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REVIEW

SPECIAL ISSUE
THE INTEGRATIVE BIOLOGY OF THE HEART

Measuring maximum heart rate to study cardiac thermal performance and heat tolerance in fishes

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ABSTRACT

The thermal sensitivity of heart rate (f_H) in fishes has fascinated comparative physiologists for well over a century. We now know that elevating f_H is the primary mechanism through which fishes increase convective oxygen delivery during warming to meet the concomitant rise in tissue oxygen consumption. Thus, limits on f_H can constrain whole-animal aerobic metabolism. In this Review, we discuss an increasingly popular methodology to study these limits, the measurement of pharmacologically induced maximum f_H ($f_{H,max}$) during acute warming of an anaesthetized fish. During acute warming, $f_{H,max}$ increases exponentially over moderate temperatures (Q_{10}~2–3), but this response is blunted with further warming (Q_{10}~1–2), with $f_{H,max}$ ultimately reaching a peak (Q_{10}≤1) and the heartbeat becoming arrhythmic. Because the temperatures at which these transitions occur commonly align with whole-animal optimum and critical temperatures (e.g. aerobic scope and the critical thermal maximum), they can be valuable indicators of thermal performance. The method can be performed simultaneously on multiple individuals over a few hours and across a broad size range (<1 to >6000 g) with compact equipment. This simplicity and high throughput make it tractable in lab and field settings and enable large experimental designs that would otherwise be impractical. As with all reductionist approaches, the method does have limitations. Namely, it requires anaesthesia and pharmacological removal of extrinsic cardiac regulation. Nonetheless, the method has proven particularly effective in the study of patterns and limits of thermal plasticity and holds promise for helping to predict and mitigate outcomes of environmental change.

KEY WORDS: Electrocardiogram, Cardiac arrhythmia, Temperature tolerance, CT_{max}, Plasticity, Thermal acclimation

Introduction

The essential response of heart rate to thermal variation

The controlling influence of temperature on heart rate (f_H) has fascinated comparative physiologists for well over a century. Early interrogations of the relationship between temperature and biological rate functions often focused on f_H for practical and functional reasons (Crozier, 1926; Cyon, 1866; Fry, 1947; Glaser, 1929; Martin, 1883; Zimmer, 1998). From a practical perspective, the heartbeat could be directly observed at early life stages in many species and in *ex vivo* preparations, or readily assessed from recordings of blood pressure, blood flow, cardiac electrical activity and plethysmography. From a functional perspective, the vital role of f_H in meeting the oxygen requirements of aerobic metabolism and the sensitivity of f_H to temperature were well established (Fick, 1870; Murlin and Greer, 1914). These considerations remain highly relevant for fishes because we now know that elevating f_H is the primary mechanism through which fishes increase convective oxygen delivery to meet the inexorable rise in oxygen demand that occurs with acute warming. Beyond advancing basic knowledge, studying cardiac thermal performance has become increasingly important given the pressing need to understand species- and context-specific physiological responses to thermal variation in a rapidly changing world (Anttila et al., 2014a; Comte and Olden, 2017; Eliason and Anttila, 2017; Eliason et al., 2011, 2013; Farrell, 2016; Farrell et al., 2009).

When examining the mechanisms that shape an organism's ability to match its oxygen supply to the exponential rise in demand during warming, it is useful to consider each component of the Fick principal for oxygen uptake ($\dot{M}_{O_2}$; see Glossary):

$$\dot{M}_{O_2} = f_H \cdot SV \cdot (Ca_{O_2} - Cv_{O_2}), \quad (1)$$

where Ca_{O_2}–Cv_{O_2} (oxygen content of arterial and venous blood, respectively) is the amount of oxygen extracted from circulating blood and the product of stroke volume (SV; see Glossary) and heart rate (f_H) is cardiac output ($\dot{Q}$). Trail-breaking research in fishes and other animals ranging from crustaceans to mammals has demonstrated that when hearts are warmed, resting and intrinsic f_H ($f_{H,rest}$ and $f_{H,intrinsic}$; see Glossary) initially increase with temperature coefficients similar to that for whole-animal $\dot{M}_{O_2}$ (Crozier, 1926; Cyon, 1866; Fry, 1947; Glaser, 1929; Henderson, 1927; Knowlton and Starling, 1912; Martin, 1883). However, at high temperatures, f_H invariably reaches a plateau or decreases, with the heartbeat ultimately becoming arrhythmic, causing a collapse in f_H. In fishes, SV does not appreciably increase with warming and can actually decline, thus limitations in f_H also constrain $\dot{Q}$ (Brodeur et al., 2001; Ekström et al., 2014; Eliason and Anttila, 2017; Eliason et al., 2013; Farrell, 2009; Gamperl et al., 2011; Steinhausen et al., 2008). Furthermore, fish can only partially offset limitations in $\dot{Q}$ by increasing the extraction of oxygen from the blood, because at low Cv_{O_2}, oxygen diffusion into vital tissues – including the myocardium – is limited (Ekström et al., 2016; Farrell and Clutterham, 2003; Lannig et al., 2004). As such, the thermal limitations of f_H can impair whole-animal $\dot{M}_{O_2}$. A heat-induced cardiac collapse (see Glossary) has now been documented in a broad range of fishes, including polar stenotherms such as the Arctic cod (*Boreogadus saida*) (Drost et al., 2016b), notable eurytherms such as the goldfish (*Carassius auratus*) and Atlantic killifish (*Fundulus heteroclitus*) (Ferreira et al., 2014;

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Glossary

Adrenergic tone

The extent to which heart rate is increased through neural and humoral activation of cardiac β-adrenergic receptors.

Arrhenius breakpoint temperature (T_{AB})

The breakpoint identified in a segmented regression (breakpoint analysis) of ln(biological rate) against the inverse of temperature in Kelvin. For $f_{H,max}$, the breakpoint analysis is performed on the rising phase of the $f_{H,max}$ thermal performance curve (Table 1).

Atrioventricular-block type arrhythmia

A cardiac arrhythmia where the conduction between the atria and ventricle of the heart is impaired, leading to prolonged duration between atrial (P wave) and ventricular (QRS complex) depolarizations or sequential P waves when a QRS complex is missing.

Cardiac collapse

Precipitous decline in heart performance commonly observed as a marked drop in heart rate with or followed by cardiac arrhythmia.

Cholinergic tone

The extent to which the parasympathetic nervous system slows heart rate through the release of acetylcholine by the vagus nerve.

Critical thermal maxima (CT_{max})

Upper temperature at which a free-swimming fish loses equilibrium during an acute warming protocol.

Differential versus single-ended recordings

Differential recordings take the difference between a positive and negative electrode relative to a ground (three electrodes total). A single-ended recording takes the difference between a single recording electrode and the ground (two electrodes total). Differential recording can minimize noise and give greater control over ECG quality if needed.

Fick principle

Equation stating that an animal's oxygen uptake is equal to its cardiac output (stroke volume×heart rate) multiplied by the difference in oxygen content between the arterial and venous blood.

Holding temperature

Temperature that an animal is housed at before testing. Often synonymous with 'acclimation temperature'.

Incremental Q_{10}

Q_{10} values calculated over a specified narrow temperature increment (e.g. 1 or 2°C) during warming.

Maximum heart rate ($f_{H,max}$)

The maximum frequency a heart can contract at under a given set of environmental conditions.

Peak $f_{H,max}$

The highest maximum heart rate recorded during acute warming.

Pejus temperature

Transition temperature(s) on a thermal performance curve (e.g. for aerobic scope) where performance falls below a predefined percentage of the optimum (e.g. 90%, 80%).

Resting heart rate ($f_{H,rest}$)

Heart rate recorded when an animal is at rest and quiescent.

Scope for heart rate ($f_{H,scope}$)

Difference between maximum and resting heart rate ($f_{H,max}$–$f_{H,rest}$).

Stroke volume (SV)

The volume of blood ejected from the heart on each heartbeat.

Safi et al., 2019), and numerous species in between (Anttila et al., 2014a; Casselman et al., 2012; Chen et al., 2015b; Eliason and Anttila, 2017; Eliason et al., 2011, 2013).

Because $f_{H,rest}$ increases with temperature, maintaining scope for f_H above $f_{H,rest}$ to support vital functions including swimming and digestion (Eliason et al., 2013; Grans et al., 2009; Steinhausen et al., 2008) requires a proportional increase in maximum heart rate ($f_{H,max}$; see Glossary). Fry (1947) first demonstrated that a fish can maintain or increase scope for f_H by increasing $f_{H,max}$ with acute warming, but only over temperatures that would be considered moderate for a given species. At warm temperatures $f_{H,max}$ increases to a lesser extent than $f_{H,rest}$ and scope for f_H (see Glossary) is lost before cardiac function collapses altogether (Fig. 1) (Eliason et al., 2013; Farrell, 2009; Fry, 1947; Steinhausen et al., 2008). Thus, the inability to increase $f_{H,max}$ with warming at high temperatures limits cardiac scope (scope for $\dot{Q}$), which constrains the aerobic metabolic scope (AS) available for functions beyond rest (Fry, 1947). Based on this relationship, characterizing the thermal response of $f_{H,max}$ to acute warming can reveal temperatures at which sub-lethal and lethal limitations may restrict maximum tissue oxygen supply and AS. Such information is valuable for mechanistic predictions of how fish distributions will be affected by a rapidly changing world (Comte and Olden, 2017; Pacifici et al., 2015).

