Received: 4 February 2025 | Accepted: 26 June 2025

DOI: 10.1111/2041-210X.70103

Methods in Ecology and Evolution

REVIEW

Measuring critical thermal maximum in aquatic ectotherms: A practical guide

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Funding information
European Research Council; Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery; Deakin University; Marie Skłodowska-Curie Actions, Grant/Award Number: 893895; Future Fellowship, Grant/Award Number: FT180100154; Australian Government

Handling Editor: Clive Trueman

Abstract

1. Critical thermal limits, commonly quantified as CT_{max} (maximum) or CT_{min} (minimum), are core metrics in the thermal biology of aquatic ectotherms. CT_{max}, in particular, has recently surged in popularity due to its various applications, including understanding and predicting the responses of animals to climate warming.
2. Despite its growing popularity, there is a limited literature aimed at establishing best practices for designing, running and reporting CT_{max} experiments. This lack of standardisation and insufficiently detailed reporting in the literature creates challenges when designing CT_{max} studies or comparing results across studies.
3. Here, we provide a comprehensive, practical guide for designing and conducting experiments to measure critical thermal limits, with an emphasis on CT_{max}. Our recommendations cover 12 topic areas including apparatus design, masking (blinding), warming rates, end points, replication and reporting. We include diagrams and photos for designing and building critical thermal limit arenas for field or lab applications. We also provide a reporting checklist as a reference for researchers when carrying out experiments and preparing manuscripts.
4. Future studies incorporating critical thermal limits would benefit from transparent reporting of warming/cooling rates (raw data, supplementary graphs) and

photo/video evidence showing arena designs and critical thermal limit end points. We also provide directions for empirical research that will help further inform the measurement of critical thermal limits, including biotic factors like stress and digestion, warming/cooling rates, the effects of body mass on heat transfer and the physiological mechanisms underlying thermal tolerance.

KEYWORDS
climate change, experimental design, fish, global warming, heat shock, heat stress, heat wave

1 | INTRODUCTION

As climate change causes more frequent and severe aquatic heat waves (e.g. Tassone et al., 2023; Woolway et al., 2021), scientists are increasingly focused on understanding the thermal biology of aquatic animals. Tolerance to acute temperature changes, as experienced during heat waves, is typically assessed through experiments that involve gradual increases in temperature (e.g. degrees per minute or per hour). Experimentally testing acute thermal tolerance has more direct ecological relevance for some species and contexts than others (e.g. rockpools can undergo extreme changes over a timescale of minutes; Desforges et al., 2023; Smit & Glassom, 2017). Nevertheless, standardised testing of tolerance to acute heat stress has been useful across a range of disciplines and aquatic ecosystems (Pörtner et al., 2005; Sunday et al., 2012; Terblanche et al., 2011).

Researchers have been interested in describing lethal temperatures for fishes and other ectotherms for over 150 years using a variety of measurements and end points (history reviewed by: Beitinger et al., 2000; Lutterschmidt & Hutchison, 1997). However, critical thermal maximum (CT_{max}) has been the most widely used metric to quantify the acute upper thermal tolerance of ectotherms for decades (Beitinger et al., 2000; Desforges et al., 2023; Ern et al., 2023). CT_{max} is typically reported as the temperature at which an animal loses motor control—often following a distinct period of agitation—during thermal ramping. This method is useful across a range of applications and research questions, such as predicting species distributions (Sunday et al., 2012; Woodin et al., 2013), investigating impacts of stressors (Haney & Walsh, 2003; Patra et al., 2007), quantifying acclimation and adaptation (Comte & Olden, 2017; Fangue et al., 2006; Ruthsatz et al., 2024) and investigating physiological mechanisms underlying thermal tolerance (Cuenca Cambronero et al., 2018; Ern et al., 2023). For aquatic ectotherms, CT_{max} experiments involve placing animals into an arena for observation, warming the water in the arena at a steady rate, and then recording the temperature at which each individual reaches its end point (loss of motor control, e.g. loss of equilibrium [LOE], state of immobility, or onset of spasms; Cowles & Bogert, 1944). CT_{max} has long been popular among ecologists and physiologists, likely owing to its simplicity and the fact that it is a non-lethal assay. However, over the past 15 years, interest in the CT_{max} of aquatic ectotherms has grown dramatically (Figure 1), suggesting that many researchers are now trying the method for the first time.

Despite its popularity and potential for standardisation, CT_{max} experiments vary greatly in how they are conducted and reported. As a result, the data from CT_{max} experiments likely vary in how interoperable and reusable they are. Papers have focused on a range of aspects of CT_{max} methodology, including warming rates (Galbreath et al., 2004; Mora & Maya, 2006), within-individual repeatability (Grinder et al., 2020; Morgan et al., 2018) and behavioural end points (Cowan et al., 2023; Lutterschmidt & Hutchison, 1997). Lacking, however, is a comprehensive, practical guide for measuring CT_{max} across a range of aquatic ectotherms.

This paper builds on the authors' experience with CT_{max} methodology and presents a set of best practices for conducting CT_{max} trials on aquatic ectotherms. With the guidance presented here, we hope to standardise the methodology and reporting of CT_{max} experiments to enhance the replicability, reliability and comparability of CT_{max} data. While there are other useful measures of maximum thermal limits (e.g. incipient lethal temperature [ILT], Thermal Death Time; Fry, 1947; Jørgensen et al., 2021) including field data on thermal occupancy (Bear et al., 2007; Challice et al., 2019; Dallas & Ketley, 2011; Webb et al., 2020), CT_{max} is our focus here. Most of our recommendations are transferable to other methods for estimating thermal limits like median lethal temperature (LT_{50}) experiments, critical thermal minimum (CT_{min}), or CT_{max} measurements in terrestrial ectotherms. Our set of best practices is primarily based on experiments with fishes, but we include insights from studies on aquatic invertebrates (Section 6).

2 | OVERVIEW OF CT_{max} APPARATUS DESIGN

In a CT_{max} trial, water is continuously heated in an arena where animals can be visually tracked and scored. There is no standard design for a CT_{max} apparatus; a customised aquarium tailored to the experiment's context and species is often necessary. Here we describe a template that can be modified as needed. Our design (Figure 2) features a tank with a mesh separator dividing the animal arena from a small section of the tank (the 'equipment section') containing the heating element(s), a submersible pump for mixing (Section 5), and an air stone for aeration. Most of the tank is

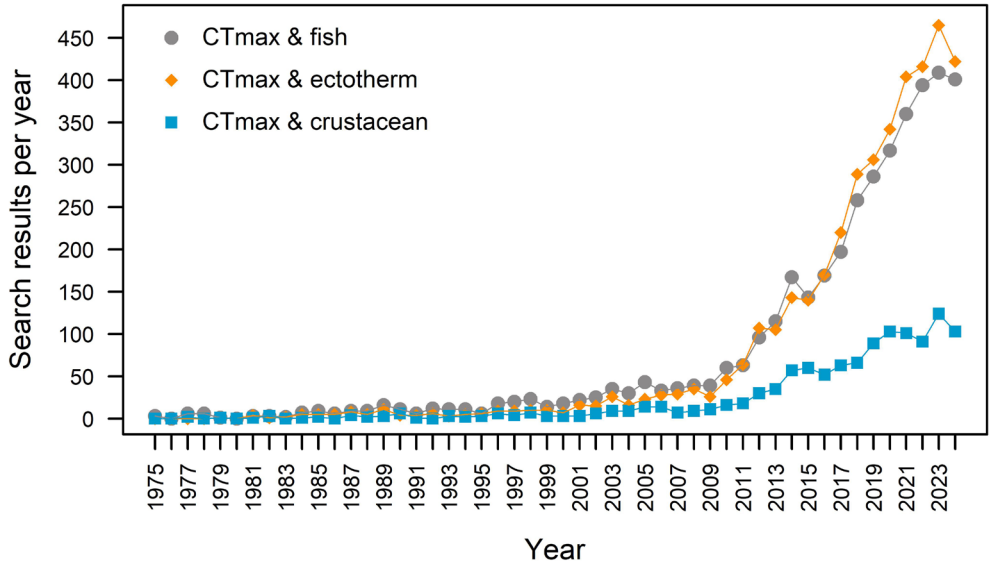

FIGURE 1 The number of scientific articles mentioning CT_{max} was collected for each year between 1975 and 2024 using Google Scholar (2025-01-21 search terms: 'CT_{max} & fish', 'CT_{max} & ectotherm', 'CT_{max} & crustacean'), indicating a surge in interest in CT_{max} over the past *ca.* 15 years.

