

# Exploring nature's natural knockouts: *In vivo* cardiorespiratory performance of Antarctic fishes during acute warming

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**Summary statement:** Routine cardiac output in the haemoglobinless icefish, *Chaenocephalus aceratus* is lower than previously reported. They show a large cardiorespiratory scope during acute warming and activity, and show the same cardiac breakpoint temperature as the red-blooded Antarctic fish *Notothenia coriiceps*.

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## Abstract

We tested the hypothesis that Blackfin icefish (*Chaenocephalus aceratus*), one of the six species in the family Channichthyidae (the icefishes) that do not express haemoglobin and myoglobin, lack regulatory cardiovascular flexibility during acute warming and activity. The experimental protocols were designed to optimize the surgical protocol and minimize stress. First, minimally invasive heart rate ( $f_H$ ) measurements were made during a thermal ramp until cardiac failure in *C. aceratus* and compared with the closely related red-blooded Black rockcod (*Notothenia coriiceps*). Then, integrative cardiovascular adjustments were more extensively studied using flow probes and intravascular catheters in *C. aceratus* during acute warming (from 0 to 8°C) at rest and after imposed activity. *C. aceratus* had a lower routine  $f_H$  than *N. coriiceps* (9 min<sup>-1</sup> vs. 14 min<sup>-1</sup>) and a lower peak  $f_H$  during acute warming (38 min<sup>-1</sup> vs. 55 min<sup>-1</sup>) with a similar cardiac breakpoint temperature (13 and 14°C, respectively). Routine cardiac output ( $\dot{Q}$ ) for *C. aceratus* at ~0°C was much lower (26.6 ml min<sup>-1</sup> kg<sup>-1</sup>) than previously reported, likely because fish in the present study had a low  $f_H$  (12 min<sup>-1</sup>) indicative of a high routine vagal tone and low stress. *C. aceratus* increased oxygen consumption during acute warming and with activity. Correspondingly,  $\dot{Q}$  increased considerably (maximally 86.3 ml min<sup>-1</sup> kg<sup>-1</sup>), as did vascular conductance (five-fold). Thus, unlike earlier suggestions, these data provide convincing evidence that icefish can mount a well-developed cardiovascular regulation of heart rate, cardiac output and vascular conductance, and this regulatory capacity provides flexibility during acute warming.

## Introduction

The notothenioid suborder of fishes that are dominant in the frigid Southern Ocean include the remarkable haemoglobinless (Hb<sup>-</sup>) Channichthyids (icefishes) (O'Brien and Crockett, 2013). The Hb<sup>-</sup> state renders icefishes unique amongst adult vertebrates (Ruud, 1954) and a suite of compensatory cardiovascular modifications accompanies the corresponding reduction in blood oxygen carrying capacity when compared with closely related red-blooded nototheniids (Axelsson, 2005), such as an enlarged heart (Holeton, 1970; Harrison et al., 1991) and greatly increased baseline vascular conductance (Hemmingsen and Douglas, 1977), which together permit a much greater cardiac output ( $\dot{Q}$ ) than other fishes (Hemmingsen et al., 1972; Hemmingsen, 1991; Axelsson, 2005).

It is generally believed that icefishes can tolerate the Hb<sup>-</sup> condition in part because of the extremely cold and stable temperature (~0°C) of the Southern Ocean, which not only increases the solubility of oxygen in seawater and the blood plasma (Ruud, 1954) but also results in a stable and low metabolic rate. Consequently, the fact that the area around the West Antarctic Peninsula, which is where many species of icefishes are found, is among the world's most rapidly warming biomes (Meredith and King, 2005; Clarke et al., 2007), is of considerable concern. In this region, average surface water temperatures have risen by approximately 1°C over the past 50 years, and this rate is expected to continue for the next century (IPCC, 2014), which could create a precarious situation for icefishes.

The lack of haemoglobin (and myoglobin) in icefishes is expected to be particularly problematic as the demand for oxygen transport increases during warming (Ruud, 1954; O'Brien and Crockett, 2013; Buckley et al., 2014; Beers and Jayasundara, 2015), but we do not understand how they are likely to respond. In fact, the only published study on oxygen consumption ( $\dot{M}_{O_2}$ ) of *C. aceratus* discovered that  $\dot{M}_{O_2}$  increased only between 1 and 4°C, but not with additional warming to 10°C (Hemmingsen and Douglas, 1972). Icefishes also show a lower critical thermal maximum (CT<sub>MAX</sub>) than sympatric Hb<sup>+</sup> nototheniids (Beers and Sidell, 2011). While these observations suggest that warming could create problems with internal oxygen transport, CT<sub>MAX</sub> was insensitive to hyperoxia (Devor et al., 2016). Added to this uncertainty, little certainty surrounds the regulatory flexibility of cardiovascular function in icefishes because previous measurements of  $\dot{Q}$  are conflicting.

For example, previous indirect estimates of routine  $\dot{Q}$  in *C. aceratus* (using the Fick principle) vary almost three-fold from 60 ml min<sup>-1</sup> kg<sup>-1</sup> (Holeton, 1970) to 100-150 ml min<sup>-1</sup> kg<sup>-1</sup>

(Hemmingsen et al., 1972). Furthermore, this range for routine  $\dot{Q}$  estimates could be even wider given the direct measurement of blood flow in the two cranial pairs of gill arches (10-15 ml min<sup>-1</sup> kg<sup>-1</sup>), which equates to routine  $\dot{Q}$  being 20 – 30 ml min<sup>-1</sup> kg<sup>-1</sup> by assuming equal blood flow in all branchial arteries (Hemmingsen et al., 1972). Despite this rather large range, the paradigm of a high routine  $\dot{Q}$  in icefishes (80-100 ml min<sup>-1</sup> kg<sup>-1</sup>) relative to Hb<sup>+</sup> species still persists in the literature (Hemmingsen, 1991; Tota et al., 1991; Zummo et al., 1995; Acierno, et al. 1997; Feller and Gerday, 1997; Sidell and O'Brien, 2006; Axelsson, 2005; Garofalo et al., 2009). Certainly, maximum  $\dot{Q}$  of icefishes determined *in vitro* with perfused heart preparations can be high (maximum  $\dot{Q}$  values between 100 and 300 ml min<sup>-1</sup> kg<sup>-1</sup> in *Chionodraco hamatus* at 3°C; Acierno et al., 1997; Tota et al., 1991). Nonetheless, our own recent work *in vitro* with perfused heart preparations found a lower maximum  $\dot{Q}$  in both *C. aceratus* (70 ml min<sup>-1</sup> kg<sup>-1</sup>) and *Chionodraco rastrospinosus* at 1°C (126 ml min<sup>-1</sup> kg<sup>-1</sup>) (Egginton et al., submitted). Thus, if routine  $\dot{Q}$  for icefishes is close to maximum  $\dot{Q}$ , an obvious concern is that regulatory capacity is necessarily limited and there can be little cardiovascular flexibility during environmental challenges.

The aim of the present study was to test the hypothesis that *in vivo* cardiovascular function in *C. aceratus* lacks regulatory flexibility during acute warming. Comparisons could then be made with cardiorespiratory regulatory capacity of Hb<sup>+</sup> nototheniids (e.g., Axelsson et al., 1992; 1994; Franklin et al., 2007; Campbell et al., 2009; Egginton and Campbell, 2016).

Previous studies have concluded that when Antarctic fishes are acutely warmed, they primarily adjust heart rate ( $f_H$ ) to regulate cardiac output ( $\dot{Q}$ ) (Axelsson et al., 1992; Campbell et al., 2009), much like other fishes (Farrell, 2009; Farrell and Smith, 2017). Also, while some studies report a high routine vagal tone in Antarctic notothenioids (Axelsson et al., 1992; Egginton and Campbell 2016), Hemmingsen et al. reported only a small increase in  $f_H$  after atropine injection (from 16 to 18 beats min<sup>-1</sup>) that could indicate stress (Hemmingsen et al., 1972). Therefore, we tested the possibility that earlier *in vivo* cardiovascular studies were affected by stress that would have an impact on other measured variables such as  $\dot{Q}$  and cardiac power output. We used a minimally invasive method (electrocardiogram (ECG) measurements during an acute thermal ramp with and without autonomic vagal blockade with atropine) to provide insights into routine  $f_H$ , the level of vagal tone and the contribution of  $f_H$  in setting upper thermal tolerance (Farrell, 2009; Farrell, 2016). The results from these minimally instrumented animals were used as a baseline for the more heavily instrumented animals that were part of the first comprehensive assessment of *in vivo* cardiorespiratory response of *C. aceratus* during activity and acute (sub-critical) warming. By simultaneously measuring a number of key

cardiorespiratory variables, we could directly assess the flexibility and interplay of autonomic regulation and oxygen transport and address if the Hb<sup>-</sup> condition reduces cardiac scope and thus their ability to tolerate temperature changes.

## Materials and methods

### *Experimental Animals*

Given our concerns about physiological measurements being made on overly stressed animals, the ECG measurements on blackfin icefish (*Chaenocephalus aceratus* Lönnberg; N=12, body mass (BM)=1.55±0.21 kg (mean ± SD)) were also performed on the closely related Hb<sup>+</sup> black rockcod (*Notothenia coriiceps* Richardson; N=13, BM=1.24±0.32 kg), which allows comparison with previously published data on other Hb<sup>+</sup> Antarctic fishes. An additional group of *C. aceratus* were also instrumented for ECG measurements and used to ascertain autonomic tones (n=4, body mass 1587.5±387.0 kg). Finally, a separate group of *C. aceratus* (N=16, BM=0.85±0.21 kg) were used for the more invasive cardiorespiratory study. Adult fish were captured at 100-200 m depth using an otter trawl and baited benthic pots (for *N. coriiceps*) deployed from the ARSV *Laurence M. Gould* in Dallmann Bay (64°10'S, 62°35'W) and off the south-western shore of Low Island (63°24'S, 62°10'W). Fish were held on the ship in circulating, aerated seawater tanks for up to two days before being transferred to and held at ambient seawater temperature (0±1°C) at Palmer Station, Antarctica for at least 72 h and up to 3 weeks before experiments commenced. *N. coriiceps* were fed blocks of fish muscle fillet every other day, but not 24-48 h prior to surgery, whereas *C. aceratus* did not feed in captivity.

### *Surgical instrumentation for in vivo upper thermal tolerance test while measuring the electrocardiogram*

Instantaneous electrocardiograms (ECG) were recorded with minimal surgical intervention using laterally staggered bipolar electrodes (7-strand Teflon coated wire, 0.2 mm diameter; A-M Systems, USA). Fish were first anaesthetised in ice-chilled seawater containing MS-222 (100 mg l<sup>-1</sup>) until unresponsive to touch but retaining opercular movements, weighed and maintained in an anaesthetized state (gills irrigated with aerated MS-222, 50 mg l<sup>-1</sup>) while electrodes were inserted through the ventral muscle overlying the pericardial cavity, taking care not to pierce the pericardial membrane (Campbell et al., 2004). To assess the influence of autonomic tone on thermal sensitivity, a catheter (PP10) was inserted through skin at the base

of the pectoral fins, fed into the pericardial region, and externally secured to ECG wires. All surgery was performed in a cold room at  $\sim 5^{\circ}\text{C}$ , and completed within  $\sim 10$  min, with forced ventilation in a holding tank used to minimise the delay before regular ventilation was re-established. Fish recovered for 48-72 h in cradles covered with plastic netting suspended in darkened flow-through tanks until  $f_{\text{H}}$  was stable and consistently low. The cradles allowed space for fish to move without them turning around, and gave ready access to electrodes/cannulae in an unobtrusive manner.