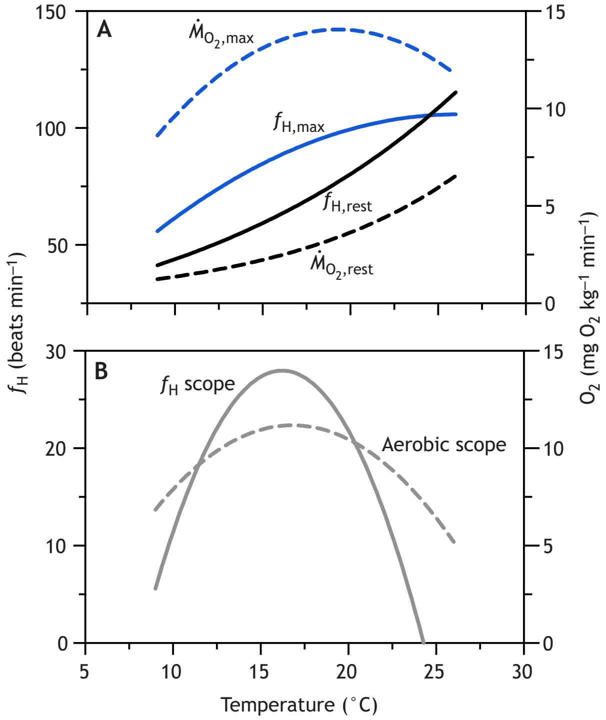

Fig. 1. Responses of oxygen uptake rates and heart rate to temperature in sockeye salmon (*Oncorhynchus nerka*). (A) Maximum and resting heart rate (f_H; solid lines) and oxygen uptake ($\dot{M}_{O_2}$; dashed lines) are shown with (B) the resulting changes in f_H and aerobic scope in adult sockeye salmon. Equations are adapted from Eliason et al. (2013).

These observations led Casselman et al. (2012) to develop a high-throughput method whereby pharmacologically induced $f_{H,max}$ is monitored in anaesthetized fish during acute warming to identify constraints on $f_{H,max}$ (referred to throughout this Review as 'the method' or 'the $f_{H,max}$ method'). When the relationship between $f_{H,max}$ and temperature was expressed on an Arrhenius plot (natural log of the rate versus the inverse of temperature in Kelvin), they identified an initial breakpoint (Arrhenius breakpoint temperature, T_{AB}; see Glossary), above which the slope declined. This T_{AB} aligned well with the optimal temperature for the scope for f_H and AS, leading to the proposition that identifying T_{AB} could replace more laborious whole-animal assessments of AS in some circumstances. This $f_{H,max}$ method, with some modifications, is being increasingly used to study cardiac thermal performance – and heat tolerance more generally – in a broad range of scenarios. It has now been applied in >40 studies and across >20 species as a proxy for, or in complement to, assessments of whole-animal performances. In this Review, we provide an overview of this method and discuss its strengths, limitations and application to assess cardiac thermal limits.

A method for rapidly screening maximum heart rate during acute warming

Overview of the method

As an overview, fish are placed under anaesthesia with assisted gill ventilation, fitted with electrocardiogram (ECG) electrodes and injected with drugs to block cardiac cholinergic tone (see Glossary) and maximally stimulate adrenergic tone (see Glossary) to a induce stable $f_{H,max}$. Fish are then acutely warmed until the heartbeat loses rhythmicity (Fig. 2B–G). The analysis of the response of $f_{H,max}$ to acute warming yields multiple metrics that characterize cardiac thermal sensitivity and heat tolerance (Table 1, Fig. 2E–G). The method was originally developed to test two fish simultaneously (Casselman et al., 2012). However, once proficient, users can increase to as many fish as can be practically managed. For instance, up to six fish have been assessed simultaneously (Adams et al., 2022; Gilbert et al., 2022b; Gilbert and Farrell, 2021). Although the protocol is conceptually simple, there are numerous considerations for new users, and for new species and contexts. These considerations are highlighted below.

Anaesthesia

Fish are immersed in a water bath at the fish's holding temperature (see Glossary) containing an anaesthetic concentration sufficient to induce stage III anaesthesia (i.e. cessation of bodily and opercular

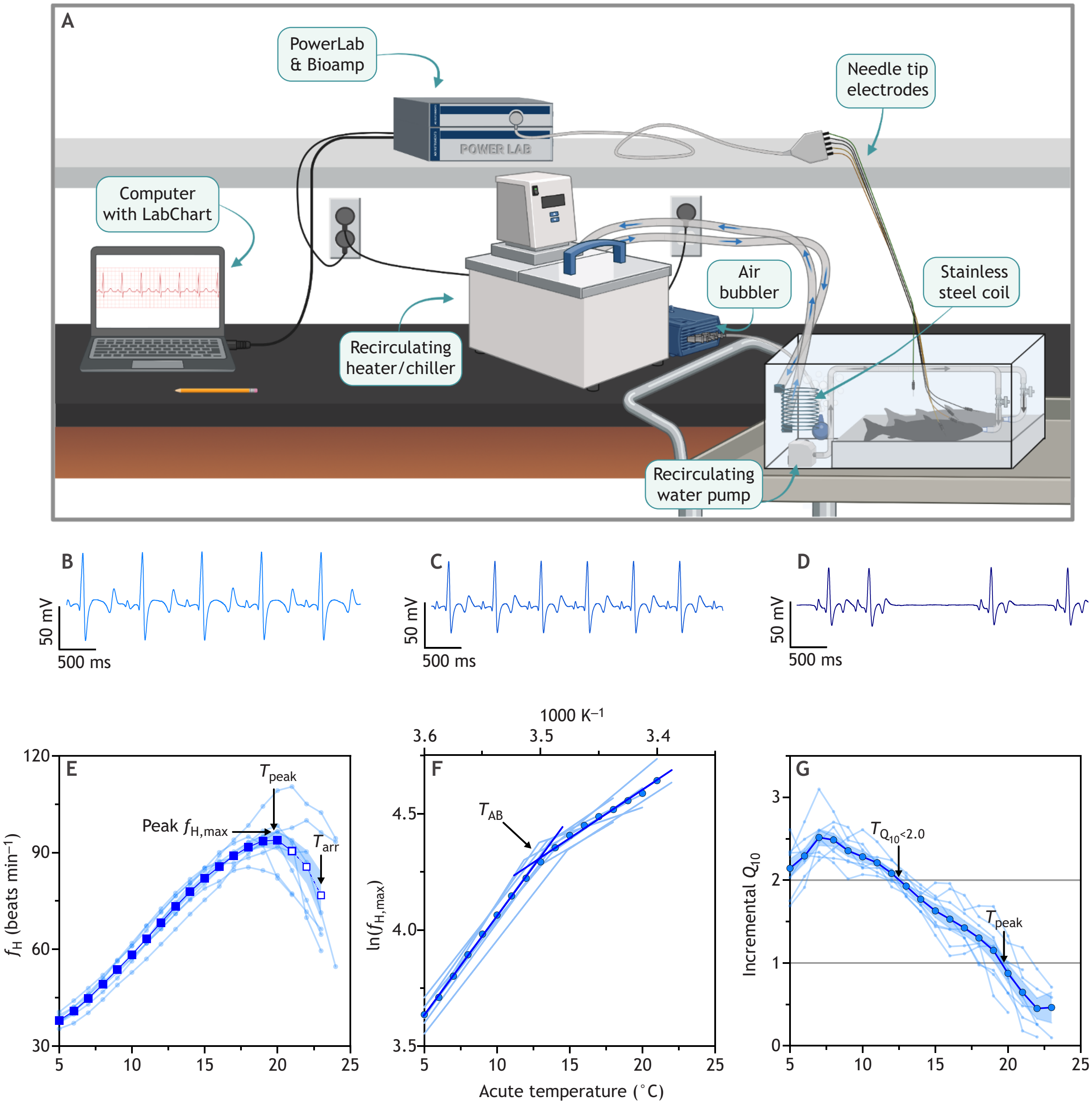

Fig. 2. Assessment of maximum heart rate ($f_{H,max}$) during acute warming. (A) A common laboratory configuration, (B–D) representative ECG recordings and (E–G) resulting data. ECG recordings are from large (3.2 kg) anadromous Arctic char (*Salvelinus alpinus*) at (B) 15°C, (C) temperature at peak $f_{H,max}$ (T_{peak}) and (D) temperature at the onset of cardiac arrhythmia (T_{arr}), and were recorded in a remote Arctic field setting (Gilbert et al., 2022a). (E) The mean (dark blue connected points, shaded area is s.e.m.) $f_{H,max}$ response to acute warming is shown with representative individual data (light blue lines; n=8 for display purposes) from the same study, along with the resulting (F) Arrhenius breakpoint and (G) incremental Q_{10} analysis. Labeled arrows indicate the mean thermal limits and peak $f_{H,max}$.