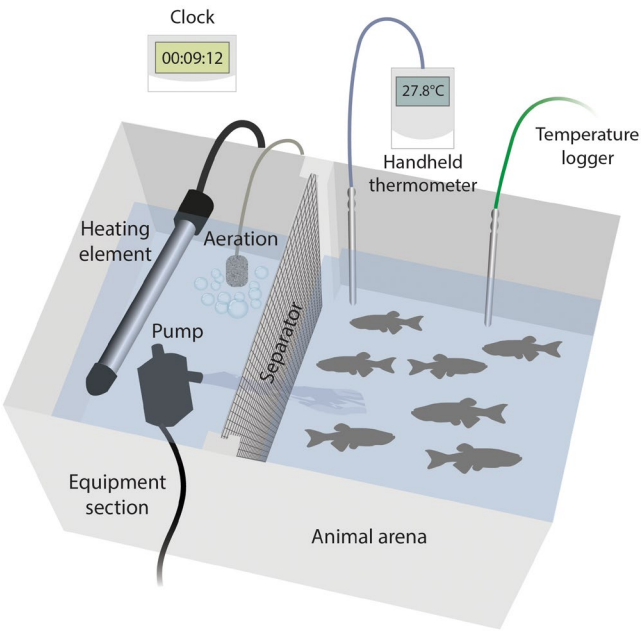

FIGURE 2 Diagram of a template design for a CT_{max} apparatus. Aeration, mixing mechanisms (pump), and heating elements are in an 'equipment section' that is physically separated with a screen from the part of the arena containing the animals ('animal arena'; here with fish). A submerged temperature sensor (or data logger) is used to log temperatures, in addition to a temperature sensor with a display that is used to score CT_{max} in real time. The time and temperature should only be visible to the data recorder (and/or video camera), not to the person observing animals for end points (see Section 7).

reserved for the animals ('animal arena'), providing sufficient water volume for horizontal and vertical movement. The animal arena should have a background colour that contrasts with the animals (e.g. Figure 3b–d), to facilitate manual or automated visual tracking. The tank should contain at least one temperature logger in the animal arena (see Sections 4 and 13), and at least one temperature sensor connected to a digital display so that temperature can be read (Section 5) and recorded by experimenters (and/or on video) in real time as animals reach their end points (Section 7). Temperature sensors should be positioned so that they are not in contact with the walls or bottom of the tank as these may warm at a different rate than the water. The partition between the animal arena and the equipment arena should have small enough openings that animals cannot get through it while allowing ample water circulation. We have used both perforated sheets (e.g. 3 mm thick) and rigid mesh of varying dimensions, made of either plastic or metal, as materials for creating the partition (in North America, McMaster-Carr Supply Company can supply a variety of options for these: www.mcmaster.com). This design allows for the animal arena to be free from most equipment, tubing and other obstructions, which helps with observing and netting individuals. It also prevents animals from hiding behind equipment and reduces the risk of harmful contact with a heating element or a pump.

In our design, a uniform heating rate among replicate trials (Section 4) is achieved by using consistent heating power (i.e. number and type of heaters), a fixed water volume and, ideally, a fixed surrounding air temperature (but see Section 11). As such, the CT_{max} tank should be drained between trials and then refilled with a standardised volume of water, using the same water source used for housing the animals (e.g. by having a 'fill to' water level mark) (Section 11). Ideally, the room temperature should be kept constant and the tank should be made of thick or insulated material to reduce heat exchange with the surrounding environment. For example, CT_{max} tanks can be constructed using insulated containers, or by adding insulating foam to the outside of the tank (Figure 3a–c).

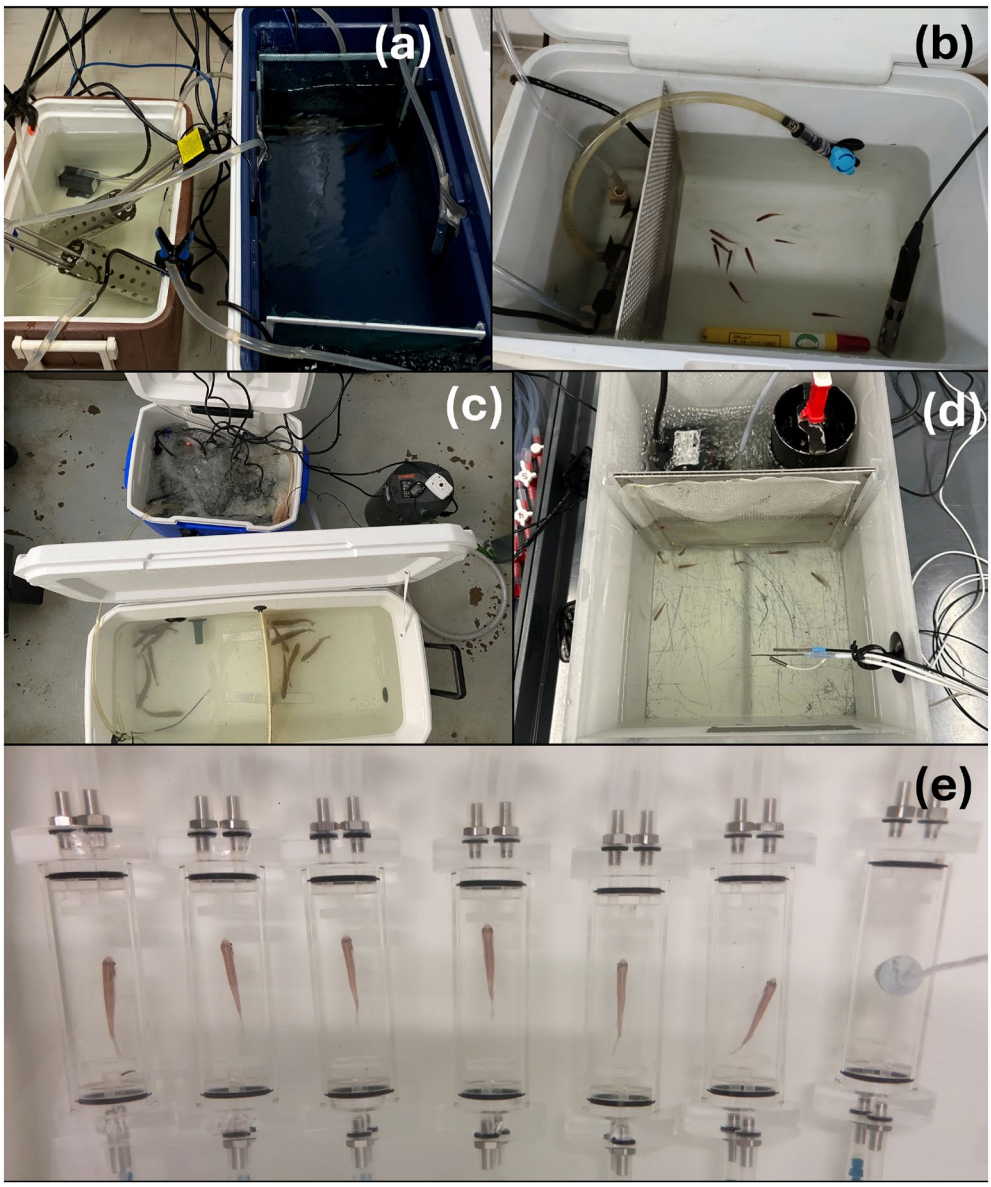

FIGURE 3 Images of CT_{max} apparatus designs. (a) A sump connected design, with the sump tank (left) containing heating elements and a pump to recirculate water to the animal arena (right) used for pumpkinseed (*Lepomis gibbosus*). Note the dark background of the arena in this example makes it more difficult to observe the fish (a contrasting background is preferred). (b) CT_{max} apparatus similar to our template design (Figure 2). A YSI Pro-solo oxygen and temperature sensor is placed in the animal arena (right) along with an additional temperature logger (RBRsolo^3 T, https://rbr-global.com/). The hose and valve going from the left side of the partition to the right are connected to a submersible pump to facilitate water mixing. Setup used for several species, here bluntnose minnow (*Pimephalus notatus*). (c) A split design similar to (a), in which a sump (top) contains heaters, aeration, and pumps to constantly exchange water with the animal arena (bottom). In this example, there is a partition in the animal arena to keep two replicate groups of brook trout (*Salvelinus fontinalis*) separated. The template design in Figure 2 is based on the arena in (d). In addition to a temperature sensor connected to a handheld display (not shown), there is a second thermistor and an oxygen sensor from a FireSting-O_2 meter (PyroScience, https://www.pyroscience.com/). The black metal cylinder in the top section of the arena in (d) is designed to hold a 300 W heating element and connected to the submersible pump to ensure water flow over the heating element and then out of the metal cylinder towards the animal arena without melting and damaging the tank. (d) Shows a CT_{max} trial on a group of zebrafish (*Danio rerio*) while (e) displays zebrafish housed individually in flow-through respirometry chambers. The setup includes six trial chambers and one control chamber equipped with a temperature probe, each receiving water from the same flush pump.

