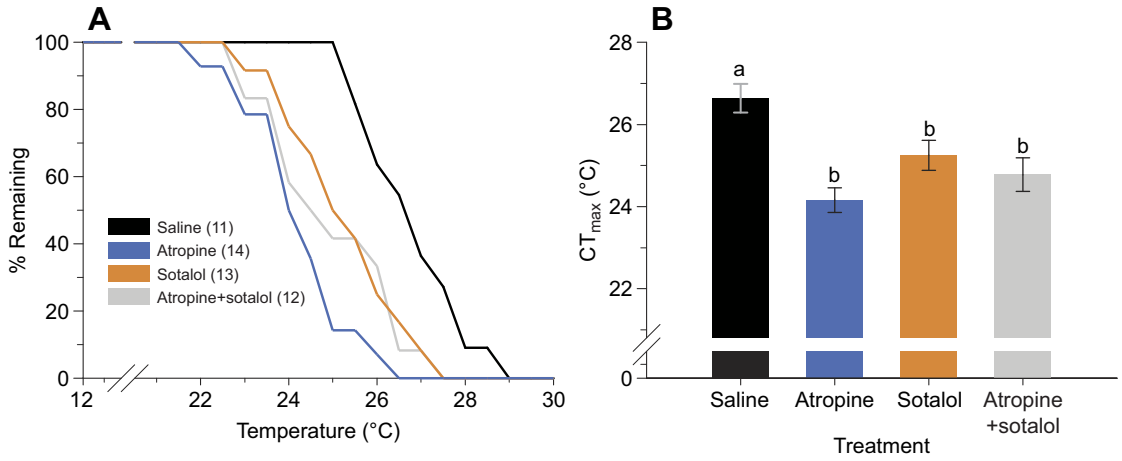

Fig. 4. Thermal survival curves and CT_{max} for rainbow trout with and without autonomic blockade. (A) Survival curves. (B) CT_{max}. Dissimilar letters indicate significant differences in CT_{max} between treatment groups (Holm-adjusted pairwise comparisons; α=0.05). Starting sample sizes for each treatment group are shown in parentheses.

Off-target effects of sotalol such as partial potassium channel blockade (Lynch et al., 2008) or actions on other aspects of circulatory physiology (e.g. blood pressure, vascular tone and red blood cell β-NHE activity) (Perry and Bernier, 1999; Sandblom and Axelsson, 2011) may have contributed, in part, to the reduction in peak f_H and CT_{max} observed with sotalol treatment. However, given the similarity in the change in peak f_H *in situ* and *in vivo* with β-adrenergic stimulation, and the fact that in mammals inhibition of K^+ channels by sotalol does not typically occur until much higher doses than were used here (Lynch et al., 2008), we expect that the off-target effects of β-adrenergic blockade were marginal.

The relative β-adrenergic sensitivity of the rainbow trout heart tends to decrease with increasing temperature (Ask et al., 1981; Ekström et al., 2014; Eliason and Anttila, 2017; Keen et al., 1993; Sandblom and Axelsson, 2011; Shiels et al., 2003). For instance, Graham and Farrell (1989) found that for *in situ* perfused rainbow trout hearts, maximum adrenergic stimulation increased routine f_H and maximum $\dot{Q}$ by 56% and 61% at 5°C, but by only 20% and 23% at 15°C. Both our study and that of Ekström et al. (2014) partially support this generalization, as we did not find a significant β-adrenergic tonus at a critically warm temperatures, while β-adrenergic tone appeared to remain stable at sub-critical temperatures. Our study also suggests that while inotropic and chronotropic effects may be relatively diminished at high temperatures, β-adrenergic stimulation has a vital stabilizing role on heart function that was not captured in past assessments of β-adrenergic sensitivity. This stabilization does not directly increase f_H at critically warm temperatures but rather allows the heart to function at a higher temperature with a higher peak f_H. Thermal history must also be taken into consideration in discussions of β-adrenergic sensitivity (Eliason and Anttila, 2017). Many studies used to develop the generality that cardiac β-adrenergic sensitivity is lower at high temperatures have been conducted in warm-acclimated fish rather than acutely exposed fish. With acclimation to warm temperatures rainbow trout tend to down-regulate myocardial β-adrenoceptor expression (Gamperl et al., 1998; Keen et al., 1993). As such, β-adrenergic stimulation may not have the same cardio-protective effects at high temperatures after warm acclimation, and conversely may be relatively more important for acute cardiac heat tolerance following cold acclimation.