Table 1. Definitions and suggested interpretation for transition temperatures or thermal limits identified through the assessment of maximum heart rate ($f_{H,max}$) during acute warming

Temperature	Definition	Suggested interpretation
T_{AB}	Arrhenius breakpoint temperature above which the slope of $f_{H,max}$ versus temperature on an Arrhenius plot is distinctly limited	Above T_{AB}, the increase in $f_{H,max}$ is generally limited relative to $f_{H,rest}$ resulting in a loss of scope for f_H; commonly corresponds with optimal or pejus temperatures around peak aerobic capacity (Casselman et al., 2012)
T_{Q10}	Temperature at which the incremental Q_{10} temperature coefficient is limited to values below the selected threshold, commonly 1.9 or 2.0 for the remainder of the acute warming challenge	Similar to T_{AB}, above T_{Q10}, the increase in $f_{H,max}$ with further warming is limited relative to what is typical for $f_{H,rest}$ and routine oxygen which can in turn constrain the f_H and aerobic scope for vital functions (Fig. 1)
T_{peak}	Temperature at peak $f_{H,max}$ during warming	$f_{H,max}$ cannot increase any further and so f_H scope and thus aerobic scope become critically limited as $f_{H,rest}$ rises while $f_{H,max}$ does not (Fig. 1); whole-animal performance is vulnerable to oxygen limitation under elevated aerobic workloads
T_{arr}	Temperature at the onset of cardiac arrhythmia	f_H cannot be sustained and cardiac collapse has occurred or is imminent; whole-animal performance and survival are time limited in agreement with the common proximity to CT_{max} (Fig. 4)

movement; Coyle et al., 2004) in ∼5 min. Anaesthetized fish are weighed during transfer to a sling immersed in a bath that recirculates a lower, maintenance concentration of anaesthetic over the gills which is continuously pumped via a mouthpiece inserted loosely into the mouth of the fish (using a cut-off large gauge needle, pipette tip or tubing, depending on fish size). The temperature of the maintenance bath can be lower than the holding temperature, particularly if multiple acclimation temperatures are being tested, so long as the temperature difference is not so large that it impacts subsequent response to warming (pilot tests should assess any concerns; Gilbert and Farrell, 2021; Safi et al., 2019). Tricaine methanesulfonate (TMS; also known as tricaine, MS-222 and ethyl 3-aminobenzoate) is the most commonly used anaesthetic and, as originally applied, does not appear to adversely impact the response of $f_{H,max}$ to warming (Casselman et al., 2012; see 'Critiques and limitations' below; Fig. 3). Likewise, the assumption should be that appropriate anaesthetic concentrations vary among taxa and should be independently determined or verified in preliminary assessments. If the initial concentration is too high, ventilation can cease before the anaesthetic has equilibrated throughout important body compartments. The maintenance concentration of anaesthetic – typically between 50 and 80% of the initial concentration – may also require pilot experiments. The guiding principle is to use the minimum concentration to prevent the resumption of opercular and body movement to ensure fish welfare, while not having excess anaesthetic that could impair heart function or be lethal. Some anaesthetics including TMS may require buffering of water pH.

Electrocardiogram

ECG electrodes are placed on anaesthetized fish in the holding sling. Electrode materials, placement and method of placement can all vary based on the specific experimental requirements, and many options exist for the equipment and software to acquire ECGs. Pilot assessments are typically needed to determine the optimal electrode placement because it can vary substantially among species, life stages and recording modes. A precise electrode placement with less exposed recording surface is often required for small fish or in saltwater. Thus, for small fish, a needle electrode (e.g. MLA1213, ADInstruments, Colorado Springs, CO, USA), affixed to a rod with only a small portion of the electrode exposed, can be gently placed on the ventral surface of a supine fish directly over the heart with a micromanipulator (Marchant and Farrell, 2019; Safi et al., 2019). The second reference electrode can be placed more posteriorly on the body of the fish. For larger fish, needle electrodes or inexpensive small-gauge silver plated wire (e.g. 30AWG silver plated copper wire; R-30W-0050, Jonard Industries, Tuckahoe, NY, USA) can be used and gently inserted in the skin on the ventral surface. In adult salmonids, for instance, electrodes can be placed diagonally across the heart on the ventral surface, with the reference electrode placed on the body or nearby in the bath as depicted by Cotter and Rodnick (2007). Recordings can be made as differential or single-ended (see Glossary) as needed. High ECG quality and detail is needed to perform subsequent ECG waveform analyses and establish the exact type of arrhythmias observed at high temperatures.

Resources on common ECG acquisition, processing and analysis practices are available directly from equipment and software providers. Multiple amplifiers (e.g. Animal BioAmp, ADInstruments; DP-300 series, Warner Instruments, Warner Instruments, Hamden, CT, USA) and data acquisition platforms (e.g. PowerLab with Labchart software, ADInstruments; MP160 with AcqKnowledge software, BIOPAC Systems, Inc., Santa Barbara, CA, USA) are suitable for this application. Modern ECG acquisition configurations will generally have options for analog and digital filters. Analog filters are applied at the level of the amplifier and permanently modify the input signal, whereas digital filters are applied within the acquisition software and can be adjusted in real-time or after the signal is acquired. Most ECG information is acquired at between 1 and 50 Hz. Analog filters can be applied conservatively around this range (∼0.1 to 100 Hz) to improve the signal-to-noise ratio while preserving all useful information. A digital band-pass filter then obtains a narrower range (∼5 to 50 Hz) to reversibly improve signal quality so that ECG waveforms can be more clearly assessed. Some amplifiers and software have an optional 50 or 60 Hz notch filter (or 'mains filter') that removes noise commonly associated with power line interference (line frequency varies by country). Finally, excessive electrical noise may be introduced by certain equipment in the bath or surrounding electronics. Suspected issues can be identified by briefly turning off electronics one at a time and, once identified, the equipment can be replaced or repositioned as needed.

Pharmacological interventions

Once water temperature and ECG recordings have stabilized (usually within a few minutes), $f_{H,max}$ is induced pharmacologically using intraperitoneal injections of the muscarinic-acetylcholine receptor antagonist atropine sulphate (to block parasympathetic inhibition of f_H), and a β-adrenoreceptor agonist such as isoproterenol (to mimic sympathetic acceleration of f_H via β-adrenoceptors). Alternative

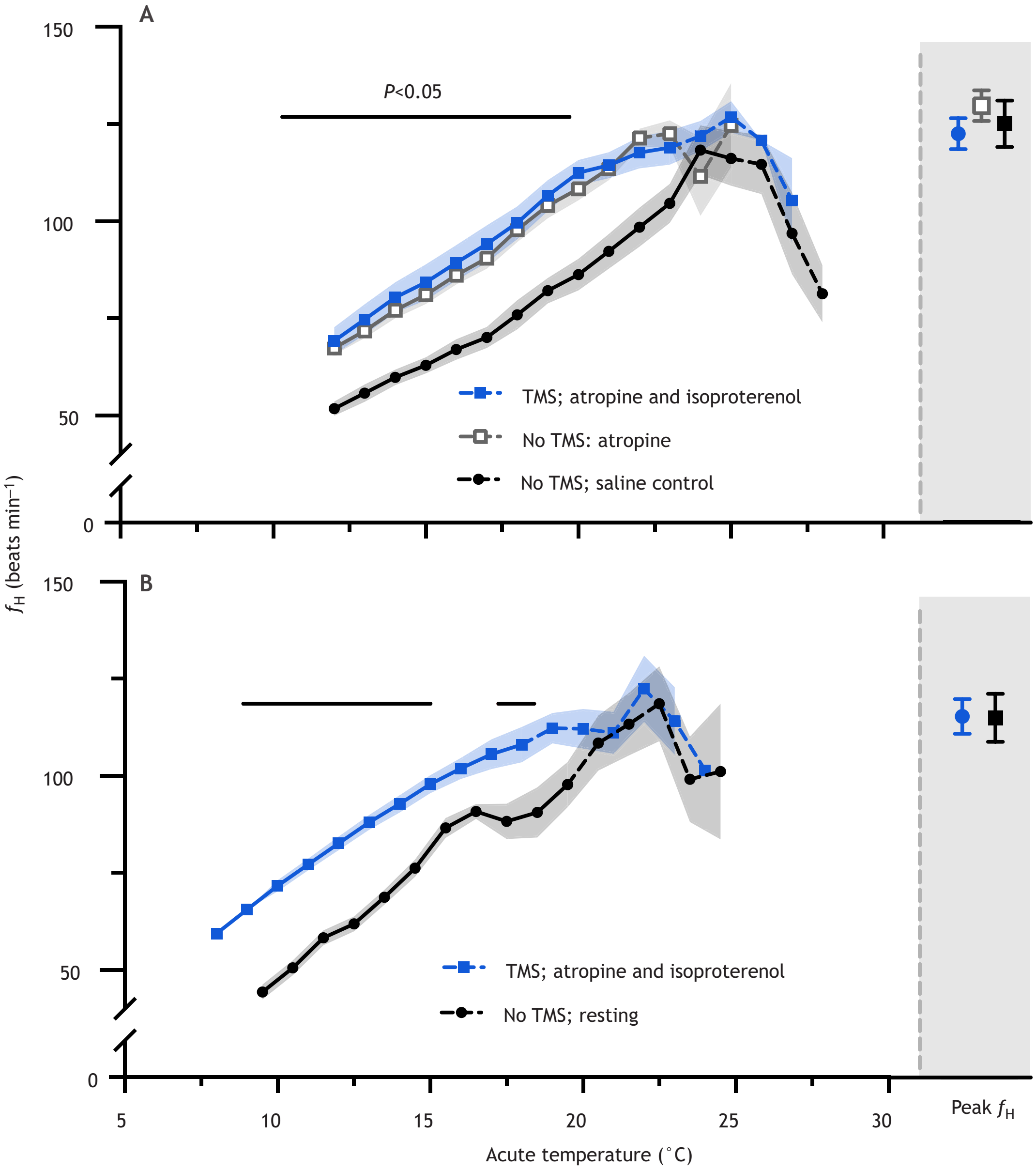

Fig. 3. Heart rates (f_H; mean±s.e.m.) during acute warming. Data are shown for anaesthetized (tricaine methanesulfonate; TMS) or non-anaesthetized (no TMS) rainbow trout (*Oncorhynchus mykiss*; A) and Arctic char (*Salvelinus alpinus*; B) with or without treatment with atropine and isoproterenol. The mean ±s.e.m. peak f_H achieved during acute warming is shown with grey background. Data for non-anaesthetized rainbow trout are from Gilbert et al. (2019), with data for anaesthetized rainbow trout (M.J.H.G., unpublished data) collected on the same cohort of fish during the same time frame (n=6). Data for captive saltwater-acclimated, non-anaesthetized and wild upriver-migrating anaesthetized Arctic char are from Penney et al. (2014) and Gilbert et al. (2020), respectively. Arctic char from these two studies are presumed to have similar acclimation temperatures (~10°C; Gilbert et al., 2020 examined wild fish so the precise acclimation temperature was unknown), were of similar size, had an identical peak f_H during acute warming (115 beats min^{-1}) and had an identical critical thermal maximum (CT_{max}=23°C). The horizontal lines indicate temperatures during acute warming at which $f_{H,max}$ in anaesthetized fish was significantly greater (P<0.05; Holm adjusted pairwise t-tests) than routine f_H in non-anaesthetized fish, indicating a positive scope for heart rate, which deteriorates at high temperatures as the responses converged.