Modifications to this suggested template are often necessary to accommodate factors like animal size. For example, if the mesh size needs to be small due to small animals tested, it may be useful to pump water via a tube from the equipment section to the animal arena to promote mixing and thermal homogeneity (Figure 3b). It is important to ensure that water mixing is not so vigorous as to force the animals to swim actively to maintain position (Section 5). Alternatively, a second tank (sump) can be connected by tubing to the animal arena

with constant water exchange between the two—the sump tank then serves as the 'equipment section' (Figure 3a,c). This latter design may allow a higher total water volume and necessitate more heating power, along with more powerful submersible pumps to ensure sufficient mixing between the sump and the animal arena. Submersible pumps do generate some heat and sound, which can influence heating rate and potentially stress animals. Pumps should therefore be kept consistent (position, make, model) among replicate trials.

Another CT_{max} apparatus design is to house animals individually or in groups within replicate chambers (i.e. multiple animal arenas) that are immersed in a common temperature-controlled water bath that contains the heater and mixing pump (e.g. Healy & Schulte, 2012). With the latter design, each chamber is fitted with at least one temperature sensor, and the chambers allow water to flow through, using a pump via tubing. Housing animals individually allows easier quantification of individual agitation temperatures from video (Firth et al., 2021; Wells et al., 2016), a task that can be more difficult when many individuals are moving around in the animal arena. Furthermore, individual housing chambers may allow for the CT_{max} trial to be combined with measurements of other physiological responses. For example, animals tethered via catheters or sensors typically require confinement.

Measurement of CT_{max} can also be done with animals confined individually in respirometry chambers so that oxygen uptake can be estimated (Ern et al., 2016, 2017; Figure 3e). Depending on the size of the respirometry chambers, this technique might restrict movement with unknown (likely species-specific) consequences for CT_{max}. Fish exposed to low levels of water oxygen (hypoxic, e.g. <70% air saturation) during typical CT_{max} trials with an open water surface may exhibit aquatic surface respiration (Rutledge & Beitinger, 1989) in which the fish actively ventilate their gills very close to the air-water interface, where dissolved oxygen is typically higher. Aquatic surface respiration could potentially affect CT_{max} (e.g. by affecting energy expenditure or oxygen uptake, if either of these factors were to affect CT_{max}) and can be prevented using submerged chambers. Recording the number of aquatic surface respiration events can be used to document changes in the frequency of this behaviour during thermal ramping as an additional variable to explore (Francispillai & Chapman, 2025).

A heating mantle design can be useful for testing CT_{max} in very small organisms such as aquatic larvae or embryos (Andreassen et al., 2022; Cowan et al., 2023). In this design, the water in the animal arena is not exchanged, but heated from a surrounding heating mantle (Figure 4). The setup requires aeration and recording of temperature and oxygen level directly in the animal arena but has the advantage of maintaining a still water surface, enabling clear video recording.

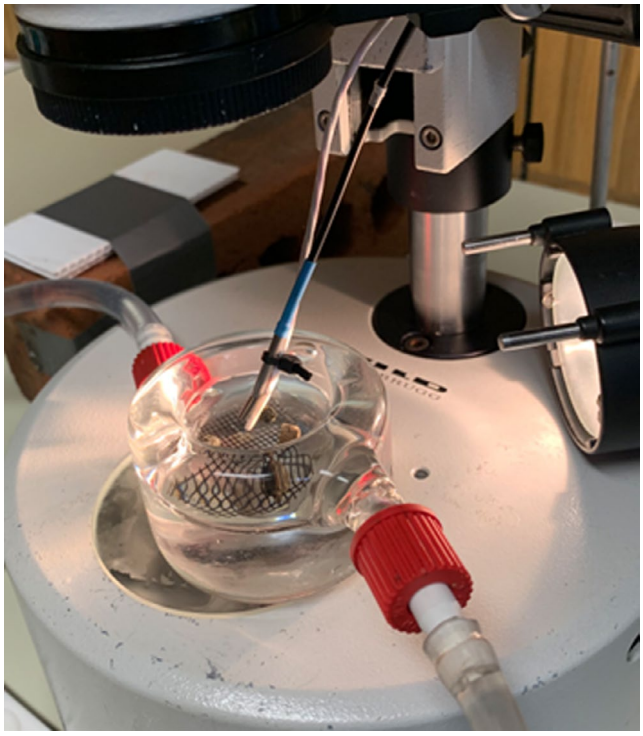

FIGURE 4 Custom-built glass chamber including a shallow well (20 mL volume) to assess CT_{max} of aquatic embryos or larvae. The chamber is a well in an enclosed water bath (heating mantle) heated by water pumped through the red nozzles. Embryos are at the bottom of the well, inside a fine mesh, kept in place by small pebbles. Larvae can be tested in the same arena without a mesh. The heating mantle is placed on a stereomicroscope, attached to a camera, to monitor individual movement. An optical oxygen probe and temperature sensor are placed in the animal arena without blocking the view of the embryos or larvae.

3 | CONSIDERATIONS FOR SELECTING A WARMING RATE

Warming rates can affect the CT_{max} values obtained from a trial (Elliot & Elliott, 1995; Moyano et al., 2017; Vinagre et al., 2015), and thus, an appropriate warming rate must be identified before the experiments begin. There are two key considerations for selecting a warming rate. First, the rate of warming should be slow enough to allow target tissue temperatures to remain close (ideally, within 0.2°C) to the temperature of the surrounding water as it warms. The processes and tissues that govern CT_{max} remain incompletely understood (Ern et al., 2023), so consideration must be given to the warming rate and the thermal inertia of different sized animals and the tissues within them (e.g. thermal inertia may be higher, and the lag time greater, for the body cavity and deep muscle of a fish in comparison with its brain or heart). Second, thermal acclimation can manifest within hours of heat exposure in some species (De Bonville et al., 2025), so the rate should be fast enough to avoid unwanted (partial) acclimation to intermediate temperatures along the temperature ramp (Åsheim et al., 2020; Jutfelt et al., 2019; Penman et al., 2023). Acclimation of animals during slower ramping rates could complicate interpretation of the resultant CT_{max} data, especially in relation to understanding the role of prior acclimation temperatures or when comparing against other studies or body sizes.

Optimal rates of warming in CT_{max} experiments may differ across taxa, life stages, body sizes, acclimation temperatures, research questions, and in relation to the natural environment of the species

(Mora & Maya, 2006; Moyano et al., 2017; Vinagre et al., 2015). A warming rate of 0.3°C min^{-1} is commonly used and generally accepted as a standard for small fish (Becker & Genoway, 1979). In Atlantic salmon parr (*Salmo salar*), warming rates ranging from 1 to 18°C h^{-1} (0.02 to 0.3°C min^{-1}) produced no differences in CT_{max} (Elliot & Elliott, 1995). Becker and Genoway (1979) found that in juvenile coho salmon (*Oncorhynchus kisutch*) and pumpkinseed (*Lepomis gibbosus*), warming rates of 0.5 and 1°C min^{-1} produced higher temperatures for LOE than did 0.3°C min^{-1}. Since rates higher than 0.3°C min^{-1} can produce delayed physiological responses (see below), when appropriate, we recommend using 0.3°C min^{-1} to allow for comparison across studies and species.