#### *Experimental protocol in vivo upper thermal tolerance test*

$\text{CT}_{\text{MAX}}$  was measured on individual fish (both *C. aceratus* and *N. coriiceps*) held in 70 L insulated experimental tanks and exposed to an acute thermal ramping treatment ( $3.3^{\circ}\text{C h}^{-1}$ ; Beers and Sidell, 2011) by recirculating water through an in-line heater (AquaLogic, San Diego, CA, USA). This warming rate was chosen to ensure that core body temperature likely had sufficient time to equilibrate with environmental temperature, rather than slower ramping to avoid the opportunity for acclimation (Beers and Sidell, 2011).  $\text{CT}_{\text{MAX}}$  is usually defined as the temperature when righting ability is lost, i.e., loss of neuromuscular coordination, but in this study we adopted a more cardio-centric endpoint of prolonged periods of ventricular asystole (defined as a period lasting at least 10-20 cardiac cycles based on the  $f_{\text{H}}$  immediately prior to asystole). Cardiac asystole was chosen as an index of  $\text{CT}_{\text{MAX}}$  because it is likely most comparable with behavioral indices (loss of righting reflex) when brain blood flow becomes compromised. Following the appearance of sustained asystole, animals were stunned and spinal cord transected; blood-free atrial and ventricular mass were then measured. The responses of intact fish were compared with those following vagal blockade (atropine,  $1 \text{ mg kg}^{-1}$ ).

Separately, an index of sympathovagal balance was calculated (Egginton and Campbell, 2016) using  $f_{\text{H}}$  measurements after vagal blockade (atropine,  $1 \text{ mg kg}^{-1}$ ) and then after complete autonomic blockade ( $1 \text{ mg kg}^{-1}$  atropine +  $1 \text{ mg kg}^{-1}$  propranolol) in four *C. aceratus* at ambient temperature ( $\sim 0^{\circ}\text{C}$ ). The acute effects of atropine (tachycardia, increased ECG amplitude) and propranolol (bradycardia, decreased ECG amplitude) were evident within 3-4 min after injection, and effects sustained for 12-24 h (i.e., substantially outlasting the experiments). Autonomic tone (atropine + propranolol) has previously been measured using this method in *N. coriiceps* (Egginton and Campbell, 2016) so instead of repeating the experiment we provide relevant comparisons with previously published data (Table 1).

ECG signals were collected using a PowerLab (AD Instruments, Oxford, UK) with an animal bio-amplifier (1 kHz sampling, 5 Hz high pass filter), and analyzed using LabChart (version 7, AD Instruments, Oxford, UK). Fishes were sensitive to external noise and so routine  $f_H$  (based on 10-20 consecutive heartbeats) was recorded at 06:00 h (local time) when fish were quiescent and external disturbance was minimal.

Heart rate often changes in a curvilinear manner with temperature hence the use of inter-beat interval (R-R), which provides a linear relationship (Vornanen et al., 2014). Heart rate variability (HRV) analysis reflects the influence of the autonomic nervous system on the sinus node, when the heart is in regular sinus rhythm. Tachograms and period histograms were used to ensure data blocks were sufficiently stable. In addition to  $f_H$  (expressed as R-R interval), HRV indices were calculated in both the time- (standard deviation of inter-beat intervals, SDNN; root mean square of successive differences, RMSSD) and frequency-domains (power spectral analysis, PSA, using Fourier analysis; low frequency/high frequency (LF/HF) ratio of power distribution around the midpoint frequency). At rest, up to 512 continuous heartbeats were sampled, but during the ramp test, this number was reduced to 256 or 128 heartbeats, as appropriate to avoid shifting baselines; spectral leakage was minimised by use of a Hanning window. A Poincaré plot of R-R intervals as a function of the subsequent R-R interval was used as an index of long-term (diagonal axis from the origin) and beat- to-beat variations (normal to the axis maxima) and provides a graphic parallel to SDNN and RMSSD indices, respectively (Campbell and Egginton, 2007).

#### *Surgical instrumentation for cardiorespiratory measurements*

*C. aceratus* were anaesthetised in ice-chilled seawater containing MS-222 (140 mg l<sup>-1</sup>) until unresponsive to touch but retaining opercular movements, and maintained in an anaesthetized state (gills irrigated with aerated MS-222, 70 mg l<sup>-1</sup>) on the surgery table (on a foam pad covered with goatskin chamois to prevent desiccation and protect the scale-less skin). A ventral mid-line incision allowed placement of a 2.5 or 4 mm transit-time flow probe (model T420, Transonic Systems Inc., USA) around the pericardium and ventral aorta, immediately distal to the *bulbus arteriosus* to measure ventral aortic blood flow (13 fish). The fish were then placed on their right side to access the branchial arteries in the left third gill arch. The efferent branchial artery was occlusively cannulated using PE-50 with a 2F polyurethane tip to measure dorsal aortic pressure ( $P_{da}$ ) in 16 fish. The afferent branchial artery in the same gill arch was cannulated in seven fish to measure ventral aortic pressure ( $P_{va}$ ) using a PE-50

cannula, which in smaller animals was tipped with heat-pulled PE-30. The left Ductus of Cuvier was non-occlusively cannulated in 5 fish to measure central venous pressure ( $P_{cv}$ ) using PE-50 tipped with 3F silicon (to prevent damage to the delicate venous vasculature), equipped with side-holes to maintain patency, which was advanced to the sinus venosus. The cannulae and electrical leads from the flow probe were sutured to the skin and the ventral incision was closed with 3-0 surgical silk. Surgery was performed in a cold room at  $\sim 2-3^{\circ}\text{C}$ , and completed within 45 – 120 min, after which the gills were irrigated with fresh seawater until spontaneous ventilation recommenced.

After surgery, the fish were placed in custom-made 12.4 litre PET (polyethylene terephthalate) respirometers (<https://skfb.ly/RMsV>), triangular in cross-section to accommodate the protruding pelvic fins that characterise *C. aceratus*. The 700 L tank that contained two identical respirometers were covered in black plastic. Icefish were permitted a minimum 40 h post-surgical recovery before experiments started. Submersible pumps (Eheim, Germany) recirculated water in the respirometers to attain complete mixing. Oxygen concentration ( $[\text{O}_2]$ , measured as % air saturation) was measured with a  $\text{PO}_2$  optode (FireSting, Aachern, Germany) placed in-line with the mixing pump. Oxygen consumption ( $\text{MO}_2$ ) was recorded as  $[\text{O}_2]$  decrease over a 10-min period, before a second Eheim pump started to flush the respirometers with fresh seawater for 15 min, thereby creating a 25-min cardiorespiratory measurement period. This regime ensured  $[\text{O}_2]$  never fell below 90% air saturation.

The flow probe was connected to a Transonic flow meter (T402; Transonic Systems, USA). The cannulae were attached *via* pinports (Instech Laboratories, Netherlands) to pressure transducers (Medizintechnik, Kirchseeon, Germany), which were regularly calibrated against a static water column. The output signals from the pressure transducers were pre-amplified by a Senselab 4CHAMP amplifier, (Somedic sales, Hörby, Sweden). The flow meter and 4CHAMP amplifier were connected to a PowerLab data acquisition system (ADInstruments, Castel Hill, Australia), which provided input to, as well as data storage on, a computer running LabChart Pro (version 7; ADInstruments, Bella Vista, Australia).

#### *Experimental protocol for cardiorespiratory measurements*

The cardiorespiratory response to acute warming was assessed using in-line heating (AquaLogic, San Diego, CA, USA) from ambient ( $0.8 \pm 0.1^{\circ}\text{C}$ ) to  $4^{\circ}\text{C}$  over approximately 3 h and then to  $8^{\circ}\text{C}$  over a further 3 h. At all three temperatures (maintained for at least 30 min at each one), routine cardiorespiratory variables were measured before inducing activity by



agitating the respirometers for 1 min. For routine cardiorespiratory measurements we ensured that the fish were not disturbed by external stimuli. Sustained activity is difficult to elicit in icefish (e.g., Egginton, 1997), but this method typically produced sustained tail flicking for several minutes without apparent exhausting the fish. Therefore, it is unlikely that this agitation method induced a maximum cardiorespiratory response.

Oxygen consumption ( $\dot{M}_{O_2}$ ) were calculated according to the formula:

$$\dot{M}_{O_2} = ((\alpha_{O_2} \times V) \times (\Delta O_2 / \Delta t)) / M_b$$

where  $\alpha_{O_2}$  is the oxygen content of seawater at a given temperature, V is volume of the respirometer,  $\Delta O_2 / \Delta t$  is the change in oxygen concentration (% air saturation) per unit time, and  $M_b$  is the fish body mass in kg.

Flow ( $\dot{Q}$ ), pressures ( $P_{da}$ ,  $P_{va}$  and/or  $P_{cv}$ ) and heart rate ( $f_H$ ; derived in real-time from the pulsatile flow or pressure trace) were measured simultaneously with  $\dot{M}_{O_2}$  at ambient temperature, 4°C, and 8°C in quiescent fish and after activity.  $\dot{Q}$  were normalized to body mass and the flow probes were calibrated for each temperature according to the instructions provided by the manufacturer. Stroke volume ( $V_s$ ) were calculated as:

$$V_s = \dot{Q} / f_H$$

In fish where  $P_{cv}$  were measured, systemic conductance ( $G_{sys}$ ) was calculated as:

$$G_{sys} = \dot{Q} / P_{da} - P_{cv}$$

Otherwise  $P_{cv}$  was assumed to be zero because measured  $P_{cv}$  was negligible.

Branchial conductance ( $G_{branch}$ ) were calculated as:

$$G_{branch} = \dot{Q} / P_{va} - P_{da}$$

Cardiac power output (CPO) were calculated as

$$CPO = (\dot{Q} * 1/60) * P_{va}$$

*Statistical Analysis for the upper thermal tolerance test*

Differences between *C. aceratus* and *N. coriiceps* were determined using a multi-factorial ANOVA, with Fisher's protected least-significant difference (PLSD) post-hoc test to discriminate amongst group means (StatView 5.0, SAS Institute Inc.) Broken-line regression analyses of  $f_H$  vs. temperature were conducted for each fish using the segmented package in R (v.3.1.0; The R Foundation) to determine the cardiac breakpoint temperature (BPT), i.e., when the increase in  $f_H$  with temperature first attenuated. The regression line fitted to this phase of increasing  $f_H$  was tested for differences in slope and intercept values using an ANOVA.

Three *C. aceratus* were excluded from subsequent analysis because routine  $f_H$  at ambient temperature was appreciably higher than other fish ( $P < 0.001$ ), suggesting stress, despite a similar peak  $f_H$  in both groups ( $P = 0.16$ ). The BPT was significantly lower in the outlier group ( $P < 0.05$ ). Even so, the outlier group showed no overt sign of stress (e.g., banding of integument pigmentation or prolonged recovery from surgery) and therefore likely would have been considered unstressed without cardiovascular (ECG) data, potentially leading to erroneous conclusions (Supplementary Fig. 1). In addition, one *N. coriiceps* were excluded due to apparent restlessness and periodic bouts of bradycardia throughout acute warming (note: BPT, HRV values also deviated from the remaining fish).