The collapse of f_H and loss of cardiac rhythmicity that occurs in fish at high temperatures is associated with a depression in ventricular excitability because the ECG often has delayed or missing QRS complexes even though the P-wave tends to remain rhythmic (Badr et al., 2016; Casselman et al., 2012; Eliason and Anttila, 2017). Vornanen (2016) has attributed this depression to an imbalance in I_{Na} and I_{K1}. Although a direct mechanism by which β-adrenergic stimulation could circumvent or rectify this I_{Na} and I_{K1} imbalance has not been studied in fish, one possible linkage is through a depression of I_{K1}, which occurs in some mammals (Koumi et al., 1995). Indeed, the K^+ channels responsible for generating I_{K1} in fish do have phosphorylation sites that could allow for β-adrenergically mediated modification (Hassinen et al., 2007; Vornanen, 2017). β-Adrenergic stimulation could also enhance rapid Ca^{2+} influx at relatively low membrane potentials through modification of T-type Ca^{2+} channels and the Na^+/Ca^{2+} exchanger (NCX), which could supplement I_{Na} and improve ventricular excitability at high temperatures. While this hypothesis will require further investigation, both T-type Ca^{2+} channels and the NCX are active in atrial and ventricular myocytes in some fish (Alday et al., 2014; Nemtsas et al., 2010; Vornanen, 2017). Furthermore, Ca^{2+} influx through NCX is enhanced with β-adrenergic stimulation in zebrafish (Xie et al., 2008), and in mammals, β-adrenergic stimulation enhances Ca^{2+} influx through both NCX and T-type Ca^{2+} channels, which contributes to cardiac excitation (Herrmann et al., 2013; Kaese et al., 2017; Li et al., 2012, 2018). Alternatively, Ca^{2+} kinetics may limit the ability of the ventricle to keep up with the pacemaker rate and an imbalance in I_{Na} and I_{K1} may not be to blame. If so, classical β-adrenergically mediated modification of Ca^{2+} cycling would account for the cardio-protective effects observed here.

As in Ekström et al. (2014), cholinergic tone in the present study persisted up to the point where f_H began to fail. This inhibitory action on pacemaker rate appeared necessary to sustain increasing f_H with temperature, as f_H began to peak at a lower temperature when cholinergic tone was blocked (−2°C). This limitation on f_H at high temperatures is unlikely to be a pacemaker limitation because Haverinen et al. (2017) showed that intrinsic pacemaker and sinoatrial rates continue to increase well above the temperature where f_H peaks, indicating the maximum pacemaker rate exceeds the maximum sustainable firing rate of atrial or, more likely, ventricular tissue (Haverinen et al., 2017). As such, we suggest that during extreme warming, cholinergic inhibition of pacemaker tissue acts as a brake to keep the pacemaker firing at a rate that is within the functional range of the ventricle. If the pacemaker rate did exceed

the physiological maxima of the ventricle, it would cause a progressive desynchrony in electrochemical events, which could account for the premature peak and decrease in f_H seen here with atropine treatment. Furthermore, the slowing of f_H through cholinergic inhibition would also allow for longer filling duration and blood residence time, which in turn could improve cardiac efficiency via the Frank–Starling mechanism and increase myocardial oxygenation (Eliason and Anttila, 2017; Farrell, 2007).