A faster warming rate may not allow a test animal's internal body temperature to equilibrate with the water temperature, especially in larger animals. Internal temperature lag (thermal inertia) depends on body size: Larger animals have more thermal inertia than do smaller animals (Kitagawa & Kimura, 2006; Nakamura et al., 2020). Thus, larger animals typically require slower warming rates if the goal is to ensure core tissues remain in thermal equilibrium with water temperature (Jutfelt et al., 2019; Morgan et al., 2018; Sandblom et al., 2016; Figure 5). For example, in ~122 g juvenile Atlantic cod (*Gadus morhua*) warmed at 0.3°C min^{-1}, the difference between the water temperature and that of the deep muscle tissue was around 1.5°C (Jutfelt et al., 2019). Here, CT_{max} may be overestimated due to the discrepancy between the water temperature and internal temperature of the fish (Figure 5). In contrast, with a 2°C h^{-1} warming rate, the internal temperature of brook trout (*Salvelinus fontinalis*; 15.1–120.4 g) did not lag behind the surrounding water (O'Donnell et al., 2024). However, for an instantaneous 8°C temperature change, the time required for internal tissues to equilibrate with the environment increased with fish size (at a rate of 7.2 s per mm of fish length; O'Donnell et al., 2024).

As noted, thermal inertia likely differs among tissues in most ectotherms. For example, the brain is highly vascularised and generally close to the body surface, allowing it to become equilibrated to external temperatures considerably faster than deep dorsal muscle (Jutfelt et al., 2019). Furthermore, thermal inertia may be influenced by the body shape (e.g. spherical vs. flat) of the animal. Hence, we discourage attempts at *post hoc* correction of CT_{max} data based on heat transfer time, given that species-specific mechanisms (and relevant tissues) involved in CT_{max} are complex and not fully understood (Ern et al., 2023). The lag between the internal and external ramping rates can be quantified with a pilot experiment (preferably on anaesthetised animals) in which thermocouples are implanted into the deep muscle tissue (Mora & Maya, 2006; Sandblom et al., 2016;). Pilot experiments should be run if possible before any new CT_{max} study, especially when measuring CT_{max} in a species for which an appropriate ramping rate has not already been identified (and even if previous studies are published, pilot experiments may be useful).

Lastly, it is important to strive for consistent and homogenous warming rates within and across studies, as CT_{max} can vary as a result of inconsistencies in warming rate (e.g. Åsheim et al., 2020; Becker & Genoway, 1979; Mora & Maya, 2006; Moyano et al., 2017). Although

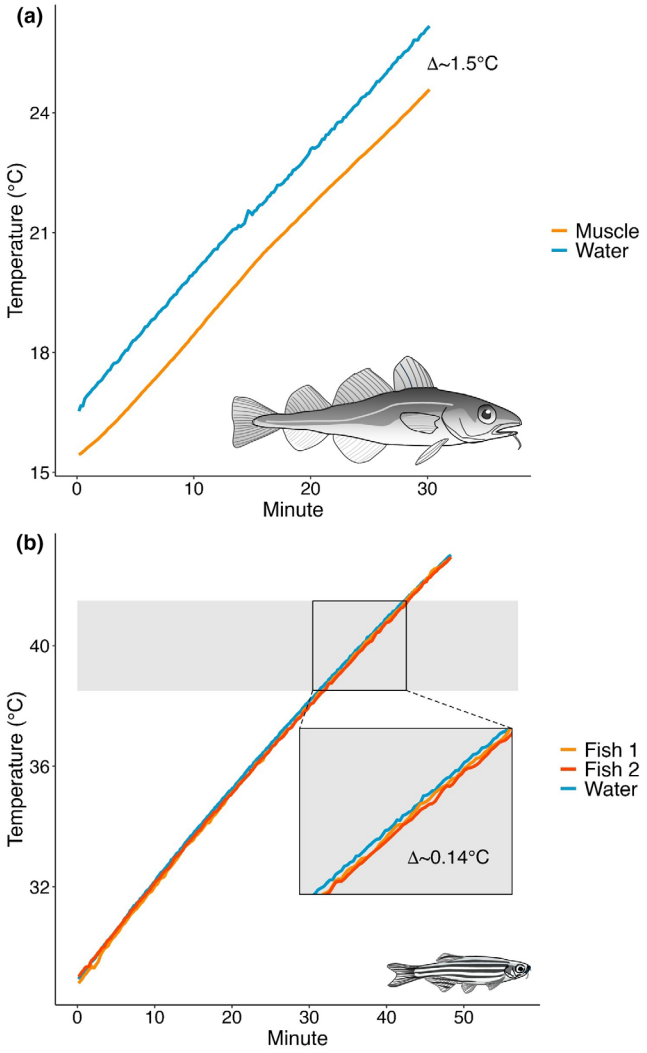

FIGURE 5 During 0.3°C min^{-1} warming, internal body temperature lags behind environmental temperature, with the magnitude of the lag dependent on body size. (a) Body temperature (°C) versus water temperature (°C) during a CT_{max} trial in the deep dorsal muscle of a 25.1 cm and 122.3 g Atlantic cod (*Gadus morhua*). The difference between the cod dorsal muscle and the water was consistently around 1.5°C once the 0.3°C min^{-1} warming was underway (Jutfelt et al., 2019). (b) Similar data (from Morgan et al., 2018) for two zebrafish (Fish 1: 2.8 cm and 0.4 g; Fish 2: 2.6 cm and 0.4 g). The grey shaded area indicates the range of expected CT_{max} for zebrafish (*Danio rerio*). Zebrafish internal temperatures were, on average, 0.14°C lower than the water temperature during the range of temperatures when CT_{max} was most likely to occur (inset). In each case, the animal was anaesthetised throughout.

it has been suggested that discrepancies in warming rates should be standardised by modelling 'thermal death time' or cumulative exposure to thermal stress (Jørgensen et al., 2021; Ørsted et al., 2022), the potential for animals to partially acclimate during slower warming rates may make the comparison of thermal death time estimates inconsistent and unreliable, plus there are ethical issues with using death as an end point. Until this method has been more thoroughly validated, we recommend adhering to a consistent 0.3°C min^{-1} heating rate or, for larger animals, the fastest warming rate that results in

minimal thermal lag of the tissues of interest and provide a rationale for the warming rate used.

4 | HOW TO ACHIEVE THE DESIRED WARMING RATE

There are a few strategies that can be used to achieve a constant warming rate within the animal arena. First, the power needed to heat the chosen water volume in the CT_{max} apparatus can be estimated as follows:

$$P = mc\Delta T, \quad (1)$$

where P is the power of the heater (in W), c is the specific heat capacity of water (approximately 4186 J kg^{-1} $^{o}C^{-1}$), m is the mass of water (in kg), and ΔT is the desired rate of heating (in °C s^{-1}). This equation does not account for heat exchange with the surrounding environment; pilot trials and adjustments are often needed. However, once the heating power and water volume are determined via pilot experiments, achieving the desired warming rate consistently for each trial is as simple as filling the chamber to the correct volume and turning on the heater(s). A drawback of this approach is that the warming rate may be somewhat different at high or low absolute temperatures, depending on the surrounding air temperature (Figure 6, and see Section 11).

A more labour-intensive approach is to manually monitor and adjust the water temperature throughout the CT_{max} trial (e.g. Åsheim et al., 2020; Stewart et al., 2023, 2024). This might include adding or removing heaters in a stepwise manner and/or adjusting the water volume. An automated proportional–integral–derivative controller can be used to precisely control the rate of warming throughout the trial (e.g. Ern & Jutfelt, 2024; McDonnell & Chapman, 2015), including automating increases to heating power at higher temperatures to maintain warming rates (this requires equipping the system with overcapacity heating relative to calculations from Equation 1). Regardless of the warming rate, researchers should log and report the raw temperature data from inside the CT_{max} arena (Figure 6; Section 13).