#### *Statistical Analysis for the cardiorespiratory measurements*

An unpaired *t*-test was used to investigate significant differences in routine  $f_H$  between *C. aceratus* instrumented with ECG electrodes and those with more extensive surgery for the cardiorespiratory measurements. The effect of temperature and activity on cardiorespiratory measurements in *C. aceratus* was investigated using a two-way repeated-measures analysis of variance (ANOVA), revealing the combined and isolated effects of warming and activity on each cardiorespiratory variable. We assumed acute warming to 8°C would impose an intolerable stress, which proved incorrect as revealed by subsequent fish responses, and so the first two fish tested were not agitated and therefore not included in the ANOVA model (to avoid Type II errors by using matched data for all measurement points). Nevertheless, these two fish are represented in the mean values in figures where data was obtained. The relative importance of  $f_H$  and  $V_S$  in meeting the increased oxygen demands following activity was elucidated at each temperature with a linear regression to correlate individual fish changes in  $\dot{Q}$ ,  $f_H$  and  $V_S$  with respect to changes in  $\dot{M}O_2$ . Statistical analysis was performed with GraphPad Prism (v. 7.0d). Statistical significance was assigned to  $\alpha \leq 0.05$  and data are presented as means  $\pm$  s.e.m.

## Chemicals

Atropine methyl nitrate, propranolol hydrochloride and tricaine methanesulfonate (MS222) were purchased from Sigma-Aldrich (St. Louis, USA).

## Results

### *Interspecific comparison of $f_H$ and thermal tolerance to acute warming*

Routine  $f_H$  was lower in *C. aceratus* than *N. coriiceps* ( $P < 0.05$ ), an absolute difference that widened at peak  $f_H$  ( $P < 0.001$ , Table 1). Consequently, the rise in  $f_H$  was slower and a lower magnitude during a thermal ramp for *C. aceratus* (0 vs. 10°C  $Q_{10} = 3.30$ ;  $\Delta f_H = 21.2 \text{ min}^{-1}$ ) than *N. coriiceps* ( $Q_{10} = 2.74$ ;  $\Delta f_H = 27.3 \text{ min}^{-1}$ ). Periodic missed and ectopic beats were noted in *C. aceratus* around 8-10°C, while ventricular asystolic events (with P-wave presence) became more frequent and prolonged closer to breakpoint temperature (BPT), suggesting a atrioventricular conduction failure (Supplementary Fig. 2).

No significant difference in BPT was found between *C. aceratus* and *N. coriiceps* (Fig. 1) despite the large significant difference in peak  $f_H$  ( $38.2 \pm 4.2$  and  $54.8 \pm 2.2 \text{ min}^{-1}$ , respectively; Table 1). Some *C. aceratus* individuals had a brief tachycardia after BPT, corresponding to the first observed change in fish behaviour. In *C. aceratus*, catastrophic cardiac failure rapidly ensued (Fig. 1), with prolonged ventricular asystolic periods (up to 8-10 sec in duration) with a regular P-wave, interspersed with bursts of ventricular tachyarrhythmias of  $>40 \text{ beats min}^{-1}$ ; a situation that points to conduction failure (Supplementary Fig. 2). In contrast, *N. coriiceps* displayed missed or ectopic beats 1-2°C above ambient, with a transient increase in HRV around mid-point of the ramp (5-10°C). Even so, regular sinus rhythm was re-established and maintained to a higher temperature than *C. aceratus*. Again, heat-induced bradycardia was evident following the BPT, with cardiac failure preceded by progressively extreme periodicity (Supplementary Figs. 2,3).

Atropine nearly doubled routine  $f_H$  at 0°C in both species, and increased peak  $f_H$  by 25% ( $P < 0.05$ ) and 5% (n.s.) in *C. aceratus* and *N. coriiceps*, respectively, but decreased both  $\Delta f_H / \Delta T^\circ\text{C}$  (Table 1) and  $Q_{10}$  (2.42 and 2.03, respectively) (Table 2). Consequently, the difference in peak  $f_H$  between *C. aceratus* and *N. coriiceps* was reduced from ~30% to ~20% after atropinisation ( $P < 0.05$ ), but peak  $f_H$  was reached at similar temperatures (n.s.). In addition, atropine substantially increased the temperatures when the first changes in the ECG

occurred in both species, making them closer to BPT; while ventricular asystolic periods progressively increased in duration, no tachyarrhythmia was observed. Vagal blockade with atropine also reduced the difference in both BPT (Fig. 1) and especially CT<sub>MAX</sub> (Table 1) between the two species.

#### *Interspecific comparison of in vivo heart rate variability*

The time and frequency domain indices of HRV showed post-surgery depression, were transiently elevated as anaesthesia wore off, and quickly stabilized within 12 h when HRV was proportional to R-R interval (Supplementary Table 1). Although *C. aceratus* recovered from surgery more slowly than *N. coriiceps* (data not shown), interspecific differences in HRV were modest (Table 2, Supplementary Table 1). Both short- (RMSSD) and long-range (SDNN) indices of HRV generally declined with rising temperature with warming to ~10°C in *C. aceratus* and to ~12°C in *N. coriiceps*. Hence, the Poincaré data cloud progressively narrowed with acute warming, but widened as CT<sub>MAX</sub> was approached (rapid collapse of *C. aceratus* after peak  $f_H$  precluded analysis at that point; Supplementary Fig. 3). While total spectral power was heterogeneous among individuals, LF/HF (a dynamic index of sympatho-vagal balance) progressively declined in both species until 5-10°C, but increased markedly at higher temperatures and especially at CT<sub>MAX</sub>, indicating an autonomic conflict consistent with signs of ECG disruption (Table 2, Supplementary Table 1). Only two *C. aceratus* reached 15°C with sinus rhythm intact, at which point all HRV variables (e.g. SDDNN, RMSSD, spectral power) were markedly different from those at 5 or 10°C (Supplementary Table 1). Such changes became accentuated between BPT and CT<sub>MAX</sub>.

Vagal blockade not only eliminated differences in BPT and CT<sub>MAX</sub> between *C. aceratus* and *N. coriiceps*, but also resulted in extremely low spectral power during the thermal ramp for both species, without changing the species difference in peak  $f_H$  (Table 1). All HRV indices were dramatically reduced by atropine, until just before BPT. The mid-range elevation of LF/HF by 10°C in atropinised *C. aceratus* is consistent with greater thermal sensitivity (likely sympathetic in origin), whereas LF/HF was minimal at this point in *N. coriiceps* (Table 2).

#### *Routine cardiorespiratory status in C. aceratus*

Routine  $\dot{Q}$  in *C. aceratus* at 0.8°C was  $26.6 \pm 2.1$  ml min<sup>-1</sup> kg<sup>-1</sup> at a  $f_H$  of  $11.5 \pm 0.9$  min<sup>-1</sup>. Routine  $f_H$  measured in *C. aceratus* equipped with only ECG electrodes for the thermal tolerance test was not significantly different ( $P=0.21$ ;  $9.0 \pm 1.1$  min<sup>-1</sup>; Table 1), suggesting that

fish had recovered sufficiently from the more extensive surgical interventions to restore the high vagal tone characterizing unstressed fish.

Routine  $\dot{M}_{O_2}$  ( $24.5 \pm 0.8 \text{ mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$ ), as well as  $P_{va}$  ( $2.06 \pm 0.05 \text{ kPa}$ ) and  $P_{da}$  ( $1.81 \pm 0.06 \text{ kPa}$ ), were similar to previously reported values (Holeton, 1970; Hemmingsen and Douglas, 1972; Hemmingsen et al., 1972).  $P_{cv}$  has not been previously measured in *C. aceratus*, but the near ambient central venous pressure ( $0.06 \pm 0.03 \text{ kPa}$ ) was consistent with very low caudal venous pressures reported previously (Hemmingsen and Douglas, 1972).

#### *Cardiorespiratory effects of acute warming in C. aceratus*

Acute warming of *C. aceratus* from  $0.8^\circ\text{C}$  to  $8^\circ\text{C}$  increased routine  $\dot{M}_{O_2}$  by 2.4-fold to  $59.5 \pm 2.4 \text{ mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$  (Fig. 2;  $P < 0.001$ ) and  $\dot{Q}$  by 2.6-fold to  $69.9 \pm 4.7 \text{ ml min}^{-1} \text{ kg}^{-1}$  ( $P < 0.001$ ). The increase in  $\dot{Q}$  with temperature was entirely attributable to an increase in  $f_H$  to  $29.1 \text{ min}^{-1}$  ( $P < 0.001$ ), which compensated for a significant but small decrease in  $V_S$  ( $P = 0.02$ ) which was most pronounced between  $0$  and  $4^\circ\text{C}$  (Fig. 2). Importantly,  $f_H$  similarly increased from  $9.0 \pm 1.1$  at  $0.6^\circ\text{C}$  to  $28.0 \pm 3.0 \text{ min}^{-1}$  at  $8^\circ\text{C}$  in ECG-instrumented *C. aceratus* (cf values for *N. coriiceps* were  $13.5 \pm 0.6$  and  $38.2 \pm 1.7 \text{ min}^{-1}$ , respectively; Table 1), again suggesting that these fish had recovered sufficiently from extensive surgical interventions.

Despite the large increase in  $\dot{Q}$  in *C. aceratus* during warming, both  $P_{da}$  and  $P_{va}$  decreased with increasing temperature (Fig. 3;  $P = 0.001$  and  $P = 0.002$ , respectively) because both  $G_{sys}$  ( $P < 0.001$ ) and  $G_{branch}$  ( $P = 0.001$ ) increased significantly with temperature and prominently at  $8^\circ\text{C}$ .  $P_{cv}$  tended to decrease with temperature ( $P = 0.06$ ), while CPO increased significantly (Fig. 3;  $P = 0.02$ ).

#### *Effects of acute warming associated with enforced activity*

At ambient temperature, enforced activity was associated with significant ( $P < 0.001$ ) increases in  $\dot{M}_{O_2}$ ,  $\dot{Q}$ ,  $f_H$  and  $V_S$  in *C. aceratus* (Fig. 2; Supplementary Fig. 4). Although  $P_{da}$  decreased during activity ( $P = 0.02$ ), as with warming,  $P_{va}$  increased with activity ( $P < 0.001$ ) because  $G_{sys}$  ( $P < 0.001$ ) increased with activity but not  $G_{branch}$  ( $P = 0.68$ ). There were no significant changes in  $P_{cv}$  from rest to activity, even though CPO increased (Fig. 3;  $P = 0.04$ ).