Autonomic regulation facilitates CT_{max}

The decline in cardiac thermal tolerance that we observed in the absence of β-adrenergic stimulation and cholinergic inhibition was associated with a decrease in CT_{max}. Likewise, Ekström et al. (2017) found that coronary ligation reduced cardiac thermal tolerance and was also associated with a reduction in CT_{max}. It is important to note that in contrast to the present study, Ekström et al. (2014) did not find any reduction in CT_{max} with either atropine or sotolol treatment. However, given that they also did not identify any significant limitations in f_H with these treatments, this is not surprising. The mechanism by which a reduction in cardiac performance would reduce CT_{max} has not been explicitly tested. Yet, it is reasonable to postulate that one contributing factor would be impaired oxygen delivery to vital tissues. Given that maintaining equilibrium does not have a substantial aerobic cost, there is no *a priori* reason to expect that CT_{max} would necessarily be determined by oxygen availability. Instead, without additional stressors, loss of equilibrium at high temperatures may occur as a result of a direct temperature induced loss of nervous function (Friedlander et al., 1976) or some other indirect effect (e.g. acidosis). However, if this is the case, additional stressors (e.g. environmental hypoxia), compromised oxygen delivery (e.g. through coronary ligation) or elevated aerobic demand (e.g. elevated activity; Peterson and Anderson, 1969) could impose an oxygen limitation on CT_{max}, as was observed by Ern et al. (2017) when environmental P_{O_2} was reduced. As such, coronary ligation (Ekström et al., 2017) and autonomic blockade could sufficiently limit heart function and associated tissue oxygenation to reduce CT_{max}. Correspondingly, peak $\dot{M}_{O_2}$ in all treatment groups in the present study tended to be lower than the control, although this difference was only significant when the two antagonists were applied together. That said, given that $\dot{M}_{O_2}$ was not significantly greater in the control group at any given temperature, this reduction in peak $\dot{M}_{O_2}$ may be a result of reduced CT_{max} rather than a direct cause. To test this, tissue oxygen supply and other aerobically sensitive measures of thermal tolerance (e.g. aerobic scope or swimming performance) should be tested with and without autonomic regulation in the future to determine the extent to which oxygen delivery is compromised at high temperatures in the absence of autonomic regulation.

Conclusion

Our findings clearly demonstrate that, as previously hypothesized (Ekström et al., 2014; Eliason and Anttila, 2017; Farrell, 2007), β-adrenergic stimulation and cholinergic inhibition of the heart can facilitate acute cardiac and whole-organism heat tolerance. Autonomic regulatory physiology, however, varies extensively with thermal history as well as across genera, among closely related species and even between strains (Eliason et al., 2011; Gamperl et al., 1998; Sandblom and Axelsson, 2011; Sandblom et al., 2016b; Wood et al., 1979), which may explain why Ekström et al. (2014) did not find significant effects of autonomic blockade on cardiac or whole-organism heat tolerance whereas we did, despite using a similar methodology. Given these marked differences and the well-documented diversity in autonomic regulatory physiology, further investigation is warranted to determine how cardiac autonomic regulation influences heat tolerance with environmental variation among teleosts.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.J.H.G., A.P.F.; Methodology: M.J.H.G., A.P.F.; Software: M.J.H.G.; Validation: M.J.H.G., V.R., S.M.M.; Formal analysis: M.J.H.G., V.R., S.M.M., A.P.F.; Investigation: M.J.H.G., V.R., S.M.M.; Resources: M.J.H.G.; Data curation: M.J.H.G.; Writing - original draft: M.J.H.G.; Writing - review & editing: M.J.H.G., A.P.F.; Visualization: M.J.H.G.; Supervision: M.J.H.G., A.P.F.; Project administration: M.J.H.G., A.P.F.; Funding acquisition: A.P.F.

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Supplementary information

Supplementary information available online at
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