5 | THERMAL HOMOGENEITY

Thermal homogeneity across the animal arena (e.g. <0.1°C in variation) is a critical assumption of CT_{max} because the animals will not consistently be in the direct vicinity of temperature probe(s). Commonly used approaches to heating (e.g. titanium heating rods) can lead to thermal heterogeneity with insufficient mixing of

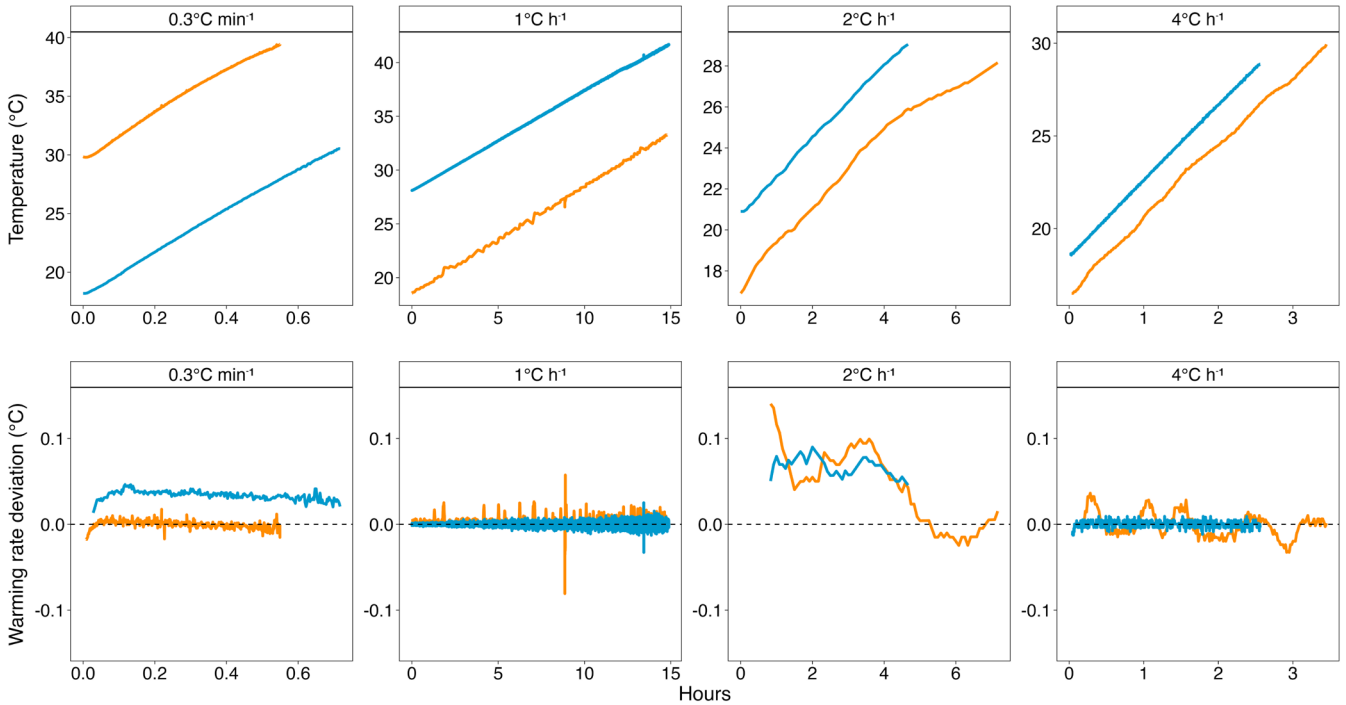

FIGURE 6 Warming rate data from CT_{max} trials at four warming rates: 0.3°C min^{-1} (18°C h^{-1}), 1°C h^{-1}, 2°C h^{-1}, and 4°C h^{-1} (4°C h^{-1} data from Stewart et al. (2024), all other data are unpublished data of the authors) that varied in how well they adhered to the desired warming rates. The top row depicts actual warming rates, and the bottom row the deviation from the desired warming rate in degrees Celsius per unit of time (unit of time matching the panel label, i.e. per minute for the left panel, per hour for the others). Each panel shows the temperature change during two different trials, distinguished by colour. Blue lines depict examples of warming rates that were more consistent throughout the duration of the trial. Orange lines depict examples of less optimal or more variable warming rates, with the 2°C h^{-1} example being the only shown here that is truly problematic. Black dashed lines indicate a warming rate deviation of 0°C (i.e. the ideal warming rate). Warming rate deviation data are smoothed using a rolling mean over 10 recording intervals. Recording intervals varied by trial: From left to right, temperature was recorded at 3 s, 1 s, 5 min and 15 s intervals. See Tables S1 and S2 for warming rates (slopes and R^2 values).

the water. In turn, some areas of the arena may become warmer than others, which could cause over- or underestimation of CT_{max} among individuals based on their positioning. Aquatic ectotherms behaviourally thermoregulate and thus are likely to seek out cooler areas of an arena once the water temperature becomes supra-optimal.

Thermal homogeneity within the testing arena can be maintained with a submersed water pump. Vigorous aeration also helps promote thermal mixing. However, the rate of water movement within the arena should not be so high that the animals have to swim actively against a current, which could cause exhaustion prior to CT_{max} (e.g. Blasco et al., 2020). Thus, the flow rate of the pump should be minimised to the lowest level needed to ensure thermal homogeneity (see below). Baffles can be used to disperse the flow, and observations of animal behaviour can be used to determine if water velocities are too high. In arenas with high rates of water mixing, placing fish in chambers with a lower flow, continuously running flush pump can protect them from excessive water movement and ensure uniform flow rates for all fish (Figure 3e). Assessment of thermal homogeneity can be made easier by using multi-probe temperature logging systems (e.g. PicoTech TC-08, Pico Technology, Cambridgeshire, UK). Confirmation of thermal homogeneity should occur with every new CT_{max} arena setup.

6 | DETERMINING A REPEATABLE END POINT

Though some approaches use lethal end points (e.g. IULT or LT_{50}), the prevailing definition of upper or lower thermal tolerance is an 'ecological death' of the organism due to loss of motor functions and, consequently, an inability to escape the harmful (lethal) conditions or avoid predators (Lutterschmidt & Hutchison, 1997; Vernon, 1899). When selecting an end point, it is important to remember that CT_{max}, unlike LT_{50}, is a non-lethal assay of an organism's thermal tolerance; the animal should recover and survive upon being returned to cooler water. Rates of survival after CT_{max} should therefore be reported. Post-CT_{max} mortality can occur minutes to days following a trial (De Bonville et al., 2024).

Loss of motor function is typically assessed through behavioural end points: LOE (Beitinger et al., 2000) is the most common (at least in fishes), but loss of swimming (Geerts et al., 2015) and loss of response to touch (Andreassen et al., 2022) are also used. Cessation of movement of embryos (Cowan et al., 2023) and loss of responsiveness of larval fish (Andreassen et al., 2022) are comparable alternatives to LOE. Assessing embryo CT_{max} requires that embryos are mature enough to have spontaneous activity and exhibit movement in response to a thermal stimulus. While differences in methodological approaches can make it difficult to compare CT_{max} across studies, behavioural and morphological differences among species or life stages can demand different end points. For example, during warming, flatfish and many invertebrates often lay still on the bottom, gastropods such as abalone attach themselves to the arena walls, and pipefish tend to coil themselves around each other or the temperature sensor (personal observations by the authors). Due to variation among species and life stages in end points, it is important to: (1) run pilot trials to map out species-specific behavioural responses to the warming; (2) accurately determine and define the end point (i.e. the behavioural response and duration threshold to identify CT_{max}); (3) ensure the end point definition is clearly communicated and agreed upon among all researchers performing experiments (or, ideally, only use one observer for all replicate trials); and (4) report details on the behaviour observed at the agitation temperatures and the end point (Section 13). Video recording can also help to determine consistent end points.

The LOE or loss of responsiveness end points are often preceded by hyperactivity (i.e. agitation), then progressive loss of movement and/or increasing lethargy that eventually leads to loss of equilibrium/responsiveness (e.g. Friedlander et al., 1976; Kochhann et al., 2021; Lutterschmidt & Hutchison, 1997). Some animals also appear to lose equilibrium then quickly regain motor functions when startled (Åsheim et al., 2024). Thus, there may be a trade-off between the accuracy of the CT_{max} measurement and the risk of recording a premature LOE when determining the duration of the behavioural end point for CT_{max}. Whether or not the behaviour of individuals close to the end point differs among experimental treatment groups should be assessed during pilot tests. Researchers have also utilised different threshold times at LOE (e.g. 1 vs. 3 vs. 10s of LOE) when identifying an animal's end point; therefore, these details should be reported.

The sample size within a CT_{max} trial should be small enough to allow the observer to carefully monitor the behaviour of all the animals. At a 0.3°C min^{-1} warming rate, it is common for all animals in a trial to reach CT_{max} within as little as 3 minutes (i.e. a range of 0.9°C in CT_{max} values). In such a scenario, observing too many animals at once makes it difficult to precisely assess end points for each individual, in turn making CT_{max} times and temperatures less accurate. When using 0.3°C min^{-1}, we recommend limiting sample sizes to approximately 8–10 animals per trial. If the behavioural end point requires the observer to flip over or test responsiveness in animals, a lower sample size per trial is likely necessary (e.g. 4–6 per trial). With slower warming rates, animals typically reach CT_{max} over a longer period, effectively allowing for a higher within-trial sample size while maintaining the same level of careful observation. As a result, slower warming rates may mean that 15–20 animals can be accurately tested per trial. As many as 30 animals were feasible at a warming rate of 4°C h^{-1} (Stewart et al., 2024) because the slower rate meant that fish reached their CT_{max} end points across a span of ~20 min.