Individual changes ( $\Delta$ ) in  $\dot{Q}$  and  $\dot{M}_{O_2}$  with temperature showed a strong positive correlation as *C. aceratus* transitioned from rest to activity at all test temperatures (Fig. 4). At ambient temperature, a strong relationship ( $R^2 = 0.87$ ) existed between  $\Delta f_H$  and  $\Delta \dot{M}_{O_2}$ , without

a significant correlation between  $\Delta V_S$  and  $\Delta \dot{M}_{O_2}$ . At 4°C, however, the relationship between  $\Delta f_H$  and  $\Delta \dot{M}_{O_2}$  weakened and a significant relationship between  $\Delta V_S$  and  $\dot{M}_{O_2}$  developed. By 8°C a strong correlation between  $\Delta V_S$  and  $\Delta \dot{M}_{O_2}$  ( $R^2 = 0.73$ ) emerged while the association between  $\Delta f_H$  and  $\Delta \dot{M}_{O_2}$  had disappeared, reflecting the necessarily diminished potential change in  $f_H$  as peak heart rate was approached (Fig. 4).

## Discussion

In quiescent, undisturbed *C. aceratus* at 0.8°C,  $\dot{Q}$  was 26.6 ml min<sup>-1</sup> kg<sup>-1</sup>. This value accords well with the partial measurements of flow inferred from Hemmingsen et al. (1972), but is much lower than the Fick estimate (100-150 ml min<sup>-1</sup> kg<sup>-1</sup>; Høletoen, 1970; Hemmingsen et al., 1972). This difference could be a consequence of well-vascularized icefish skin (Jakubowski, 1982), which provides an alternative pathway for oxygen uptake for which the Fick principle does not account (Farrell et al., 2014). Even though Hemmingsen et al. (1972) recognized this potential error, the considerably greater Fick-derived estimates were accepted without addressing the discrepancy with their direct flow measurements, a surprising conclusion considering that the same authors previously suggested that cutaneous oxygen uptake could constitute up to 40% of total  $\dot{M}_{O_2}$  (Hemmingsen and Douglas, 1970). Similarly high (35%) cutaneous oxygen uptake has been independently measured in another scaleless Antarctic fish (*Rhigophila dearborni*; Wells, 1986). However, Høletoen (1976) disputed such a significant role for a cutaneous oxygen supply in *C. aceratus* by arguing that branchial gas exchange must dominate because of a greater PO<sub>2</sub> diffusion gradient (i.e., between water and venous rather than arterial blood), a shorter blood-to-water distance, and gills receiving the entire  $\dot{Q}$  vs. only a small portion to the skin. Thus, alternative factors, such as post-surgical stress, may explain the difference between current and earlier measurements of routine  $\dot{Q}$ .

Cardiac rhythmicity, which was linked with ventilatory activity, was evident in both *C. aceratus* and *N. coriiceps*, thus demonstrating good surgical recovery (see Campbell et al., 2004). The resting  $f_H$  (16-18 beats min<sup>-1</sup>) reported by Hemmingsen et al. (1972) was close to the maximum  $f_H$  we recorded during activity at a similar temperature, again indicating that post-surgical stress could explain the extremely high Fick-derived estimates of routine  $\dot{Q}$  for *C. aceratus* at ~1°C (Hemmingsen et al. 1972). However, this concern would equally apply to the fish instrumented with electromagnetic flow probes in the same study and that yielded a

lower routine  $\dot{Q}$ . Our assumption of equal blood flow in all branchial arteries may therefore not be correct, underestimating the inferred  $\dot{Q}$ , such that correspondence between our data and the estimated blood flows could be coincidental. Post-surgical stress was less likely an issue in Holeton's (1970) study, which reported the lowest  $\dot{M}_{O_2}$  measured in the period of 2-10 days post-surgery and a relatively low routine  $f_H$  (13.8 beats  $\text{min}^{-1}$ ), while  $\dot{Q}$  was calculated as 61  $\text{ml min}^{-1} \text{kg}^{-1}$  using the Fick principle. This  $f_H$  was lower than that of Hemmingsen et al. (1972) but, rather than being close to our routine value (11.5 beats  $\text{min}^{-1}$ ), it is closer to our value immediately following the 10 min of enforced activity (14.7 beats  $\text{min}^{-1}$ ). We therefore conclude that a combination of cutaneous  $O_2$  supply skewing the Fick-calculation, post-surgical stress, and perhaps other methodological differences account for the previous overestimations of routine  $\dot{Q}$  in *C. aceratus*. This is an important conclusion, as we now have clear evidence that the low routine  $\dot{Q}$  provides a large scope for change and that the cardiorespiratory regulation may permit physiological flexibility during acute temperature challenges, something that appeared unlikely based on earlier measurements of routine  $\dot{Q}$ .

#### *Interspecific comparison of in vivo cardiac electrical activity*

*In vivo* electrocardiograms (ECG) have not been reported previously for *C. aceratus*, and the similarity of the ECGs between *C. aceratus* and *N. coriiceps* allowed our detailed assessment of disturbances to cardiac conduction, which may be central to the failure of the heartbeat at high temperature in other fish species (Vornanen et al., 2014). Even though  $CT_{MAX}$  may vary with the extent of facilitated  $O_2$  transport (Beers and Sidell, 2011), the current observations suggest that for acute warming this effect is modest, which is consistent with recent work showing that hyperoxia failed to increase upper thermal tolerance of icefish (Devor et al., 2016).

Reduced HRV is often used as an index of diminished cardiovascular regulation in mammals, although changes in blood pressure may also induce changes in short-term HRV due to baroreflex control. Acute warming of both *C. aceratus* and *N. coriiceps* dramatically reduced HRV at around 10°C, before a marked elevation prior to BPT and  $CT_{MAX}$ , at which point ventricular asystole occurred, likely a result of conduction failure. In combination, these data suggest a biphasic response to thermal stress. Using the LF/HF ratio as a simplified readout of sympathovagal balance, the pharmacological blockade shows it to be a reasonable index of autonomic regulation in these species, as seen in other comparative studies (Campbell et al., 2004). Our data demonstrate that *C. aceratus* uses vagal tone to suppress thermally-induced

tachycardia more so than *N. coriiceps*, but similar to other Hb<sup>+</sup> nototheniids (Axelsson et al., 1992; Franklin et al., 2001). While this vagal reflex during acute warming could minimise cardiac  $\dot{M}_{O_2}$ , acidosis or oxidative stress, it is likely to incur a cost of reducing the limits to thermal tolerance (*cf.* Sandersfield et al., 2015). Thus, acute cardiac stress may have chronic downstream effects that have not been explored to date. Poincaré plots represent a more global view of HRV, and at this level there appears to be little difference between species, highlighting that additional influences on cardiac performance need to be considered (see below). For example, *C. aceratus* is relatively unresponsive to adrenaline (Egginton et al., submitted), consistent with a low synthesis rate, and attenuated stress-induced release of catecholamines (Whiteley and Egginton 1999), thereby obviating a humoral arm of cardiovascular control that could be available to other notothenioids, albeit to a lesser extent than in temperate species.

Regardless, it is clear that *C. aceratus* acutely stressed to temperatures well beyond current environmental conditions can mount a robust cardiovascular response, one that is similar to Hb<sup>+</sup> nototheniids. An important difference is that while *N. coriiceps* retains a modest scope for  $f_H$  changes at the BPT (26%), *C. aceratus* retains a significantly higher scope for  $f_H$  change (81%) leading to a lower peak  $f_H$ . Thus, the cardio-protective strategy of imposing an autonomic brake on  $f_H$  in *C. aceratus* appears to be more robust, failing only *in extremis*. Indeed, indices of HRV (e.g. RMSSD, an index of the integrity of vagus-mediated autonomic control) are consistent with this suggestion. The HRV response to elevated temperature was similar among individual fish and between species, e.g., RMSSD tracked the change in SDNN in both *N. coriiceps* and *C. aceratus*, consistent with maintaining tight regulation of cardiac rhythmicity even in the Hb<sup>-</sup> fish.

As noted above, icefish are clearly capable of developing a robust cardiorespiratory response to acute warming. The initially high  $Q_{10}$  likely reflects thermal history, as speciation in the extremely cold and stable aquatic environment is assumed to allow specialisations that may blunt physiological plasticity (Hochachka & Somero, 2002). However, *N. coriiceps* displays greater vagal withdrawal in response to a temperature ramp, possibly because extreme sensitivity to metabolic acidosis accompanying the lack of proton buffering by Hb would otherwise drive a greater vagal suppression of thermally induced tachycardia in icefish. The reduction in  $Q_{10}$  for  $f_H$  with atropine suggests a similarity in fundamental thermal sensitivity that is over-ridden by vagal tone during temperature increase, revealing a similarity of intrinsic  $f_H$  seen among notothenioids of varying ecotype (Campbell et al., 2009).



*The cardiorespiratory effects of acute warming and activity in C. aceratus*

In *C. aceratus*, both resting and active  $\dot{M}_{O_2}$  increased almost linearly between 0.8 and 8°C, whereas Hemmingsen and Douglas (1972) reported that  $\dot{M}_{O_2}$  only increased from 1 to 4°C and remained unchanged up to 10°C, consistent with stressed animals reaching  $\dot{M}_{O_2\max}$  earlier in a temperature ramp. However, we used fish that were rapidly warmed (i.e., over several hours) compared with the earlier study where fish were incrementally warmed by 0.3 – 1°C per day and maintained at a given temperature for at least 48 h prior to  $\dot{M}_{O_2}$  measurements. Thus, fish may have become less stressed by the slower warming speed, which permitted an overall reduction in oxygen demand, as seen in some temperate species (Gräns et al., 2014). However, it is unlikely that the fish were acclimating to a new temperature over a few days, as *N. coriiceps* does not exhibit reduced  $\dot{M}_{O_2}$  following acclimation (6 - 9.5 weeks) from 0 to 5°C (Egginton and Campbell, 2016; Joyce et al., in preparation).

At 0.8°C,  $\dot{Q}$  reached 44.7 ml min<sup>-1</sup> kg<sup>-1</sup> during enforced activity which is about 60% of the maximum value for  $\dot{Q}$  in the *in situ* perfused heart preparation (70 ml min<sup>-1</sup> kg<sup>-1</sup>; Egginton et al., submitted), an expected difference considering the relatively moderate activity imposed likely did not exploit maximal cardiac capacity. Also, we measured mean  $\dot{Q}$  concomitantly with  $\dot{M}_{O_2}$  over a period of several minutes, while peak  $\dot{Q}$  was transitory and higher than the mean value (Supplementary Figure 4; peak  $\dot{Q}$  immediately following agitation was 52.9 ± 4.5 ml min<sup>-1</sup> kg<sup>-1</sup>). Importantly, peak  $f_H$  (17.6 ± 0.7 min<sup>-1</sup>) following agitation was identical to that measured in *in situ* perfused hearts at the same ambient temperature (Egginton et al., submitted), an indication of a complete loss of vagal tone with just moderate activity.

The implication of these data is that *C. aceratus* at 0.8°C has a considerable cardiac scope and that the heart is not pumping near its maximum capacity when the fish is in a quiescent, unstressed state. While an abnormally high cardiac preload may be used with perfused hearts to generate maximum  $\dot{Q}$ , the maximum  $\dot{Q}$  measured *in vivo* at high temperatures (86.3 ml min<sup>-1</sup> kg<sup>-1</sup>) was close to the maximum  $\dot{Q}$  (98 ml min<sup>-1</sup> kg<sup>-1</sup>) reported for perfused hearts at 4°C. Thus, we confirmed that the icefish heart is not only capable of pumping large volumes of blood at a near-freezing temperature, but also retains a cardiac scope by maintaining vagal tone to increase  $f_H$ . Furthermore, the Hb<sup>-</sup> *C. aceratus* can increase  $\dot{Q}$  with activity even at elevated temperature *in vivo* by elevating  $f_H$ . In fact, despite their similar routine  $\dot{Q}$ , maximum  $\dot{Q}$  in *C. aceratus* clearly exceeded that of the more active Hb<sup>+</sup> nototheniid *P. borchgrevinki* (~60 ml min<sup>-1</sup> kg<sup>-1</sup>; Franklin et al., 2007).