injection methods (e.g. intramuscular or intravascular) may be favoured in some scenarios such as with very small or very large fish, but the dosage, effect strength and effect duration should be verified before they are used. Under anaesthesia and without these pharmacological interventions, the measured f_H cannot be considered as the resting, routine or maximum level. Cholinergic (via the vagus nerve) and adrenergic tone (via circulating catecholamines and sympathetic innervation, if present) vary considerably among fishes and with acclimation temperatures (Wood et al., 1979; Axelsson et al., 1987; Altimiras et al., 1997; Axelsson, 2005). Drug doses can vary with species, duration of the measurement period and temperature range. If appropriate dosages are not already established for a given scenario, pilot tests must be performed to determine these. Doses in the range of 1.2–10.0 mg kg^{-1} for atropine and 4.0–8.0 µg kg^{-1} for isoproterenol have been used successfully across a broad range of contexts (see https://doi.org/10.6084/m9.figshare.25661178.v1). In some instances, isoproterenol has been omitted because it did not change $f_{H,max}$ after atropine injection (Anttila et al., 2014a,b). Successful drug effects are indicated when a second, similar dose elicits no further change in f_{H}. Likewise, additional drug doses can be administered at any time during pilot tests, particularly near the end, to test whether the pharmacological effect has been maintained (Casselman et al., 2012). Alternative cardioactive drugs, applied following the same principles, can address other mechanistic questions. For instance, Marchant and Farrell (2019) used specific channel blockers to examine pacemaker mechanisms for $f_{\mathrm{H,max}}$ under warming.

Warming rates

The original method used a warming rate 10°C h^{-1} for ∼20 g fish (Casselman et al., 2012), applied in a stepwise manner (1°C step every 6 min). The first guiding principle was that the thermal increments ensured sufficient resolution (e.g. >10 data points) for precise identification of thermal limits without unduly prolonging test duration. For fish with limited warming tolerance above the experimental starting temperature, analytical resolution can be improved using finer temperature increments (e.g. 0.5°C; Drost et al., 2016b) over the same duration. The second guiding principle was to allow the heart temperature to equilibrate with the bath (Casselman et al., 2012), as indicated by $f_{\mathrm{H,max}}$ stabilizing before the end of each 1°C increment. For ∼20 g fish, Casselman et al. (2012) verified that a slower warming rate produced the same results. For small volumes of water, a typical lab heater-chiller device (Fig. 2) can achieve this heating rate. With larger fish, the water volume is larger, manually controlled heaters are added and slower warming rates are needed to increase temperature equilibration times (e.g. 5–6°C h^{-1} in ∼0.5 to >3 kg salmonids) (Gilbert et al., 2022b, 2020). Because gills are highly effective heat exchangers (Stevens and Sutterlin, 1976), the blood supply returning to the heart may warm it faster than more insulated or less perfused body compartments such as the peritoneal cavity, where body temperature is commonly measured (e.g. Sandrelli and Gamperl, 2023). The coronary circulation present in some fish also returns warmed blood directly from the gills to a portion of the heart. Thus, a stable $f_{\mathrm{H,max}}$ may be a better indicator of cardiac temperature equilibration than measures of core body temperature, especially in large fish with coronary circulation. Nonetheless, future research on the topic could help refine the method and improve our general understanding of how fish experience acute thermal variation.

Data extraction and analysis

Real-time monitoring of $f_{\mathrm{H,max}}$ is achieved through automated heartbeat or cyclic measurement detection algorithms in data acquisition software. The $f_{\mathrm{H,max}}$ at each temperature increment is reported as an average over a specific period (e.g. 1 min, 30 s or 10 beats) towards the end of the increment. Thermal sensitivity (or dependence) of $f_{\mathrm{H,max}}$ can be characterized in two ways. First, an Arrhenius breakpoint temperature can be identified for each individual fish using segmented regression analysis of the natural logarithm of $f_{\mathrm{H,max}}$ against the inverse of temperature in Kelvin (1/K) (Casselman et al., 2012) (Fig. 2F, Table 1). This regression is more accurate if the analysis is limited to temperatures over which $f_{\mathrm{H,max}}$ is increasing. T_{AB} calculations are sensitive to the number of datapoints available and their distribution around the breakpoint. Insufficient data can be an issue if the fish's experimental starting temperature is close to or above T_{AB}. In this case, to increase the data available below T_{AB}, the starting temperature can be lowered (if confirmed that performance is not impaired), or a finer temperature increment can be used (e.g. 0.5°C).

A complementary or alternative analysis of the change in thermal sensitivity involves calculating the 'incremental Q_{10}' (see Glossary), the Q_{10} temperature coefficient for every 1–2°C of warming (Fig. 2G, Table 1). This incremental Q_{10} decreases with warming as $f_{\mathrm{H,max}}$ approaches its peak, and the temperature at which it falls below a specified Q_{10} threshold (T_{Q10}; see Table 1; Anttila et al., 2013a) for the remainder of the trial can be used to summarize this decline. A Q_{10} threshold of ∼1.9–2.0 is commonly selected to indicate a decreased thermal sensitivity because physiological rate functions during acute temperature changes typically have Q_{10} values ≥2. The T_{Q10} tends to be slightly (<1°C) higher than T_{AB} (Fig. 4C), but agreement depends on the selected Q_{10} threshold.

With warming above T_{AB} and T_{Q10}, $f_{\mathrm{H,max}}$ reaches a plateau or a peak ($Q_{10} \leq 1$), termed peak $f_{\mathrm{H,max}}$, and the temperature is T_{PEAK} (Table 1). Further warming induces cardiac arrhythmia, at an upper thermal limit termed T_{ARR} (Fig. 2B–E, Table 1). Arrhythmia is generally unambiguous (Fig. S1), and identified as sudden intermittent drops in beat-to-beat $f_{\mathrm{H,max}}$ (an irregular pattern of 2 or 3 missed heartbeats near the previous rhythmic frequency followed by a gap is common) (Anttila et al., 2013a; Casselman et al., 2012). Atrioventricular-block type arrhythmias (see Glossary) are common and identified by the presence of a P wave with a missing QRS complex if the ECG waveforms are analyzed (Fig. S1) (Gilbert et al., 2022a; Haverinen and Vornanen, 2020; Vornanen, 2020).

To assess thermal acclimation responses, relationships between thermal limits and trends across studies, we compiled data from all studies that cited the study first proposing the method (Casselman et al., 2012), and in which animals were held under their treatment temperature conditions for >1 week before testing. The compiled data include 'control' treatments (i.e. no co-occurring stressor effects) and treatments that represent natural sources of variation within a population (i.e. size, life stage, diet). The data include mean values for metrics from the $f_{\mathrm{H,max}}$ assessment and additional relevant metadata, and are available from figshare (https://doi.org/10.6084/m9.figshare.25661178.v1).

Thermal limits and performance metrics: interpretation and associations

The four thermal limits commonly identified using the $f_{\mathrm{H,max}}$ method, in the order that they occur during warming, are T_{AB} or T_{Q10}, T_{peak} and T_{arr} (Table 1, Figs 2, 4 and 5). These thermal limits represent transitions at which $f_{\mathrm{H,max}}$ (T_{peak} and T_{arr}) or the response of $f_{\mathrm{H,max}}$ to warming (T_{AB} and T_{Q10}) become limited and are thus also called 'transition temperatures'. These metrics have ecological relevance to cardiac thermal performance, sensitivity and tolerance. Their definitions and suggested interpretations are summarized in Table 1. Below T_{AB}, for example, $f_{\mathrm{H,max}}$ increases proportionally or to a greater extent with warming than $f_{\mathrm{H,rest}}$ (Fig. 1A). Thus, scope for f_{H} can be maintained to around T_{AB} but declines above T_{AB} (Fig. 1B). Also, T_{AB} can correspond with the optimal or upper pejus temperature (see Glossary) for aerobic scope (Anttila et al., 2013b; Casselman et al., 2012; Chen et al., 2015b), although this is not always the case (Ferreira et al., 2014; Kraskura et al., 2023). Why these relationships vary among species and contexts is a natural avenue for future research.