CT_{max} protocols can be established for slow-moving or sessile organisms. For example, a study on abalone (*Haliotis rubra*×*H. laevigata*) used a customised tank to ensure animals remained attached to vertical walls (Holland et al., 2024). The CT_{max} end point was taken as the temperature when animals lost pedal adherence (Holland et al., 2024). The end points of less mobile invertebrates that attach to the edges of tanks can be observed as the animal suddenly falling off a vertical surface to which it was attached. Mobile

macroinvertebrates such as decapods often swim, allowing similar CT_{max} end points as fish. In rusty crayfish (*Faxonius rusticus*), CT_{max} was typically preceded by the animal bursting up off the bottom of the arena then drifting back to the bottom with negative equilibrium (Chasse et al., 2025). For some species, such as flatfish or some crab species, it may be necessary to intermittently turn animals' upside down to assess their righting response. However, doing so too frequently could lead to exhaustion and earlier CT_{max} and should only be done at predetermined intervals for consistency.

7 | MINIMISING OBSERVER BIAS AND MAXIMISING SCORING CONSISTENCY

Predetermined expectations about the outcome of an experiment can lead to conscious or unconscious observer bias (Tuyttens et al., 2014). Evidence that observer bias affects results and interpretation has been documented broadly across the life sciences and is likely a problem in much of experimental biology (e.g. Holman et al., 2015; Tuyttens et al., 2014). Masking (also known as blinding) the observer from the treatments is commonly used as a technique for reducing observer bias (Holman et al., 2015). Without masking, false positive findings are more likely, and treatment effects tend to be overestimated (Holman et al., 2015). To apply masking to CT_{max} trials, the observing researcher scoring the animals should ideally be unaware of the temperature in the animal arena by ensuring the display temperature (Figure 2) is not visible to them. This can be achieved by having two researchers performing the experiments: one observing the animals, and one recording data. With this setup, the animal observer monitors the arena for behavioural end points and calls out when an animal is removed and placed into a numbered, individual recovery tank. Ideally, the animal observer is also masked to any treatments of the animals being tested.

If having two researchers running the CT_{max} trials is a limitation, an alternative is to film the trials and assess the temperature and time at the end point from masked videos. There may be other concerns or issues that prevent researchers from incorporating masking (Karp et al., 2022). Information revealing the temperature or treatment may be visible (both visually and auditorily, e.g. from seeing heaters or other equipment turning on and off). This can be counteracted by ensuring transparent recording of data, for example by video recording the setup and trials (Clark et al., 2017).

8 | REPLICATION AND INTER-TRIAL VARIABILITY

CT_{max} trials are usually conducted on groups of animals (e.g. 10 animals at a time), giving rise to the possibility that animals within a trial are not independent. Trial effects, where replicate CT_{max} trials on animals from the same treatment show markedly different mean temperatures or different variance, do occur. However, testing animals individually requires many parallel arenas or more time to achieve similar sample sizes. We recommend assessing the number of replicate trials that are necessary to reliably detect a treatment effect, given that most studies will likely continue to test groups of animals together for efficiency. Studies that use only one CT_{max} trial per treatment may report erroneous treatment effects (type I errors) simply due to variation among trials (trial effects, analogous to tank effects). To illustrate this point, we re-examined data from previous CT_{max} experiments to answer the following question: how many replicate trials are needed to reliably detect a difference (or lack thereof) in CT_{max} between two groups of animals?

We modelled the likelihood of detecting differences in the mean CT_{max} among replicate trials using Bayesian linear regression models (see Supporting Information for detailed Methods and Results). In a dataset consisting of 12 experiments (12 species), each consisting of four replicate CT_{max} trials ($n = 5$–10 individuals per trial; overall $N = 417$; subset of data from Raby et al., 2025), in seven of the 12 cases, there was no evidence of a trial effect. That is, trials 2–4 did not differ in their mean CT_{max} values from the first trial. For these cases, conducting only one or two CT_{max} trials per treatment would have reached the same conclusions as experiments testing four trials per treatment (low risk of type I error). In three of the 12 case studies (25%), the trial mean CT_{max} in at least one of trials 2–4 differed from the initial trial mean (i.e. there was a 95% likelihood that a subsequent trial mean would not equal the initial trial's mean). In two cases (bluntnose minnow and lesser pipefish), two or more subsequent trials differed from trial 1 (mean replicate differences = 0.21°C; Figure 7). These scenarios illustrate that type I errors (false positives) can occur in CT_{max} experiments involving only one trial per treatment.

In another dataset consisting of 12 replicate CT_{max} trials from eight treatment groups in zebrafish (*Danio rerio*; $n = 9$–18 individuals per trial; overall $N = 1192$; Morgan et al., 2020), there were trial effects in seven of eight cases. In these cases, an average of 3 of the 11 subsequent trials differed from the first trial (range: 1–6). Again, these results illustrate that even with well-controlled experiments on the same animals (no treatment effects), statistically significant 'unexplained' trial differences can occur. Therefore, researchers should avoid a design in which a single CT_{max} trial per treatment group is used. Determining the total sample size needed per treatment and how animals are divided among trials can be based on power analyses where effect sizes and variance can be estimated from pilot trials and data from previous research. In general, we recommend at least three replicate trials per treatment where possible because it allows for an assessment of inter-trial variability. If an initial three trials show meaningful inter-trial variability then more trials would likely be warranted.

9 | BIOTIC CONFOUNDS TO CONSIDER

Of the many considerations for designing CT_{max} experiments, confounding effects of biotic factors (covariates) are poorly understood in most cases. However, we highlight some known