Whether fish primarily regulate  $V_S$  or  $f_H$  to change  $\dot{Q}$  during activity remains a subject of debate (Farrell, 1991; Altimiras and Larsen, 2000, Sandblom et al., 2005; Shiels and White, 2008; Farrell and Smith, 2017; Nelson et al., 2017). The present study informs this discussion because *C. aceratus* increased  $\dot{Q}$  during activity by elevating  $V_S$  and  $f_H$  to different degrees across temperatures. While  $\Delta \dot{M}_{O_2}$  was closely correlated with  $\Delta f_H$ , as described previously in *N. coriiceps* (Campbell et al., 2008), this occurred only at 0.8°C. In fact,  $V_S$  increased by approximately 1 ml kg<sup>-1</sup> irrespective of the increase in  $\dot{M}_{O_2}$  across temperature, and became a primary determinant of the magnitude of  $\Delta \dot{M}_{O_2}$  with activity at 8°C. This suggests that at ambient temperature,  $f_H$  is finely tuned to preserve adequate cardiovascular oxygen transport in the face of elevated metabolic demands, but as  $f_H$  moves closer to peak capacity at high temperatures, there is less scope to increase pacemaker activity and  $\Delta f_H$  can no longer be closely correlated with  $\Delta \dot{M}_{O_2}$ . Thus, the closer alignment of  $\Delta V_S$  and  $\Delta \dot{M}_{O_2}$  with temperature and that the change in  $\dot{Q}$  with activity became predominantly volume-regulated is more a reflection of a limit on peak  $f_H$  rather than a change in the ability to regulate  $V_S$ . The present data also support the general finding that changes in  $f_H$  are prioritized over  $V_S$  when a fish is acutely warmed, which is an unresolved issue because quiescent fish with an artificially depressed  $f_H$  can elevate  $V_S$  in a compensatory fashion (Gamperl et al., 2011), yet exercising fish that are warmed can certainly increase  $V_S$  (present study; Steinhausen et al., 2007; Eliason et al., 2011; 2013). Thus, while an increase in  $f_H$  reduces cardiac filling time and thus limits the capacity to increase  $V_S$  (Altimiras and Axelsson, 2004), at high temperature in *C. aceratus*, and in exercising fishes more generally, this confounding problem must be overcome for  $V_S$  to increase and contribute to increasing  $\dot{Q}$ .

During both warming and activity we revealed surprisingly large (up to five-fold) increases in  $G_{sys}$ , which indicates vasodilatation of the peripheral vasculature. A similar change has previously been reported in swimming *P. borchgrevinki* (Axelsson et al., 1992; 1994). This change in conductance can be attributed only in part to passive distension of the vasculature when accommodating greater  $\dot{Q}$  (e.g., Wood and Shelton, 1975) because  $P_{da}$  clearly decreased. Both activity and temperature increase the metabolic demand of tissues, which may release local vasoactive factors and induce a functional hyperaemia. Indeed, in humans it is believed that peripheral vasodilatation is the primary driver of the increased  $\dot{Q}$  during exercise (Bada et al., 2012).

### *Evolutionary consequences for the loss of Hb*

In comparison with Hb<sup>+</sup> Antarctic notothenioids, our measurements of routine  $\dot{Q}$  in *C. aceratus* (at 0.8°C, 26.6 ml min<sup>-1</sup> kg<sup>-1</sup>) is not as divergent as previously reported. Indeed, routine  $\dot{Q}$  is lower (Axelsson et al., 1992, at 0°C; Franklin et al., 2007, 29 ml min<sup>-1</sup> kg<sup>-1</sup> at -0.5°C), or only marginally higher (Sandblom et al., 2012, 24 ml min<sup>-1</sup> kg<sup>-1</sup> at 0°C, Franklin et al., 2007, 22 ml min<sup>-1</sup> kg<sup>-1</sup> at -1.0°C), than that previously reported in smaller specimens of the more active, cryopelagic *P. borchgrevinki*. In a more typically benthic species, *Trematomus bernacchii*, routine  $\dot{Q}$  was lower (17.6 ml min<sup>-1</sup> kg<sup>-1</sup>; Axelsson et al., 1992). The greatest difference reported exists with another benthic species, *N. coriiceps* (6 - 7 ml min<sup>-1</sup> kg<sup>-1</sup>; Egginton, 1997; Joyce et al., in preparation) of similar body mass to *C. aceratus*. Thus, there is clearly considerable variation among notothenioid fishes. As a consequence of the lower routine  $\dot{Q}$ , the estimated routine CPO (which now also includes  $P_{cv}$ ) places *C. aceratus* squarely within the range of their Hb<sup>+</sup> relatives (Table 3). This suggests that cardiac pumping in *C. aceratus* is not necessarily twice as metabolically expensive as in Hb<sup>+</sup> nototheniids (*cf.* Sidell and O'Brien, 2006), and the loss of haemoglobin may not be costly in terms of cardiac work. Furthermore, if recent estimates of maximum  $\dot{Q}$  with *in situ* perfused hearts are accurate (Egginton et al., submitted), factorial scope for  $\dot{Q}$  is about 4 in *C. aceratus* at ~0°C, and is in line with other fish species such as rainbow trout (*Oncorhynchus mykiss*) and seabass (*Dicentrarchus laborax*) (Farrell and Smith, 2017).

Comparisons among related species provide valuable insight into the functional consequences of vicarious loss of individual gene products, or wider disruption of gene expression, such as variation in cardiac performance within the Channichthyid icefish family where six species not only lack haemoglobin but also myoglobin. While *C. aceratus* occupies a largely benthic habitat, other icefish species are more pelagic (Rutschmann et al., 2011). In *C. aceratus*, relative ventricular mass is ~0.3 % of body mass (Holeton, 1970; Robertson et al., 1998; Egginton et al., submitted), whereas in other icefish species, *C. rastrispinosus*, *C. hamatus* and *Champscephalus gunnari*, it is over 25% greater (0.38 - 0.4 %) (Tota et al., 1991; Robertson et al., 1998; Egginton et al., submitted). Although resting values have not been reported, maximum  $\dot{Q}$  in *C. rastrispinosus* (128 ml min<sup>-1</sup> kg<sup>-1</sup>) and *C. hamatus* (300 ml min<sup>-1</sup> kg<sup>-1</sup>) perfused hearts are also greater than *C. aceratus* (70 ml min<sup>-1</sup> kg<sup>-1</sup>) (Tota et al., 1991; Egginton et al., submitted). *C. aceratus* is one of six out of sixteen icefish species that does not express cardiac myoglobin (Moylan and Sidell, 2000; Sidell et al 1997), the absence of which has been suggested to limit cardiac power, particularly in the face of increased afterload

(Acierno et al., 1997). *C. rastrospinosus* (Hb<sup>-</sup>Mb<sup>+</sup>) had a larger ventricle, a higher  $f_H$ , and greater maximum  $V_S$  and  $\dot{Q}$  than *C. aceratus*, suggesting that cardiac Mb has functional relevance in supporting greater CPO *in vivo* (Egginton et al., submitted).

## Conclusions

Our measures show that routine  $\dot{Q}$  and CPO in *C. aceratus* under ambient temperatures are not as high as the values reported previously, but are still higher than benthic red-blooded (Hb<sup>+</sup>) notothenioids. In fact, routine  $\dot{Q}$  in *C. aceratus* is similar to  $\dot{Q}$  in *P. borchgrevinki*, a more active cryopelagic species. Furthermore, *C. aceratus* is also capable of increasing routine  $\dot{Q}$  several-fold to meet the increased  $\dot{M}_{O_2}$  when metabolic demands were elevated during an acute temperature challenge up to +8°C and stress induced activity. Also, there was no difference in cardiac BPT in Hb<sup>+</sup> *N. coriiceps* and Hb<sup>-</sup> *C. aceratus* during acute temperature challenges. Hemmingsen and Douglas (1972) were able to maintain *C. aceratus* ‘in satisfactory condition’ for at least 2 weeks at 8°C and only encountered aberrant behaviour at 10°C. Further, the Patagonian *Champscephalus esox* (which is also Hb<sup>-</sup>) occupies habitats in which water temperatures reach as high as 10°C, although this may be the edge of their thermal tolerance/ecological range because modelling studies conclude that at this temperature there is little scope for activity (Egginton et al., 2002).

Together these data suggest that increased temperature – at least during acute warming - is surprisingly well tolerated by *C. aceratus*. However, it remains unresolved if acute thermal tolerance leaves sufficient scope for sustained activity, digestion and reproduction, which are crucial for long-term species survival in a rapidly changing environment.

## **Ethics**

All experiments involving live animals were approved by the University of Alaska, Fairbanks Institutional Animal Use and Care Committee (570217-9).

## **Authors' Contributions**

WJ, APF, SE, ELC, KMO & MA designed the experiments. WJ, SE and MA conducted the experiments and carried out data analyses. WJ drafted the manuscript, which was revised and approved by APF, SE, ELC, KMO & MA.

## **Competing interests**

The authors declare no competing interests.