Scope for f_{H} declines beyond T_{AB} and is low or negligible but T_{peak}. Thus, despite occurring at peak f_{H}, T_{peak} does not correspond to the optimal temperature for performance. Rather, T_{peak} lies beyond the optimal window for cardiac and aerobic capacity performance and is a temperature when fish are far more likely to be experiencing limitation of their maximal oxygen supply (Table 1). For this reason, and because of its close association with T_{arr} (Figs 4 and 5), some studies end recordings at T_{peak} (Chen and Narum, 2021). The difference between the $f_{\mathrm{H,max}}$ at the acclimation temperature or the

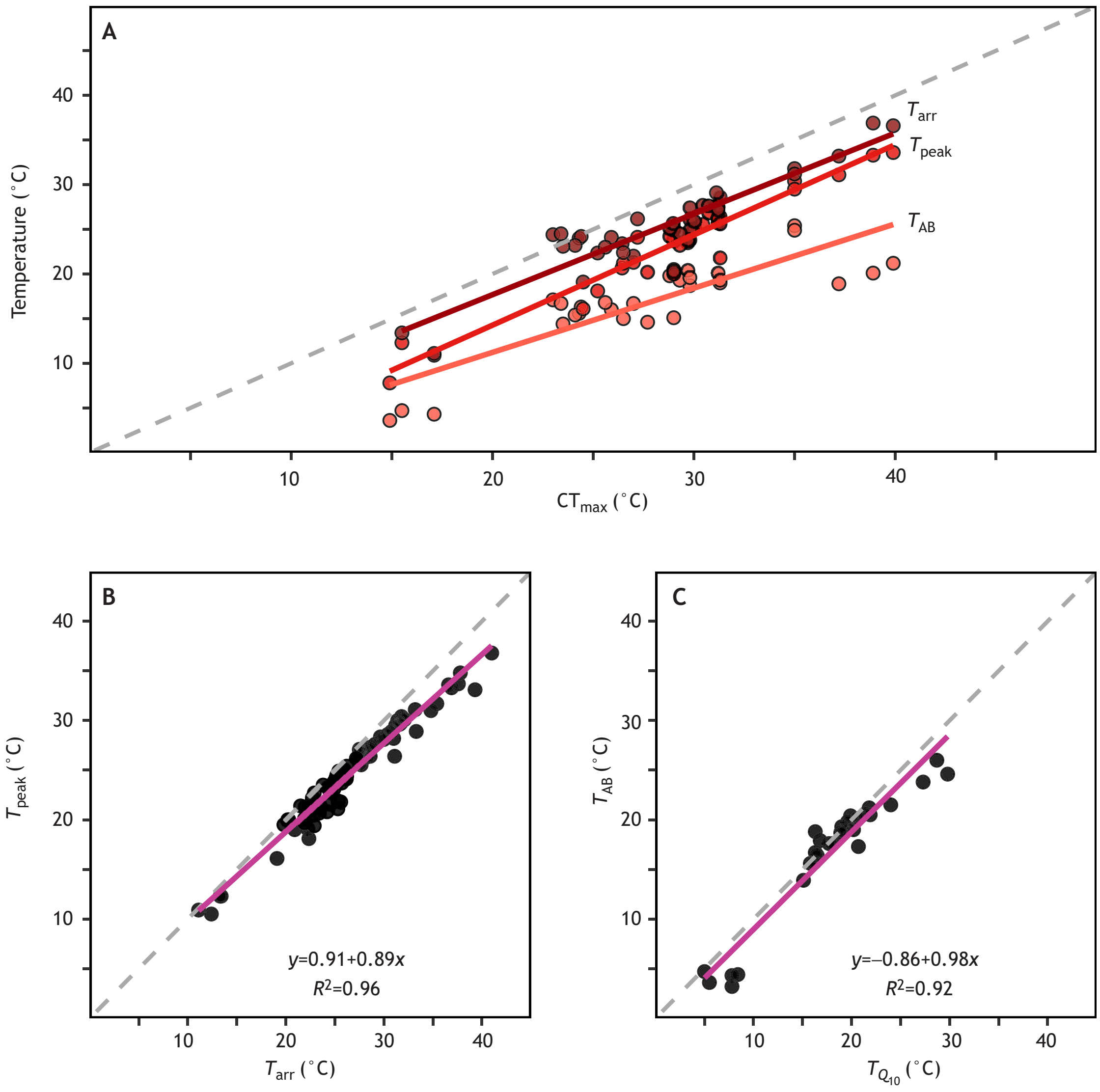

Fig. 4. Data from studies compiled as part of the data synthesis. (A) Various thermal limits calculated in the $f_{H,max}$ test in relation to CT_{max}. Color indicates the thermal limit, with individual points indicating means from various studies and solid line indicating the line of best fit. Dashed line indicates a 1:1 relationship. (B) Relationship between T_{peak} and T_{arr}. (C) Relationship between the Arrhenius breakpoint temperature (T_{AB}) and temperature at the Q_{10} threshold (T_{Q10}). Simple best-fit lines are added along with the equation and fit. Each point is a different mean value. The dashed line indicates 1:1. See https://doi.org/10.6084/m9.figshare.25661178.v1

initial test temperature and peak $f_{H,max}$ is termed $\Delta f_{H,max}$ and provides information on the ability to increase $f_{H,max}$ (Fig. S3), helping to maintain a scope for f_H during acute warming (i.e. the total thermal safety margin for $f_{H,max}$; Fig. S3). Use of the term $\Delta f_{H,max}$ is preferable because 'scope' is more commonly defined as the difference between $f_{H,max}$ and $f_{H,rest}$. The final limit above T_{peak} is T_{arr}, which indicates a thermal limit for imminent cardiac failure and is beyond functional thermal limits of a fish.

The thermal limits values for $f_{H,max}$ are all positively correlated with the critical thermal maxima (CT_{max}, see Glossary; Fig. 4A), the upper temperature at which fish lose equilibrium. Understanding such relationships is useful because CT_{max} is the most commonly used metric to characterize whole-animal heat tolerance (Desforges et al., 2023). When CT_{max} and $f_{H,max}$ values compiled from available studies (see https://doi.org/10.6084/m9.figshare.25661178.v1) were compared, we found that T_{ARR} was 11% below CT_{max}, T_{peak} was 7% below T_{arr}, and $T_{Q_{10}}$ and T_{AB} were ∼25% lower than T_{peak} (Fig. 5). However, CT_{max} is commonly assessed using higher warming rates (typically 0.3°C min^{-1}), which can affect such comparisons. Nonetheless, T_{ARR} is generally similar to, or slightly below, CT_{max}. The other thermal limit metrics for $f_{H,max}$ are typically well below CT_{max} (Figs 5B and 6D; see https://doi.org/10.6084/m9.figshare.25661178.v1) and at temperatures that are inherently encountered more often in the wild, thereby improving their direct ecological relevance. Conversely, temperatures as high as CT_{max} or T_{arr} are acutely lethal, which has required species to evolve behavioural avoidance strategies and occupy biogeographical distributions that make exposure to such temperatures rare (Payne et al., 2016). Below these critical temperatures, T_{peak}, T_{Q10} and T_{AB} are generally sublethal temperatures, and indicate a form of $f_{H,max}$ limitation that that can impair or impose trade-offs on the performance of fitness-related functions (e.g. swimming performance and feeding) (Table 1). Although limits to $f_{H,max}$ such as T_{peak} may be important in many contexts, other vital processes (e.g. feeding or digestion rate) may

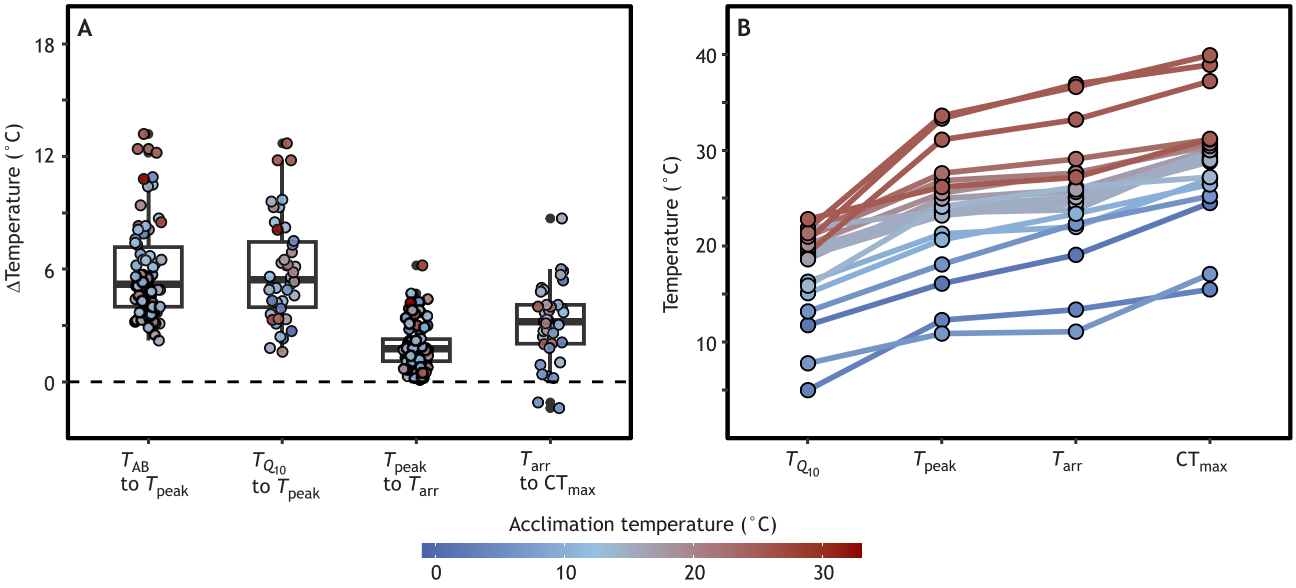

Fig. 5. Differences between and progression of thermal limits identified from the assessment of $f_{\mathrm{H,max}}$ during acute warming in anaesthetized fish. (A) Differences are within individual treatments in a study and are shown for all data included in the data synthesis. (B) The progression of thermal limits in a treatment group are shown for the subset of studies in which T_{Q10}, T_{peak}, T_{arr} and CT$_{\mathrm{max}}$ were all available. In both plots, colors indicate the acclimation temperature. See https://doi.org/10.6084/m9.figshare.25661178.v1.

independently become constrained at lower temperatures and over different time scales, all of which should be considered when making inferences about the effects of temperature on whole-animal performance.