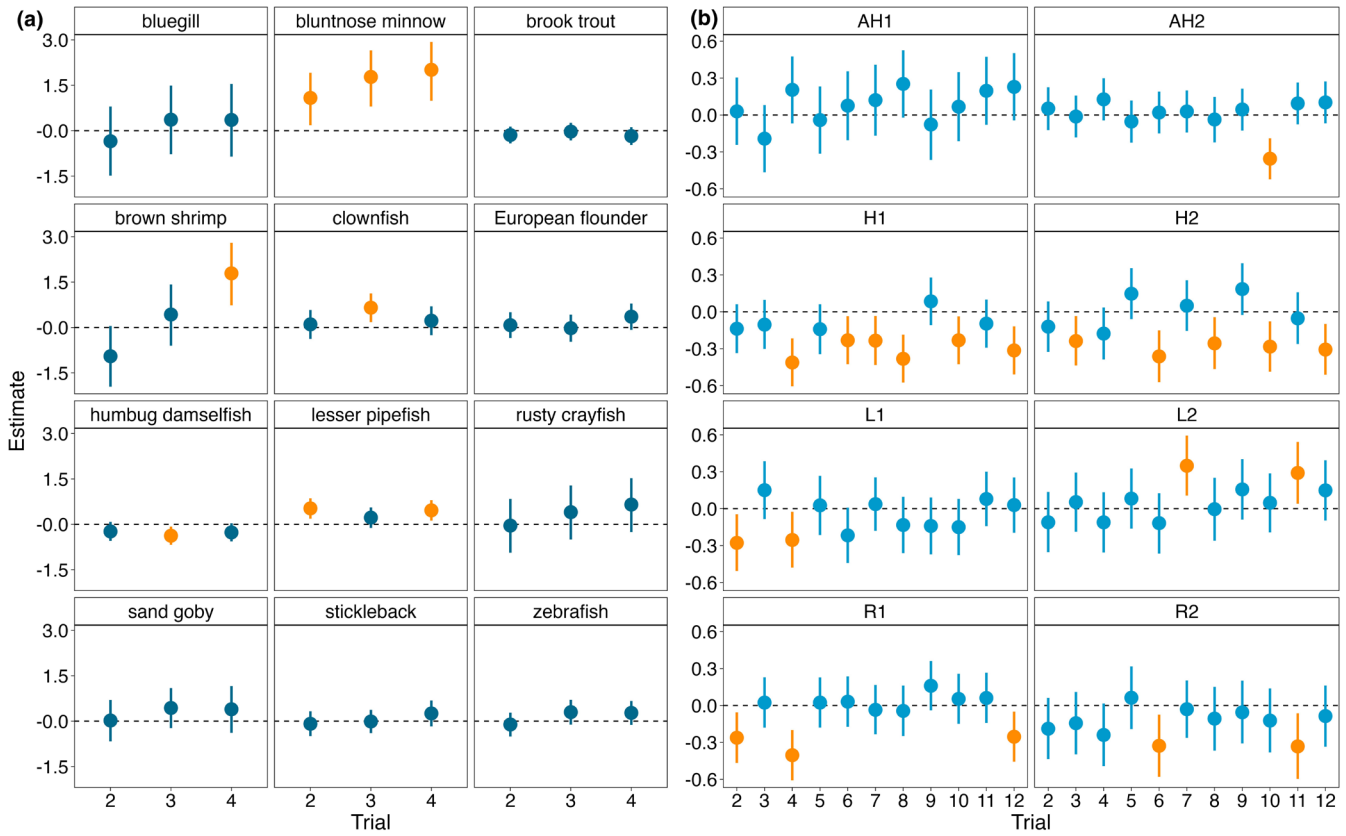

FIGURE 7 Modelled posterior predicted mean (estimate; points) and posterior distributions (95% credible intervals; lines) of replicate trial CT_{max} in reference to the initial trial in (a) twelve species of aquatic ectotherms tested under controlled conditions (Raby et al., 2025: Bluegill *Lepomis macrochirus*, bluntnose minnow *Pimephalus notatus*, brook trout *Salvelinus fontinalis*, brown shrimp *Crangon crangon*, clownfish *Amphiprion chrysopterus*, European flounder *Platichthys flesus*, humbug damselfish *Dascyllus aruanus*, lesser pipefish *Syngnathus rostellatus*, rusty crayfish *Faxonius rusticus*, sand goby *Pomatoschistus rostellatus*, three-spined stickleback *Gasterosteus aculeatus*, zebrafish *Danio rerio*), and **(b)** zebrafish tested under eight treatment conditions (Morgan et al., 2020; AH1 & AH2 = selected for increase warming tolerance after acclimation to 34°C; H1 & H2 = selected for increased warming tolerance after acclimation to 28°C; L1 & L2 = selected for decreased warming tolerance after acclimation to 28°C; R1 & R2 = control lines at 28°C, where individuals were randomly selected). Where posterior distributions do not cross 0, strong evidence of a given subsequent trial (replicate) effect exists (orange points), indicating a difference in mean from the initial trial. See Supplementary Information for methods and all model parameters.

aspects that researchers should consider. Infection with parasites and pathogens has been associated with reduced thermal tolerance in juvenile brown trout (*Salmo trutta*; Bruneaux et al., 2017), pumpkinseed (De Bonville et al., 2024), bluegill (*L. macrochirus*) and longear sunfish (*L. megalotis*; Lutterschmidt et al., 2007). If parasites are not a covariate of interest, researchers could consider treating wild animals for parasites upon arrival to the laboratory, quantifying parasite load following tests and/or excluding noticeably infected individuals (Chrétien et al., 2023). However, removing infected hosts from a study could lead to an overestimation of the CT_{max} of the natural population.

Food quality, ration size and the time elapsed since the last meal could all affect CT_{max} (Gomez Isaza et al., 2019; Verhille et al., 2016). Juvenile barramundi (*Lates calcarifer*) fed a 20% fat diet for four weeks had lower CT_{max} than those fed a diet containing 10% fat (both groups were fasted for 40–48 h prior to CT_{max}; Gomez Isaza et al., 2019). Through one anecdotal observation, we noted that juvenile European flounder (*Platichthys flesus*) that were mistakenly fed 6 h prior to a CT_{max} trial exhibited a mean CT_{max} that was 1.5°C lower than in fish fasted for 24 h (unpublished data; $p < 0.001$, partial $R^2 = 0.60$, controlling for an effect of mass). Similarly, juvenile white sturgeon (*Acipenser transmontanus*) fed larger rations had lower CT_{max} (Verhille et al., 2016). We recommend fasting laboratory animals for an appropriate and consistent duration (species and temperature dependent, typically 24 h minimum) before quantifying CT_{max}. However, we caution readers that further research is needed to assess how consistent and large an effect digestion has on CT_{max}. Independent of digestion, photoperiod and time of day could also affect CT_{max} based on evidence in common killifish (Healy & Schulte, 2012); we recommend researchers strive to constrain their CT_{max} trials to a consistent period of the day across their experiments.

Differences in the upper and lower thermal tolerance can occur between different size classes of animals (McKenzie et al., 2021). For example, Di Santo and Lobel (2017) measured CT_{max} and CT_{min} of two goby congeners (*Elacatinus lobeli* and *E. oceanops*) and found

larger fish had narrower thermal tolerance (lower CT_{max} and higher CT_{min}). Size effects can vary among life stages. In juvenile zebrafish, larger individuals had a lower CT_{min}, but there was no effect of mass on CT_{min} in adults (De Bonville et al., 2025). Overall, there are numerous examples in the literature in which body size did or did not affect CT_{max} (e.g. Clark et al., 2017; Messmer et al., 2017; Morgan et al., 2018; Recsetar et al., 2012). Thus, we recommend carefully controlling for body size (statistically or experimentally; also see Section 3; Figure 5).

Social dynamics within trial groups may alter the physiology and thermal tolerance of animals (Currie & Tattersall, 2018; Gilmour et al., 2005). In some fishes, small group sizes promote aggression and social stress. For example, subordinate salmonids, which have chronically elevated plasma cortisol, also have elevated plasma cortisol and reduced CT_{max} compared to dominants when tested in groups (Bard et al., 2021; LeBlanc et al., 2011). In mangrove rivulus (*Kryptolebias marmoratus*), social aggression was stimulated via a mirror test and resulted in lower thermal tolerance (Currie & Tattersall, 2018). Social effects on CT_{max} are likely to be species-specific. In gregarious lake sturgeon, isolation or conspecific grouping did not alter CT_{max} (Yusishen et al., 2020). Therefore, the behaviour (including sociality) and ecology of the species should be considered when choosing the appropriate testing method. Pilot experiments can be run to assess the effects of isolation vs. groups on CT_{max} results.

Habituation time to the experimental arena can help reduce stress following animal capture, transport, or transfer from holding tanks. Indeed, Bard et al. (2021) found a link between elevated cortisol and reduced CT_{max} in subordinate rainbow trout (*Oncorhynchus mykiss*). Habituation times reported in the literature range from hours (e.g. overnight) to minutes, with the optimal habituation time likely to be species dependent. Animals should be placed in the animal arena for a standardised duration before thermal ramping begins (and this duration reported in the methods). Similarly, the starting temperature of the trial could impact CT_{max} values (Cicchino et al., 2024). In most cases, the starting temperature should be the same as the holding temperature of the organisms. In studies with different thermal acclimation groups, this either means that different groups must start their CT_{max} trials at different temperatures, or they could start at a common temperature with the understanding that some groups will undergo an abrupt temperature change (and potentially commence a new thermal acclimation trajectory) immediately prior to the CT_{max} trial (e.g. De Bonville et al., 2024, 2025; Stewart et al., 2023). As a result, the time spent in the trial and the accumulated thermal injury during the trial may differ between treatment groups.

Reproductive status could affect CT_{max} (Auer et al., 2021; Dahlke et al., 2020; Pörtner & Peck, 2010) but evidence for reduced CT_{max} in spawning fish is the subject of ongoing debate (Pottier et al., 2022). A reduction in CT_{max} did occur in guppies (*Poecilia reticulata*), a livebearing fish, at a later gestational stage compared to those at an earlier or non-gestational stage (Auer et al., 2021). Thus, it may be worth considering or controlling for variation in reproductive status during experimental design.

10 | ABIOTIC FACTORS TO CONSIDER

Oxygen limitation has been proposed as a mechanism involved in thermal tolerance, and there are examples in the literature of hypoxia and hyperoxia affecting CT_{max} (McArley et al., 2022; Reemeyer & Chapman, 2024; Verberk et al., 2016). If manipulation of dissolved oxygen is not of interest to the study, a CT_{max} arena should be equipped with sufficient aeration to ensure oxygen remains close to 100% air saturation throughout the trial. Dissolved oxygen should be monitored as % air saturation because the concentration in mg L^{-1} changes dramatically with warming. Towards the end of a CT_{max} trial, both temperature and stress-induced increases in metabolism can increase the rate of oxygen consumption by animals, potentially leading to declines in dissolved oxygen in the arena if the water volume is low and aeration is insufficient. Conversely, when warming water at 0.3°C min^{-1}, arena water can become supersaturated with oxygen (e.g. 110–120% air saturation). The solubility of oxygen in water decreases as temperature increases, but the rate at which oxygen diffuses out of water is too slow to maintain air saturation at or below 100% when the water is heated quickly. Aeration should minimise both issues, and researchers should use a reliable O_2 sensor to confirm that the O_2 level remains stable (e.g. between 95 and 105% air saturation) throughout trials in pilot experiments.