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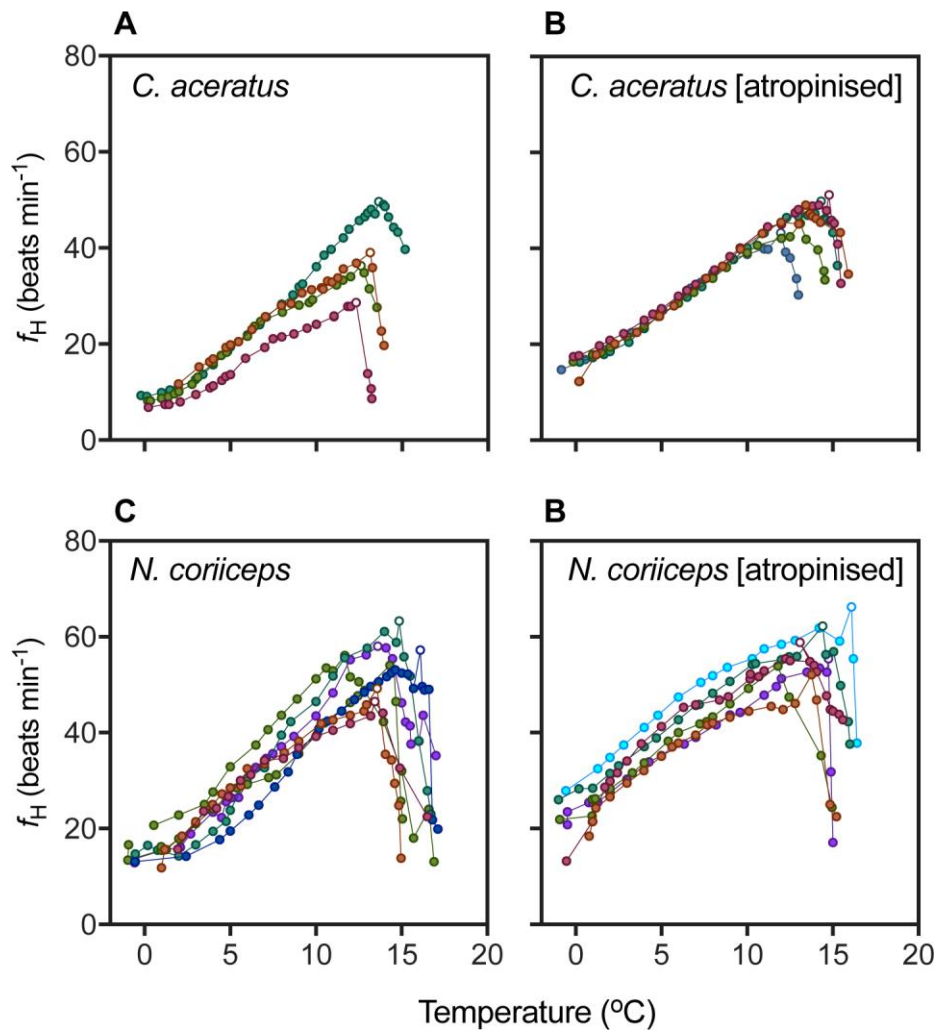
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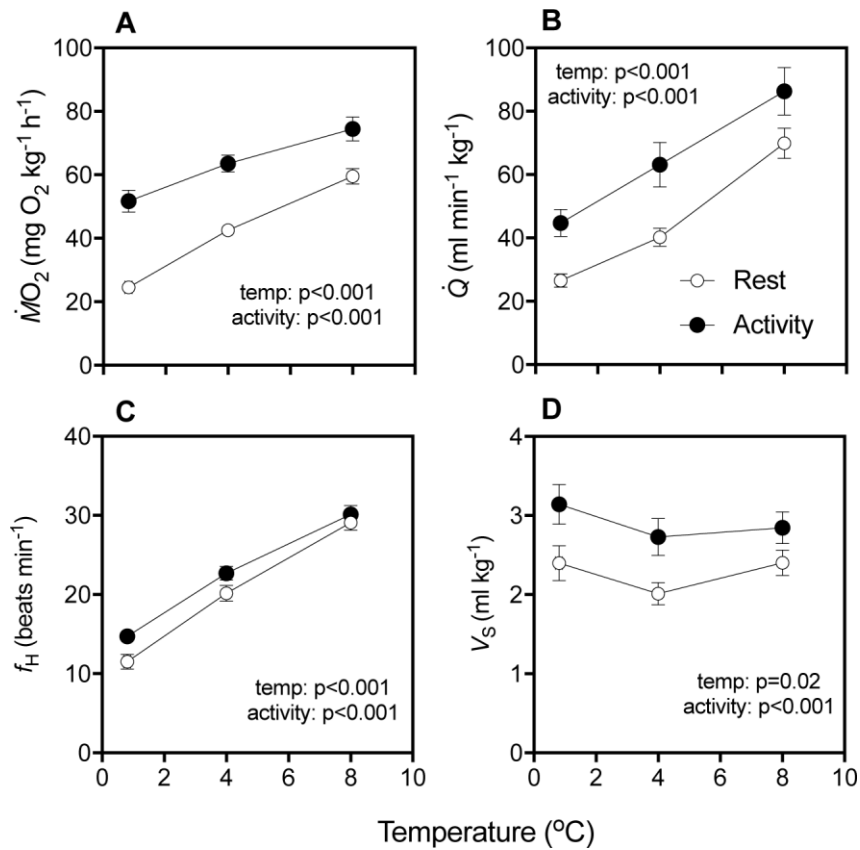
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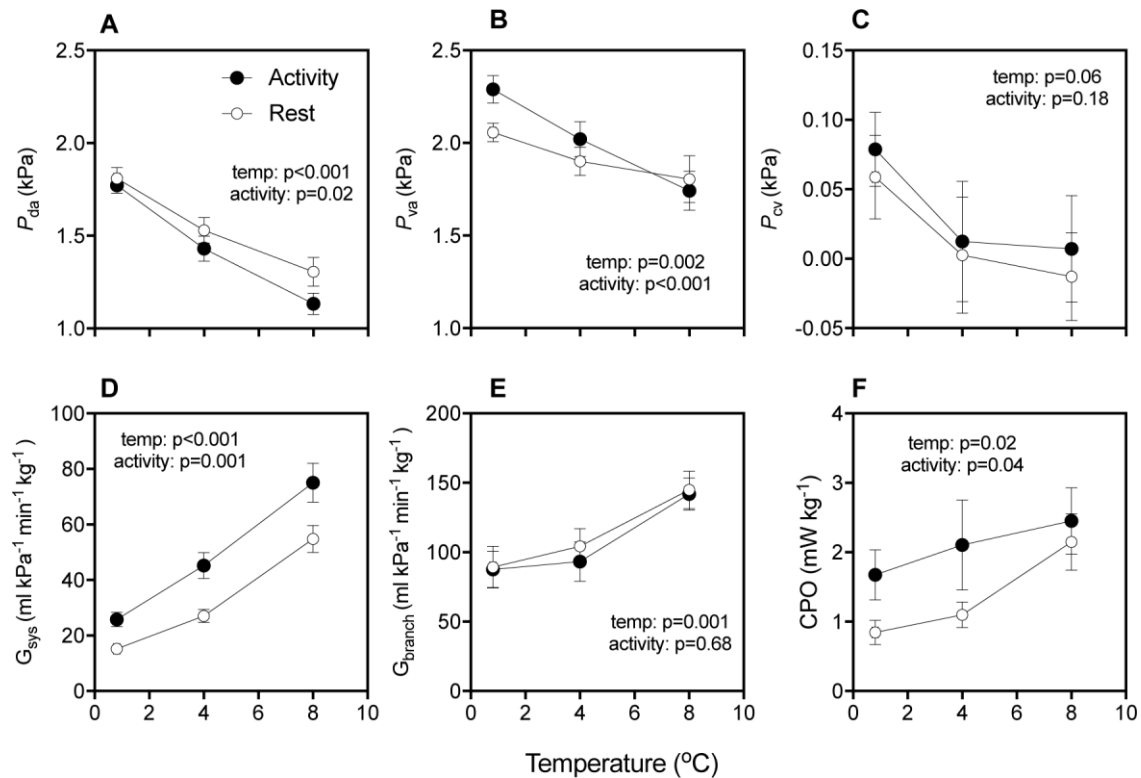
## Figures



**Figure 1.** Thermal sensitivity of notothenioid heart rate with and without autonomic blockade. A) intact *Chaenocephalus aceratus*, B) atropinised *C. aceratus*, C) intact *Notothenia coriiceps*, D) atropinised *N. coriiceps*. Individual animals are colour-coded, and calculated BPT shown as open circles. Note intraspecific variability, reduced following atropine, and the rapid onset of post-BPT bradycardia prior to cardiac failure.

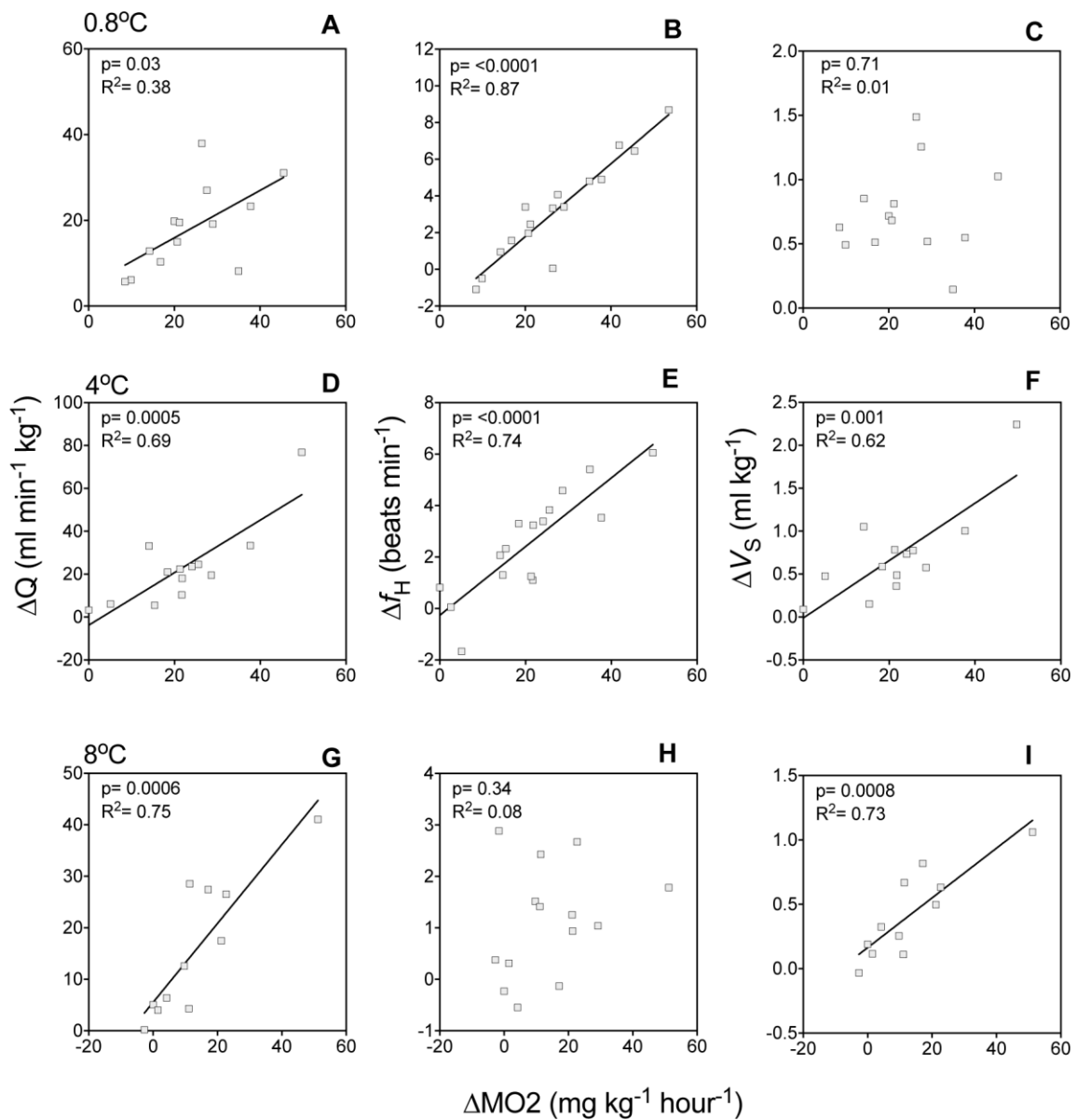


**Figure 2.** The effect of temperature and activity on oxygen consumption and cardiac function in blackfin icefish (*Chaenocephalus aceratus*). (a)  $\dot{M}O_2$ , oxygen consumption; (b)  $\dot{Q}$ , cardiac output; (c)  $f_H$ , heart rate; (d)  $V_S$ , stroke volume. N= 16 for  $\dot{M}O_2$  and  $f_H$  (except 8°C activity, N=14) and N=13 for  $\dot{Q}$  and  $V_S$  (except 8°C activity, N=11). P values according to a two-way ANOVA. Values are means  $\pm$  s.e.m.