Strengths

A crucial requirement to make predictions for how a warmer future might affect the distribution and success of fish populations is reliably characterizing their upper thermal limits over varied time scales (Comte and Olden, 2017; Desforges et al., 2023). Whole-animal CT$_{\mathrm{max}}$ has a long history and is technically simple to perform (Desforges et al., 2023). Hence, CT$_{\mathrm{max}}$ data are widely available, facilitating impactful examinations of biogeographical patterns of thermal tolerance and important comparisons of upper thermal limits among and within species (e.g. Comte and Olden, 2017; Sunday et al., 2011). Yet, the direct ecological relevance of CT$_{\mathrm{max}}$ continues to be debated, outside the context of relatively rare acute warming events that can cause mass mortality (Desforges et al., 2023). In contrast, characterizations of thermal performance curves for traits such as growth rate and AS have strong ecological relevance and have been used to set water temperature criteria by fisheries managers (Eliason et al., 2024). However, these measurements are technically more challenging and far more time-consuming than determining CT$_{\mathrm{max}}$, making them challenging to perform over broad ranges of species, life stages and environmental contexts. With the rapid rate of ongoing environmental change and over 32,000 species of fishes, the $f_{\mathrm{H,max}}$ method provides an intermediate between more detailed, laborious approaches (AS) and coarser high-throughput methods (CT$_{\mathrm{max}}$) while still providing valuable information relevant to thermal tolerance and limits to cardiorespiratory performance. Ultimately, effective conservation and management practices (e.g. setting water quality targets) are rarely based on single studies or narrow lines of evidence. Rather, multiple lines of evidence are weighed and synthesized to frame conservation challenges and potential interventions (Mayer et al., 2023). To this end, we recommend the $f_{\mathrm{H,max}}$ method as part of the toolbox.

The $f_{\mathrm{H,max}}$ method is a high-throughput assessment of a several ecologically relevant thermal limits and specific information on cardiorespiratory thermal performance. Depending on the warming rate and temperature range, 12 fish can be comfortably assessed in a day with an entire protocol taking ∼2–5 h for two to six fish. This strength enables studies with large or complex designs (see ‘Applications of the measurement of $f_{\mathrm{H,max}}$ in anaesthetized fish’ section) or that have tight time constraints (e.g. a brief field trip or window of fish availability). The method’s other advantages include that it is simple to perform, highly mobile and amenable for field studies in remote locations (Drost et al., 2014; Gilbert et al., 2020; Hansen et al., 2016). Indeed, field-based ECG recordings and the data presented here (Fig. 2B–G; Fig. S1) demonstrate the ability to obtain laboratory quality data in remote settings (Gilbert et al., 2022a). Drost et al. (2014) directly compared fish tested in a field and laboratory setting and obtained similar quality data and typical responses in both cases. However, thermal acclimation conditions differed between the field and lab, precluding direct comparison of absolute values. Successful applications also include a broad range of species, life stages and body masses (e.g. ∼0.5 g zebrafish to >3 kg adult Arctic char) (Gilbert et al., 2022a; Marchant and Farrell, 2019) (see https://doi.org/10.6084/m9.figshare.25661178.v1). Note that a study of Arctic cod (*Boreogadus saida*) demonstrated the potential for the method to be applied with larvae but required significant methodological adjustments (Drost et al., 2016a), and so further validation of TMS effects and pharmacological interventions are needed before widespread with such early life stages.

The use of pharmacological interventions and anaesthesia give rise to some benefits but also have some limitations (see ‘Critiques and limitations’). Anaesthetized fish provide a minimally invasive alternative to more invasive methods that assess cardiorespiratory performance, a marked benefit for fish welfare. Also, fewer fish are needed to generate equivalent data using the $f_{\mathrm{H,max}}$ method. In non-anaesthetized fish, 10 acute exposure temperature challenges with a typical sample size of eight requires either 80 fish, or repeatedly

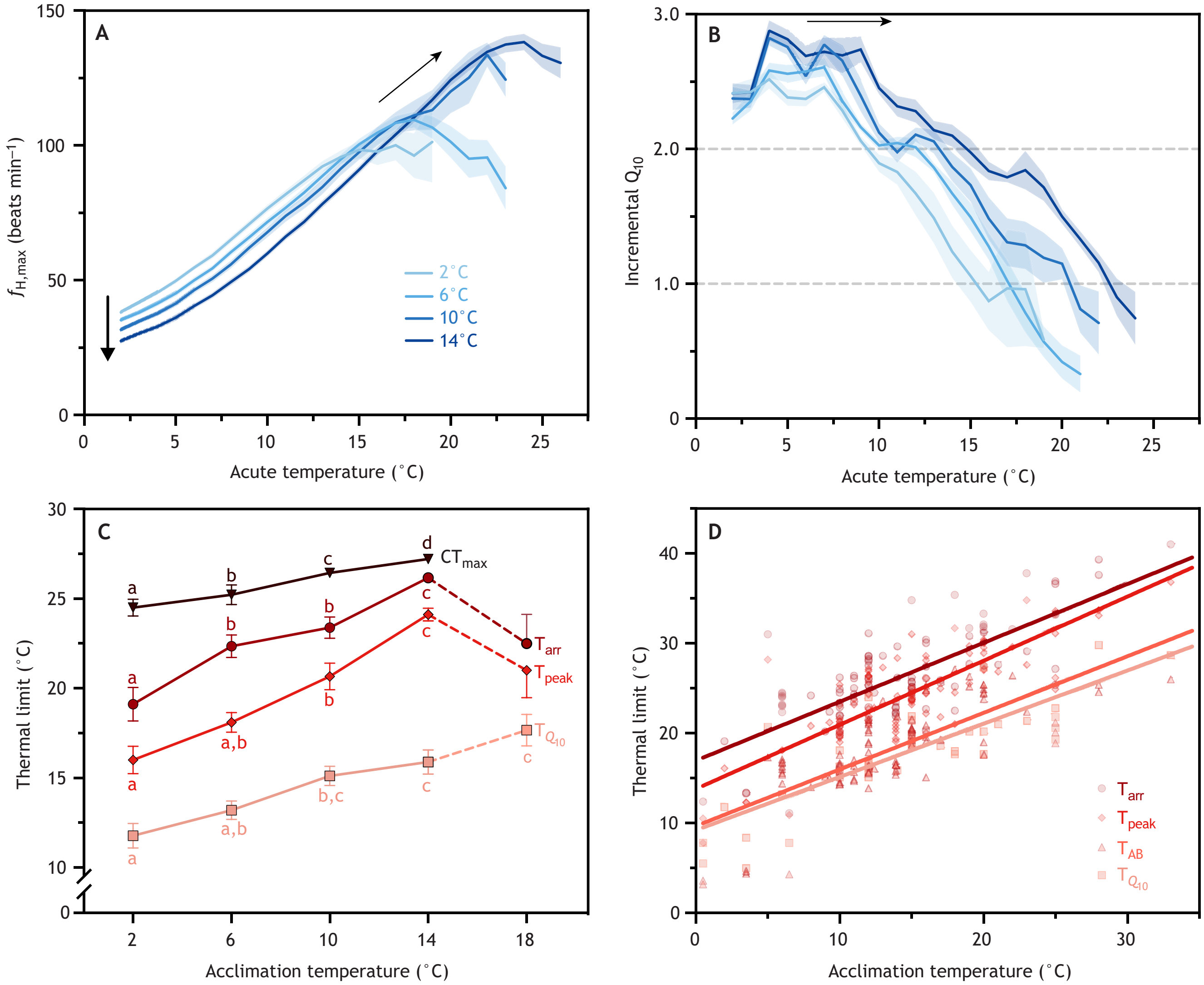

Fig. 6. Plasticity in the response of $f_{H,max}$ to acute warming and associated change in cardiac thermal limits. (A) $f_{H,max}$ and (B) resulting incremental Q_{10} values during acute warming are shown (mean±s.e.m.) for lab-reared Arctic char (472 g; *Salvelinus alpinus)* acclimated to 2, 6, 10 and 14°C (modified from Gilbert and Farrell, 2021). The arrows highlight the thermal compensation of $f_{H,max}$ and an increase in peak $f_{H,max}$ and cardiac thermal limits, which are common (but not universal) aspects of cardiac thermal plasticity. (C) The corresponding thermal limits including temperatures at the Q_{10} threshold (T_{Q10}), peak $f_{H,max}$ (T_{peak}) and the onset of cardiac arrhythmia (T_{arr}) as well as the critical thermal maximum (CT_{max}) are shown, including for fish acclimated to 18°C, a temperature at which mortality was elevated and feeding had ceased. (D) The same thermal limits (excluding CT_{max}) and the Arrhenius breakpoint temperatures (T_{AB}) are shown for all studies examined in our data synthesis (see https://doi.org/10.6084/m9.figshare.25661178.v1), with simple lines of best fit (±95% confidence intervals) for each thermal limit over acclimation temperature (T_{arr} R^2=0.57; T_{peak} R^2=0.66; T_{Q10} R^2=0.68; T_{AB} R^2=0.58). Error bars or shading are encompassed by the symbol or line if not visible. Different letters in C indicate significant differences between acclimation temperatures within a trait (P<0.05; Gilbert and Farrell, 2021).

exposing, exercising and recovering the same eight fish each 10 times, which raises additional fish welfare concerns. In contrast, eight anaesthetized fish can generate similar data in a single day using the $f_{H,max}$ method (see ‘Overview of the method’; e.g. Gilbert and Farrell, 2021; Hardison et al., 2023).

Furthermore, anaesthesia eliminates behavioural responses to warming, limiting the associated variation in f_H and ECG quality. Likewise, variation in the autonomic regulation of f_H (Casselman et al., 2012) is eliminated by artificial stimulation of $f_{H,max}$. These pharmacological interventions also mean that fish can be assessed without a prolonged recovery after capture – an important consideration for field studies. The information gained from the $f_{H,max}$ method can subsequently help streamline studies of other aspects of cardiorespiratory function. Together, these strengths make the $f_{H,max}$ method highly useful for both basic and applied research on the thermal limits to cardiorespiratory performance.

Critiques and limitations

All reductionist approaches have limitations, some of which affect how useful the results are for understanding whole-animal function. However, such methods are most useful when users recognize these limitations and apply the methods for suitable purposes (Treberg et al., 2020). The $f_{H,max}$ method focuses specifically on $f_{H,max}$ because of the central role of f_H in supporting whole-animal aerobic capacity and the cardiovascular response to acute warming (Casselman et al., 2012; Eliason et al., 2013). The $f_{H,max}$ method reveals the upper limits for f_H during acute warming. It does not, nor is it intended to, reflect the response of $f_{H,rest}$ to acute warming, except perhaps at high temperatures where the two responses converge (Fig. 1). This explicit intent has been overlooked in some critiques of the approach (Porter and Gamperl, 2023; Sandrelli and Gamperl, 2023). Alternate methods exist to measure $f_{\mathrm{H,rest}}$, but measuring $f_{\mathrm{H,rest}}$ may not distinguish between a vagal slowing of $f_{\mathrm{H,rest}}$, which may be a protective mechanism (Eliason et al., 2013) from arrhythmia-related cardiac impairment.

Anaesthesia impairing $f_{\mathrm{H,max}}$ (and its response to warming) is a concern, as examined experimentally and discussed by Casselman et al. (2012). Certainly, $f_{\mathrm{H,rest}}$ is commonly affected by anaesthesia (Cotter and Rodnick, 2006), but such studies rarely distinguish between direct effects of anaesthetics on f_{H} or cardiac function (Haverinen et al., 2018) and indirect effects. Indirect effects arise from the partial blockade of vagal tone by some anaesthetics, a release of catecholamines (Lochowitz et al., 1974; Randall, 1962) or hypoxemia driven by the decrease in ventilation, which then triggers vagal slowing of f_{H}. These indirect effects are not a factor when using the $f_{\mathrm{H,max}}$ method because vagal tone is blocked and the gills are artificially ventilated with well oxygenated water. All the same, the usual anaesthetic used (TMS) is a sodium channel antagonist and can impair cardiac sodium currents at sufficient concentrations (Haverinen et al., 2018). In zebrafish cardiomyocytes, Haverinen et al. (2018) found a reduction in sodium current at TMS concentrations >168 mg l^{-1} but none below ~100 mg l^{-1}. This higher TMS concentration is above the maintenance concentration commonly used for the $f_{\mathrm{H,max}}$ method, and available evidence for salmonids suggests minimal if any effect of a maintenance concentration of TMS on $f_{\mathrm{H,max}}$ (Casselman et al., 2012). Not surprisingly then, the response of f_{H} in non-anaesthetized, atropinized rainbow trout to acute warming (Gilbert et al., 2019) was nearly identical to that for the $f_{\mathrm{H,max}}$ method (Fig. 3). Nonetheless, researchers should use a minimal TMS maintenance concentration to avoid the untoward effects described above. Unfortunately, equivalent data are unavailable for other species.

A related concern is that anaesthesia abates any stress responses and associated additional metabolic demands that normally arise at high temperature. The fish's internal milieu may constrain $f_{\mathrm{H,max}}$ perhaps through cardiac oxygen or substrate limitations that do not occur to the same extent *ex vivo*, in fish at rest or while under anaesthesia (Eliason et al., 2013). Indeed, although $f_{\mathrm{H,max}}$ was indistinguishable for non-anaesthetized and TMS-anaesthetized fish over intermediate temperatures, $f_{\mathrm{H,max}}$ tended to be lower in non-anaesthetized fish forced to exercise near their upper thermal limits (Casselman et al. (2012). In non-anaesthetized sockeye salmon (*Oncorhynchus nerka*) at high temperatures, $f_{\mathrm{H,max}}$ during exercise fell below the $f_{\mathrm{H,rest}}$ of non-exercising fish (Eliason et al., 2013). Also, peak $f_{\mathrm{H,max}}$ is substantially reduced under hypoxia (Schwieterman et al., 2023). Thus, the $f_{\mathrm{H,max}}$ method may produce a 'best case scenario' for the response to acute warming. Congruently, warming-induced peak f_{H} in non-exercised, non-anaesthetized rainbow trout (*Oncorhynchus mykiss*) and Arctic char (*Salvelinus alpinus*) is highly similar to that from the $f_{\mathrm{H,max}}$ method (Fig. 3). Nonetheless, T_{ARR} is generally less than – or occasionally similar – to $\mathrm{CT_{max}}$ (Fig. 3), with the other thermal limits falling below T_{ARR}, so the method still provides a series of conservative estimates of acute heat tolerance.

While a concern of eliciting $f_{\mathrm{H,max}}$ pharmacologically is that it precludes normal CNS integration to warming, the benefits of autonomic control of f_{H} to both cardiac and whole-animal thermal tolerance have been explored in non-anaesthetized fish (Ekstrom et al., 2021; Gilbert et al., 2019). Stimulation of cardiac β-adrenergic receptors can improve heat tolerance and increase peak f_{H} (Ekstrom et al., 2021; Gilbert et al., 2019); the $f_{\mathrm{H,max}}$ method achieves this by injection of isoproterenol rather than a CNS-induced increase in sympathetic output to the heart. However, blocking muscarinic acetylcholine receptors with atropine can prevent the increase in T_{peak} (Gilbert et al., 2019), but does not always (Ekström et al., 2014, 2021). Even if $f_{\mathrm{H,max}}$ values from anaesthetized fish are generally consistent with available literature values (see Anttila et al., 2013a; Casselman et al., 2012), further direct investigations comparing pharmacologically and activity-induced $f_{\mathrm{H,max}}$ in anesthetized and non-anaesthetized fish, respectively, would help quantify effects of anaesthesia. To this end, Sandrelli and Gamperl (2023) compared f_{H} responses to acute warming in anaesthetized fish, non-anaesthetized fish confined in a respirometer and free-swimming fish. They discovered multiple differences in f_{H} and cardiac heat tolerance among the different methods. They applied pharmacological treatments similar to those discussed here in anaesthetized fish; however, $f_{\mathrm{H,max}}$ was not measured in non-anaesthetized fish. Other differences precluded direct evaluation of the $f_{\mathrm{H,max}}$ method including an invasive surgical implantation of ECG loggers, high initial anaesthetic concentration, caudal vein injections of the cardioactive drugs, a warming rate that the authors determined was too fast for the large fish used in the study, and a continuous warming ramp or large warming increment, although the specific details were not presented. Furthermore, intermittent ECG recordings precluded the identification of T_{arr}. Nevertheless, future studies aiming to evaluate and refine the $f_{\mathrm{H,max}}$ method or complementary high-throughput methods are welcome given the urgent need to expand our knowledge of species- and context-specific aspects of thermal physiology.

Applications of the measurement of $f_{\mathrm{H,max}}$ in anaesthetized fish

Characterization of cardiac thermal performance and heat tolerance

The initial proposed application of the $f_{\mathrm{H,max}}$ method was to rapidly assess T_{AB} as a proxy for the optimal temperature for AS because of their numerical associations (Anttila et al., 2013a; Casselman et al., 2012). It continues to be applied in that regard, but its use has quickly expanded to include assessments of upper thermal limits and the general study of cardiac thermal responses to acute warming. It has been used to study cardiac thermal performance in data-poor species (Hansen et al., 2016; Drost et al., 2014; Skeeles et al., 2020), to determine how that performance relates to environmental exposures under current and climate change scenarios (Gilbert et al., 2020; Van Der Walt et al., 2021), and to examine associations among physiological functions across levels of organization (Anttila et al., 2013a; Adams et al., 2022).

Examining intraspecific and interspecific diversity of thermal physiology

As the application the $f_{\mathrm{H,max}}$ method expands to a broader range of species and contexts, opportunities emerge for broad-scale examinations of phylogenetic and biogeographical patterns in cardiac thermal performance as previously explored for AS and $\mathrm{CT_{max}}$ (Comte and Olden, 2017; Payne et al., 2016; Sunday et al., 2019; Sunday et al., 2011). Although outside the specific scope of this Review, the compiled data (https://doi.org/10.6084/m9.figshare.25661178.v1; Fig. S2) demonstrate that sufficient data are already available to allow for interspecific comparisons of thermal physiology among species or other levels of classification. However, such interspecific comparisons within a single study are currently uncommon. One study did identify differences in cardiac thermal tolerance among closely related *Danio* species (Sidhu et al., 2014). More studies have applied the method to examine intraspecific variation in thermal physiology among genetic crosses, and strains within multiple salmonid species (Anttila et al., 2014a; Chen et al., 2013, 2015b, 2018a,b; Chen and Narum, 2021; Gradil et al., 2016; Muñoz et al., 2014a,b). For instance, Chen et al. (2018b) found that even when reared in a common environment, redband trout (*Oncorhynchus mykiss*) from populations obtained from cool montane habitats had a lower peak $f_{\mathrm{H,max}}$ (see Glossary) than a population obtained from a hot desert environment. This difference corresponded with population differences in aerobic thermal performance and gene expression.