**Figure 3.** The effect of temperature and activity on vascular pressures, conductances and cardiac power output. (a)  $P_{da}$ , dorsal aortic pressure; (b)  $P_{va}$ , ventral aortic pressure; (c)  $P_{cv}$ , central venous pressure; (d)  $G_{sys}$ , systemic conductance; (e)  $G_{branch}$ , branchial conductance; (f) CPO, cardiac power output.  $N = 16$  for  $P_{da}$  (except 8°C activity,  $N = 14$ ),  $N = 7$  for  $P_{va}$ ,  $N = 5$  for  $P_{cv}$  (except 8°C activity,  $n = 4$ ),  $N = 13$  for  $G_{sys}$  (except 8°C activity,  $N = 11$ ),  $N = 6$  for  $G_{branch}$  and CPO. P values according to a two-way ANOVA. Values are means  $\pm$  s.e.m.





**Figure 4.** The relationship between the changes in cardiac output, heart rate and stroke volume with change in oxygen consumption from rest to activity at ambient temperature (0.8°C; a,b,c), 4°C (d,e,f) and 8°C (g,h,i).  $\Delta \dot{Q}$ , change in cardiac output (a, d, g);  $\Delta f_H$ , change in heart rate (b, e, h);  $\Delta V_S$ , change in stroke volume (c, f, i);  $\Delta \dot{M}O_2$ , change in oxygen consumption. N=16 (panels a,d); N=13 (b,c,e,f,); N=14 (g); N=11 (h,i). P values and  $R^2$  values calculated with linear regressions.

**Tables:****Table 1.** Thermal tolerance of *Chaenocephalus aceratus* and *Notothenia coriiceps*.

	<i>C. aceratus</i>	<i>N. coriiceps</i>
<u>Routine <math>f_H</math> (beats <math>\text{min}^{-1}</math>)</u>		
Intact	9.0±1.1 (4)	13.5±0.6 (7)*
Atropinised	16.8±1.5 (5)	24.6±2.2 (5)
<u>Cholinergic tonus (%)</u>		
	55.3±9.9 (4)	52.4 <sup>§</sup>
<u>Adrenergic tonus (%)</u>		
	11.7±1.6 (4)	12.7 <sup>§</sup>
<u>8°C <math>f_H</math> (beats <math>\text{min}^{-1}</math>)</u>		
Intact	28.0±3.0 (4)	38.2±1.7 (7)*
Atropinised	33.2±0.6 (5)	39.0±1.2 (6)*
<u>Peak <math>f_H</math> (beats <math>\text{min}^{-1}</math>)</u>		
Intact	38.2±4.2 (4)	54.8±2.2 (7)**
Atropinised	47.7±1.5 (5)	58.1±2.2 (6)*
<u>Break point temp (°C)</u>		
Intact	13.0±0.3 (4)	14.0±0.5 (7)
Atropinised	13.5±0.6 (5)	14.0±0.6 (6)
<u>CT<sub>MAX</sub> (°C)</u>		
Intact	14.1±0.4 (4)	16.7±0.3 (7)
Atropinised	15.1±0.5 (5)	15.8±0.3 (6)

Routine  $f_H$  and autonomic tonus were obtained at  $\sim 0.6^\circ\text{C}$ . BPT analysis calculated from linear regression intercepts using transformed  $f_H$  and temperature data to determine the  $f_H$  at  $8^\circ\text{C}$  and temperature at which a peak  $f_H$  occurred;  $\text{CT}_{\text{MAX}}$  was defined as the temperature at which persistent ventricular asystole occurred. Mean  $\pm$  s.e.m. (N); \*  $P < 0.05$ , \*\*  $P < 0.001$  vs. *C. aceratus* (ANOVA with Tukey post-hoc test). <sup>\$</sup> values from Egginton & Campbell 2016.

**Table 2.** Time- and frequency domain analysis of heart rate variability during a thermal ramp to define CT<sub>MAX</sub>.

	n	NN(ms)		SDNN(ms)		Power*10 <sup>3</sup> (ms <sup>2</sup> )		LF:HF	
		intact	(atropine)	intact	(atropine)	intact	(atropine)	intact	(atropine)
<i>C. aceratus</i> (Hb <sup>-</sup> Mb <sup>-</sup> )									
0°C	4	6838±1669	3599±336	427±121	9±9	120.7±44.7	0.07±0.10	0.96±0.52	0.58±0.46
5°C	4	3653±716	2284±60	187±182	7±10	22.1±30.2	0.02±0.04	1.65±1.01	1.11±0.88
10°C	4	2089±513	1482±128	115±78	2±1	8.5±9.2	0.004±0.002	1.01±0.82	2.08±2.11
15°C	2	2389±13.6	1415±68	2441±2032	15±9	2920.7±4062.8	0.3±0.2	0.60±0.09	2.52±1.65
CT <sub>MAX</sub>	4	2641±413	2594±1947	1752±1575	932±128	2152.1±2701.1	312.4±327.8	2.39±2.61	3.43±1.43
Q <sub>10</sub> (0,10)		3.30	2.42						
<i>N. coriiceps</i> (Hb <sup>+</sup> Mb <sup>+</sup> )									
0°C	7	3915±651	2452±204	322±143	19±15	323.8±368.5	0.3±0.3	1.38±0.69	0.98±0.92
5°C	7	2319±419	1549±124	156±68	5±3	27.0±33.0	0.01±0.01	2.71±1.46	1.45±1.47
10°C	7	1402±101	1212±117	83±45	4±2	6.0±4.9	0.01±0.01	1.30±0.57	0.87±0.74
15°C	6	1399±269	1268±273	1778±2749	58±78	253.6±294.4	0.5±0.5	0.55±0.29	2.78±5.38
CT <sub>MAX</sub>	7	3959±1784	1742±594	2816±934	2176±2157	1936.8±1817.6	1875.7±2230	1.01±0.77	1.16±0.29

Q<sub>10</sub>(0,10)      2.74            2.03

Abbreviations: NN, interbeat intervals obtained by removing artifacts from the R-R series (i.e. ‘normal’ intervals, ms); SDNN, standard deviation of NN intervals (ms); Power, total spectral power in the analysis region (ms<sup>2</sup>); LF:HF, ratio between LF power and HF power.

	$\dot{Q}$ (ml min <sup>-1</sup> kg <sup>-1</sup> )	$P_{va}$ (kPa)	$P_{cv}$ (kPa)	CPO (mW kg <sup>-1</sup> )
<i>Chaenocephalus aceratus</i>	26.6 <sup>a</sup>	2.06 <sup>a</sup>	0.06 <sup>a</sup>	0.89
<i>Trematomus bernacchii</i>	17.6 <sup>b</sup>	3.09 <sup>b</sup>	Not measured	0.91
<i>Pagothenia borchgrevinki</i>	29.6 <sup>b</sup>	3.60 <sup>b</sup>	0.11 <sup>c</sup>	1.72
<i>Notothenia coriiceps</i>	6.5 <sup>d</sup>	5.92 <sup>d</sup>	0.08 <sup>d</sup>	0.63

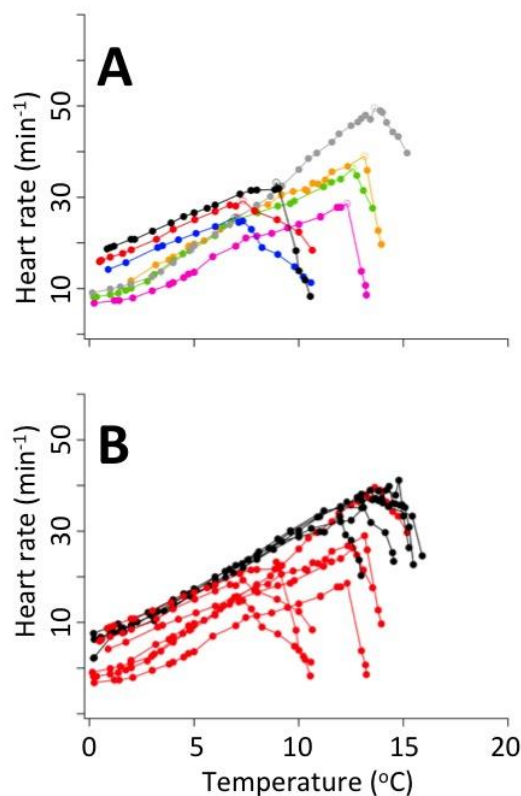
**Table 3.** Routine cardiac output ( $\dot{Q}$ ), ventral aortic pressure ( $P_{va}$ ), central venous pressure ( $P_{cv}$ ) and cardiac power output (CPO) in icefish (*C. aceratus*) and three red-blooded notothenioids. CPO estimated based on mean  $\dot{Q}$  and pressure measurements for all species and calculated as: CPO = ( $\dot{Q} * 1/60$ ) \* ( $P_{va} - P_{cv}$ ). For *Trematomus bernacchii*,  $P_{cv}$  has not been measured so we assumed it was zero. Body mass specific CPO (cardiac work) in *C. aceratus* is within the range of that in red-blooded notothenioids.

a present study

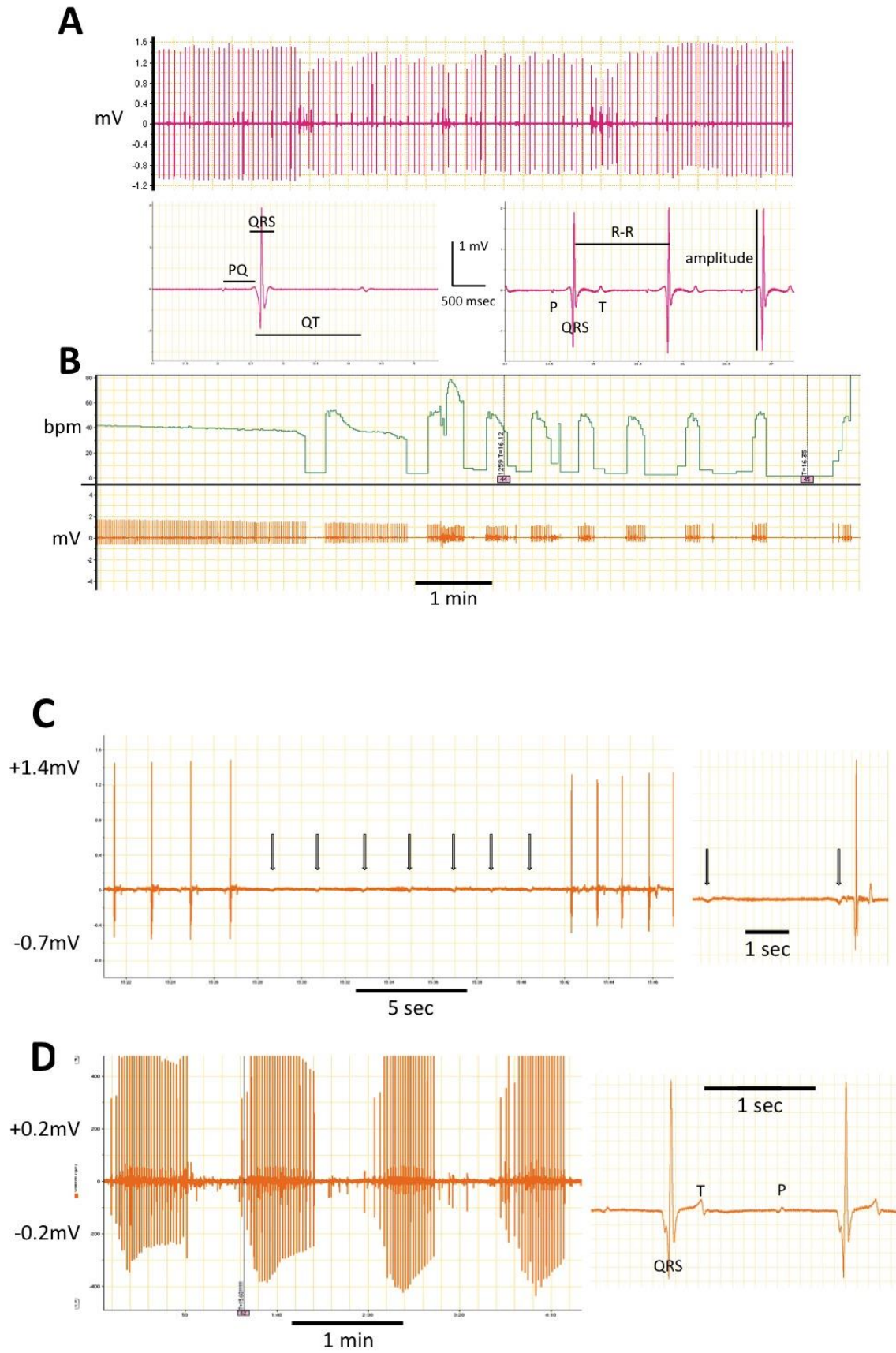
b Axelsson et al., 1992

c Sandblom et al., 2009

d WJ, MA, SE, APF, ELC, KMO, unpublished data

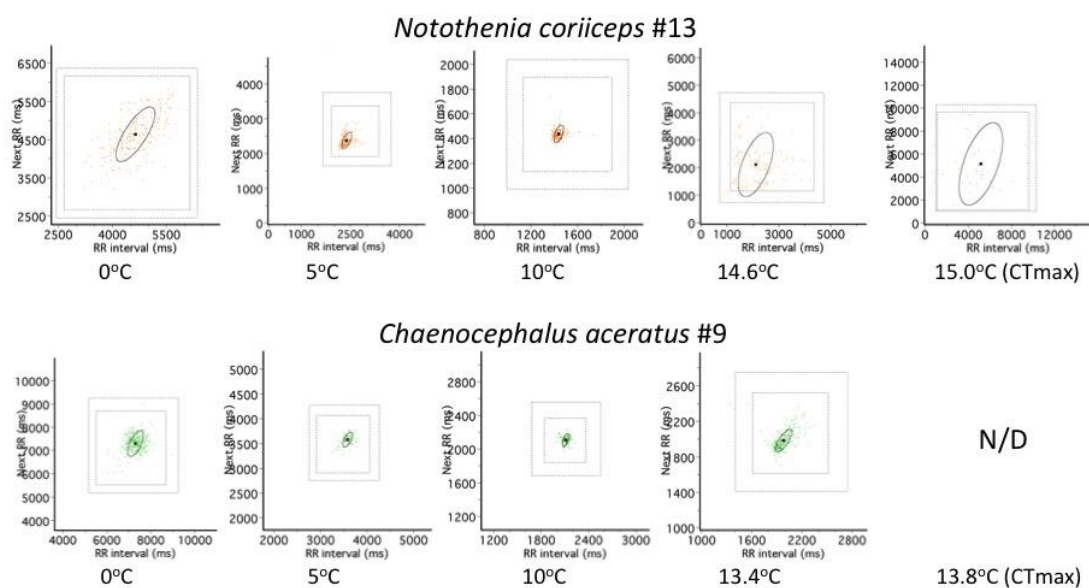


**Figure S1.** Quality control of individual *C. aceratus* suitability for analysis of  $f_H$  and HRV, using objective assessment of thermal sensitivity with minimally invasive measures. Assuming that  $f_H$  is the most labile variable, based on our existing data for these species and prior knowledge of stress in fishes, the consequences of incorporating all gathered data can be profound. A) All individuals recovered well from surgery and displayed none of the external signs of stress over the next 48-72 h, e.g. banded pigmentation, and hence were considered suitable for use in subsequent investigations. Note that those individuals with intact sinus rhythm but which started the thermal ramp with higher resting  $f_H$  demonstrated the lowest BPT, and  $CT_{MAX}$ , hence inclusion would underestimate thermal tolerance in this species. B) Heart rates of intact icefish (red circles) show an overlap of the relatively tachycardic individuals with atropinised icefish (black circles) during a thermal ramp, suggesting one contributor to greater thermal sensitivity is an unusually low vagal tone.

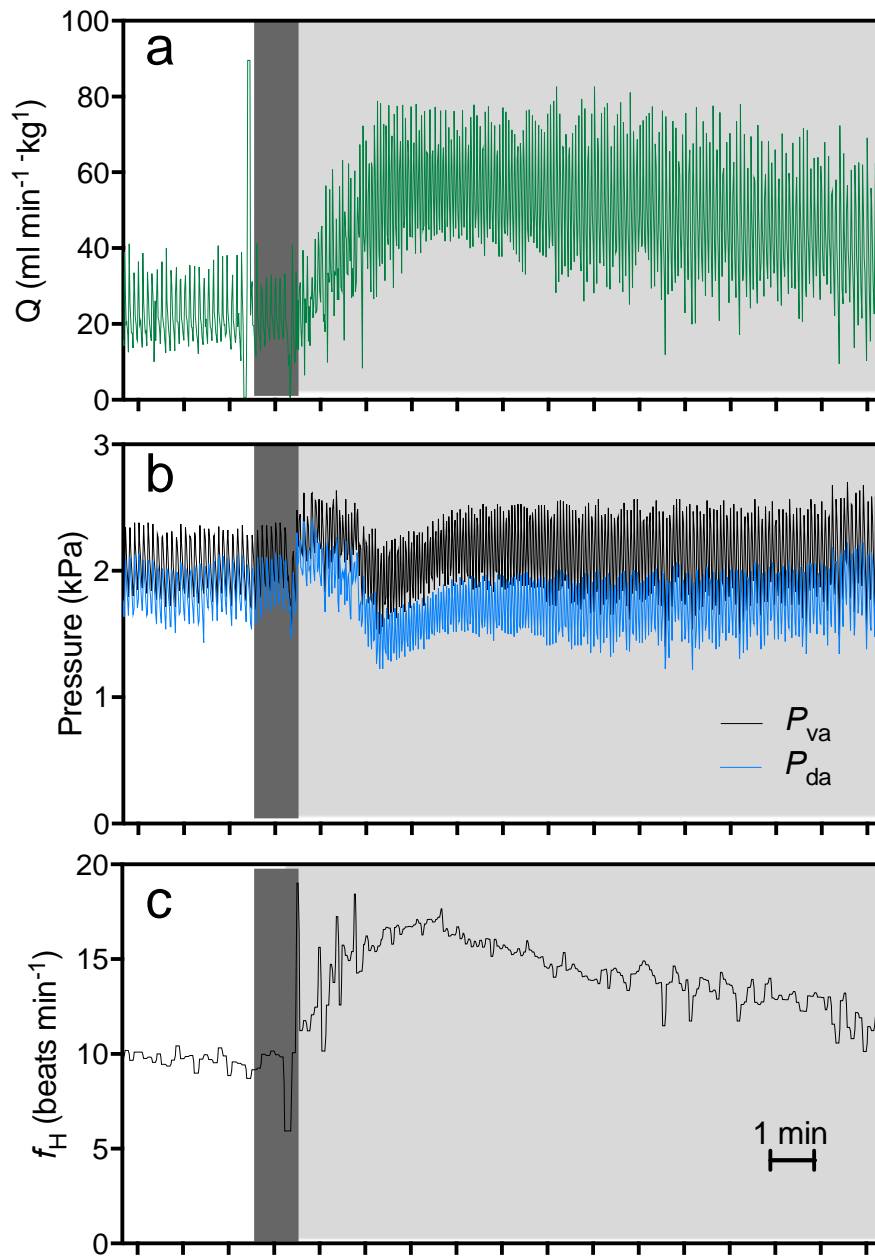




**Figure S2.** Screenshots of live recordings showing variability in cardiac electrical activity during a thermal ramp. A) *N. coriiceps* showing variable  $f_H$  at 7.6°C, transiently increasing HRV as a result of prolonged R-R interval before normal sinus rhythm was restored – insert, individual waveforms at -1.01°C and +14.6°C, respectively, indicating the distinct cycle durations evident; B) *N. coriiceps* showing atropine periodicity prior to death at 16.1°C, with progressively longer periods of asystole; C) ECG trace from *N. coriiceps* showing conduction failure at 16°C, 7 P-waves are indicated – insert, expanded view showing 2 P-waves without intervening QRS; D) *C. aceratus* with ventricular tachyarrhythmias of 42 bpm at 15.8°C – insert, expanded view showing normal ECG waveform during elevated  $f_H$ .



**Figure S3.** Representative Poincaré plots - a plot of R-R(n+1) against R-R(n) -of individual *N. coriiceps* (top) and *C. aceratus* (bottom). Note the progressive narrowing of R-R interval spread with elevated temperature, followed by widening as CT<sub>MAX</sub> is approached. The collapse of *C. aceratus* sinus rhythm rapidly after peak  $f_H$  (BPT) makes analysis difficult around CT<sub>MAX</sub>.



**Figure S4.** An original and representative trace of cardiovascular parameters before and during activity at 0.8°C. The dark grey zone represents the period during which the animal was agitated, the light grey zone represents the ensuing period of activity. (a)  $\dot{Q}$ , cardiac output; (b)  $P_{da}$ , dorsal aortic pressure;  $P_{va}$ , ventral aortic pressure; (c)  $f_H$ , heart rate.

**Table S1.** Heart rate variability (analysis of changes in beat-to-beat intervals with time) indices during recovery and thermal ramp test.

[Click here to Download Table S1](#)

Mean $\pm$ SD(n); \* $P$ <0.05, \*\* $P$ <0.001 vs. *C. aceratus*. NB: few icefish reached 15°C, CT<sub>MAX</sub> was consistently lower than this.

Abbreviations: NN, interbeat intervals obtained from the R-R series (ms); SDNN, standard deviation of NN intervals (ms); Ratio, (standard deviation of all NN intervals / standard deviation of differences between adjacent NN intervals); RMSSD, square root of the mean of the sum of squared differences between adjacent NN intervals; CVNN, coefficient of variation (ratio of SDNN to mean NN) of NN (%); NN<sub>150</sub>, proportion of adjacent NN intervals that differ by more than 150ms (%); Total power, power in the analysis region (ms<sup>2</sup>); LF:HF, ratio between LF power and HF power.