Examining context dependence of cardiac thermal performance and heat tolerance

The method has also been used to examine the effect of numerous other factors on cardiac thermal performance with both basic science and conservation motives. Kraskura et al. (2023) found that, as expected, $f_{\mathrm{H,max}}$ decreased as body mass increased (mass scaling exponent: −0.05), whereas cardiac thermal limits increased with body mass. Others have found that the specific nutrients and dietary compounds (e.g. taurine and fucoidan) can affect peak $f_{\mathrm{H,max}}$ and cardiac thermal performance (Baker et al., 2023; Dixon et al., 2023; Papadopoulou et al., 2022), as with diet more generally (Hardison et al., 2021, 2023). Researchers have also examined associations with swimming performance (Anttila et al., 2014b) and identified improved cardiac heat tolerance following exercise training in some contexts (e.g. intermediate exercise intensity) (Papadopoulou et al., 2022; Pettinau et al., 2022b). Other factors examined have included contaminant exposures (Anttila et al., 2017), genetic modification (Chen et al., 2015a), induction of triploidy (Verhille et al., 2013), varied life-history tactics (Mottola et al., 2020), hypoxia and hyperkalemia (Schwieterman et al., 2023), ocean acidification (Crespel et al., 2019) and thermal history (Eliason and Anttila, 2017). Among these, the method has been most widely and effectively applied in the study of patterns and limits in cardiac plasticity in response to varied thermal histories.

Cardiac thermal plasticity

Studies of thermal physiology often struggle to separate the consequences of temperature acclimation from the direct effect of thermal variation. A principal strength of the $f_{\mathrm{H,max}}$ method is that this separation can be made straightforward by examining the cardiac effects of acute thermal change in fish from multiple acclimation temperatures. Thermal acclimation can reset the intrinsic cardiac pacemaker rate (through changes in membrane/ion channels and pumps) and change the level of autonomic control of heart rate (Gamperl and Farrell, 2004; Sutcliffe et al., 2020; Vornanen et al., 2002a,b). Such changes are revealed in the $f_{\mathrm{H,max}}$ method through various changes in cardiac thermal limits (T_{arr}, T_{peak}, T_{Q10} and T_{AB}), peak $f_{\mathrm{H,max}}$ and $f_{\mathrm{H,max}}$ at common test temperatures (Marchant and Farrell, 2019; Eliason and Anttila, 2017) (Fig. 6). These changes are both species specific and context dependent. For example, at common, moderate test temperatures, cold-acclimated Atlantic salmon (*Salmo salar*) had a higher $f_{\mathrm{H,max}}$, along with lower thermal limits and peak $f_{\mathrm{H,max}}$ than their warm-acclimated counterparts (Anttila et al., 2014a). Although several species display the same classic thermal compensation response of $f_{\mathrm{H,max}}$ – being higher over moderate temperatures after cold acclimation (Anttila et al., 2014a; Adams et al., 2022; Drost et al., 2016b; Gilbert and Farrell, 2021; Fig. 6) – this is not universal. For example, in killifish (*Fundulus heteroclitus*), an acclimation temperature of 15°C produced the highest $f_{\mathrm{H,max}}$ at a common temperature when compared with both cold (5°C) and warm (33°C) acclimation (Safi et al., 2019). Yet, peak $f_{\mathrm{H,max}}$ and the thermal limits still increased with acclimation temperature (Safi et al., 2019). Thus, the method can be used to explore the diversity in cardiac thermal plasticity among fishes.

While cardiac thermal limits generally increase with warm acclimation, there is a ‘thermal ceiling’ for cardiac plasticity, much like for other measures of acute heat tolerance (i.e. LT_{50}, $\mathrm{CT}_{\mathrm{max}}$). In fact, acclimation close to this thermal ceiling can even lower thermal limits and reduce peak $f_{\mathrm{H,max}}$ (Adams et al., 2022; Gilbert and Farrell, 2021; Pettinau et al., 2022a; Marchant and Farrell, 2019) (Fig. 6C). For instance, Adams et al. (2022) performed the method on rainbow trout acclimated to six temperatures from 15°C to 25°C. They found that thermal limits increased with acclimation temperature up to 23°C, but increasing the acclimation temperature to 25°C decreased these limits and the peak $f_{\mathrm{H,max}}$ achieved during warming. The $f_{\mathrm{H,max}}$ test can be rapidly assessed across acclimation temperatures to identify this ceiling for species of conservation concern.

Thermal plasticity is time dependent. In $\mathrm{CT}_{\mathrm{max}}$ acclimation rate trials, for example, $\mathrm{CT}_{\mathrm{max}}$ increases logarithmically with time when moved from cold to warm (Fangue et al., 2014). The same principle applies to cardiac plasticity. By studying $f_{\mathrm{H,max}}$ throughout an acclimation (Gilbert et al., 2022b; Hardison et al., 2023) or during fluctuating temperature treatments (Schwieterman et al., 2022), researchers can (1) assess how rapidly the animals can acclimate, (2) better model performance and thermal limits of species in response to environmentally relevant temperature exposures, and (3) examine mechanisms of heart rate resetting across species and in response to secondary stressors. Examining this time course has revealed varied results in a few studies. In rainbow trout, only T_{Q10} increased whereas T_{peak} and T_{arr} were unaffected when acclimation temperature was increased from 10°C to 18°C (Gilbert et al., 2022b). In the same study, $f_{\mathrm{H,max}}$ over moderate temperatures was rapidly (∼72 h) reset to a lower level while peak $f_{\mathrm{H,max}}$ rapidly increased, but then subsided after 2 weeks of acclimation to 18°C. In opaleye (*Girella nigricans*), the fish’s thermal limits and peak $f_{\mathrm{H,max}}$ increased when warmed from 12 to 20°C for 2 weeks, but only after being fed a carnivorous or omnivorous diet (Hardison et al., 2023). When the fish were fed an herbivorous diet, their thermal limits still increased, but their peak $f_{\mathrm{H,max}}$ did not. Notably, differences in $f_{\mathrm{H,max}}$ were related to the fatty acid composition of the heart, which was affected by the fish’s diet. Future research should investigate how mechanisms of cardiac pacemaker resetting and autonomic control are influenced by thermal exposure time and extrinsic factors – such as diet – to understand the relative contributions of these factors to changes in $f_{\mathrm{H,max}}$ in wild and farmed fishes. The $f_{\mathrm{H,max}}$ method is a valuable assay for examining these mechanisms more closely across taxa, time scales and environments.

Emerging applications and future directions

Several emerging applications and future directions have the potential to expand the utility of the $f_{\mathrm{H,max}}$ method. Given the high-throughput nature of the method, it can be used to study diversity in the plasticity and drivers of cardiac thermal performance across fish taxa. For instance, mechanisms of cardiac failure may differ among species and life stages based on the extent to which they rely on coronary circulation for cardiac oxygen supply, or based on the relative composition of their myocardium (e.g. spongy versus compact; Ekström et al., 2021, 2023). High quality ECG measurements permit detailed ECG waveform analyses and the application of well-established interpretations of the relationships among waveforms to understand changes in the cardiac cycle that underly changes in heart rate and function (e.g. Badr et al., 2016). This potential has been largely unexploited so far using the $f_{\mathrm{H,max}}$ method (Pettinau et al., 2022a). Additional or alternative pharmacological interventions can target other specific ion channels or regulatory mechanisms. There is substantial room for growth in this regard in addition to the study of pace making mechanisms by Marchant and Farrell (2019), and ongoing studies which use non-selective adrenergic antagonists to study intrinsic f_{H} and the thermal plasticity of adrenergic sensitivity. Lastly, Doppler echocardiography can be used, instead of ECG electrodes, to record cardiac blood flow holistically ($\dot{Q}$ as function of SV and $f_{\mathrm{H,max}}$) and reveal additional information regarding the collapse of cardiac function at high temperatures (Muir et al., 2022, 2021). However, how SV and $\dot{Q}$ in the $f_{\mathrm{H,max}}$ preparation relates to that which occurs under routine or elevated workloads (e.g. exercise and digestion) has not been established.

Conclusions

In summary, the assessment of $f_{\mathrm{H,max}}$ in pharmacologically stimulated, anaesthetized fish is an effective high throughput method for the study of cardiac thermal performance during acute warming and the quantification of upper thermal limits. The resulting f_{H} and thermal performance metrics are valuable for the basic study of cardiac function and in an applied context for understanding how rapid, ongoing environmental change may impact fishes. Although these metrics are valuable on their own, the method also provides an excellent starting point for studying mechanisms that underly temperature effects on cardiac and cardiorespiratory performance at lower levels of organization (e.g. isolated myocytes, mitochondria and ion channels) and integrated outcomes at the whole-animal level. For instance, subsequent targeted molecular studies or whole-animal performance assessments can be performed at the specific temperatures that were identified as limiting (i.e. T_{AB}, T_{peak} and T_{arr}) in the $f_{\mathrm{H,max}}$ method without having to generate full thermal performance curves for traits that are far more time consuming to assess. Such studies are more urgent now than ever as a thorough understanding of the causes and consequences of physiological responses to variable thermal regimes will assist in predicting and mitigating outcomes of global environmental change.

Competing interests
The authors declare no competing or financial interests.

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Data availability
Mean values for metrics from the $f_{\mathrm{H,max}}$ assessment and additional relevant metadata are available from figshare: https://doi.org/10.6084/m9.figshare.25661178.v1.

ECR Spotlight
This article has an associated ECR Spotlight interview with Matthew Gilbert.

Special Issue
This article is part of the Special Issue ‘The integrative biology of the heart’, guest edited by William Joyce and Holly Shiels. See related articles at https://journals.biologists.com/jeb/issue/227/20.

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