CO_2 could build up in arena water during CT_{max} experiments because of animal respiration. This phenomenon has rarely been measured, and thus, its potential impacts on CT_{max} estimates are not well understood. A few studies have tested the effect of high CO_2 levels (acute or chronic) on CT_{max} and typically report no impact (Clark et al., 2017; Frommel et al., 2020; Montgomery et al., 2024). For example, coral reef fish acclimated to an end-of-century CO_2 level of ~1000 µatm had a similar CT_{max} to fish acclimated to a current-day coral reef CO_2 level of ~500 µatm (Clark et al., 2017). CO_2 is >20 times more soluble in water than is O_2, so vigorous aeration is unlikely to prevent its build-up during CT_{max} trials (or the build-up of other waste products like ammonia with similarly high solubility). We encourage more research to understand whether metabolic waste products interact with CT_{max} (e.g. in relation to animal biomass: water volume).

Other abiotic considerations include the water salinity, where varying effects have been found on CT_{max} (see Åsheim et al., 2024 for a review of available salinity effects). Some natural habitats have large fluctuations in conductivity (e.g. estuarine and coastal systems), which should be considered. Dissolved organic matter or carbon might influence thermal tolerance through modifications of ion movements across the gills or other epithelial surfaces. Humic substances, for instance, make up a substantial portion of dissolved organic carbon and can bind to biological surfaces (Campbell et al., 1997), altering membrane permeability and transepithelial potential in fish and invertebrate species (Glover & Wood, 2005), which may confound or alter CT_{max}. Similarly, sedimentary turbidity could impact CT_{max} by reducing the efficiency of gill oxygen uptake (Fortin-Hamel & Chapman, 2024; Francispillai et al., 2024). Finally, pH could conceivably affect thermal tolerance

and may be a relevant parameter to measure, particularly when conducting in situ measurements of CT_{max} across diverse field sites that are expected to vary meaningfully in pH (Zimmer et al., 2024).

Toxicants have the potential to act as invisible confounds via contaminated field or facility water, or from the animal arena. Toxicants that could potentially modify CT_{max} include inorganic and organic contaminants such as pharmaceuticals, metals, micro- and nanoplastics, pesticides, nanopesticides, legacy and emerging contaminants, oil and oil co-contaminants, and potential mixtures of all of the above (Chang et al., 2023; Khursigara et al., 2019; Lydy & Wissing, 1988; Patra et al., 2007).

11 | FIELD VS. LABORATORY CONSIDERATIONS

The principles involved in designing and setting up a CT_{max} experiment in the field (e.g. streamside; Firth et al., 2021; Leclair et al., 2020; Stewart et al., 2024) are no different from doing so in the laboratory in terms of the arena design, warming rates, or end points. In the field, a petroleum-powered generator may be used as the power source to run equipment (Figure S1). Depending on the species and arena water volume, researchers could even conceivably use more powerful propane water heaters (Clark et al., 2012) to provide a supply of warmed water to a larger CT_{max} arena (instead of electric heaters). Warming rates in the field can be affected by variation in air temperature (weather) and site conditions (shade, ground surface). Most of our recommendations above about achieving good warming rates apply here (see Section 4).

The advantages to the in situ (field-based) CT_{max} approach are threefold: (1) it allows for assessment of different genetically or phenotypically distinct wild populations of animals without the need to transport animals to the lab, (2) animals can be released after the experiment, which can be important for obtaining scientific collection permits for some species, and (3) it may provide 'realism' in terms of animals being in a more physiologically 'wild' state and by exposing animals to the natural, ambient water chemistry to which they are acclimated (see Section 10). In pumpkinseed (*L. gibbosus*), hypoxia tolerance changes when wild fish are acclimatised to captivity (Borowiec et al., 2016). Unfortunately, there are few studies explicitly testing how CT_{max} changes with captivity (but see Kraskura et al., 2024; Morgan et al., 2019).

In the field, a key difference from the laboratory (arguably a disadvantage) is that the animals are (typically) caught directly from the wild and then exposed to a CT_{max} test minutes or hours later (Morgan et al., 2019). As a result, the animals are likely to be in more variable physiological states than animals acclimatised to captivity. Wild-caught animals also have unknown individual thermal and ecological histories and may be in varying states of digestion, differ in parasite burden (see Section 9) or health status. This added variation may necessitate larger sample sizes to accurately estimate CT_{max}. Similarly, field sites may vary in their water chemistry or toxicants. For example, Mottola et al. (2022) simulated a storm, resulting in relevant increases to environmental copper within a field collection site, which in turn increased thermal tolerance in male three-spined stickleback by 1.5°C (*Gasterosteus aculeatus*). Researchers assessing thermal tolerance across a wide range of locations may therefore want to consider partnering with environmental chemists to quantify water chemistry and consider surrounding land use (e.g. agricultural pesticides).

CT_{max} data should always be reported relative to the acclimation temperature of the animals (see Section 13) given that acclimation can have strong short- and long-term (i.e. carry-over) effects on CT_{max} (reviewed by McKenzie et al., 2021). Acclimation temperatures are more difficult to quantify in the field than in the laboratory. We recommend deploying temperature loggers at each field site at least 5 days prior to CT_{max} (ideally 1–2 months earlier; Reemeyer et al., 2024; Stewart et al., 2024). The logged temperatures can then be used to estimate mean acclimation temperatures for a range of time windows preceding the date of CT_{max} (e.g. 5–30 days) to determine the acclimation period that is most predictive (Stewart et al., 2024). If animals are caught from a thermally heterogeneous environment (e.g. a thermally stratified lake), a single temperature logger deployed may not represent the diversity of acclimation temperatures among the animals tested. Deployment of multiple loggers could help understand the thermal habitats available, capturing the range of possible acclimation temperatures.

12 | MEASURING CT_{min}

Critical thermal minimum (CT_{min}) is less commonly measured than is CT_{max} but is conceptually and methodologically similar. However, there are methodological considerations specific to CT_{min}. The assessment of the LOE end point (Section 6) at CT_{min} is complicated by the cold-induced reductions in activity common among aquatic ectotherms, especially lethargic or winter-dormant species (Reeve et al., 2022). Some species do lose equilibrium at temperatures approaching CT_{min}. In others, a response to touch stimuli at low temperatures when animals become minimally active can be used as an end point, especially for animals with CT_{min} values close to 0°C (Ford & Beitinger, 2005). Polar species may not have a CT_{min}. Instead, they function down to the temperature at which ice forms inside their bodies. Antarctic fishes maintain high activity and aerobic scope with a body temperature equal to that of the freezing point of seawater (−1.86°C; Brijs et al., 2020; DeVries & Cheng, 2005).

Cooling water is more difficult than warming it, and chillers are more costly than heaters (the cost per watt for cooling is up to 10× that of heating). The cooling power of the chiller must account for the volume of the test arena and the temperature difference between the arena and environment. These are the same considerations as with heating, but more challenging in practice. Extra insulation of the test arena may be needed, and setting up the CT_{min} arena in a temperature-controlled room with cold air can

help. Water chillers available in the consumer aquatics industry are not capable of cooling water below 2–3°C, which is typically not an issue for warm-water species with CT_{min} above this range. However, many temperate animals are likely to have a CT_{min} approaching 0°C, especially when acclimated to moderate or cool temperatures. For example, in pumpkinseed acclimated to 10°C, CT_{min} was 0.6°C (De Bonville et al., 2024). In killifish (*Fundulus heteroclitus*) acclimated to <15°C, fish exposed to a CT_{min} assay survived until the (brackish) water froze at −1.1°C (Fangue et al., 2006).

For species with very low CT_{min}, even more complex recirculating water bath chillers are needed (potentially paired with conventional chillers to more easily reach 2–3°C). A glycol–water bath can be ultra-cooled (e.g. −20°C) to achieve temperatures reaching the freezing point of fresh or seawater (though freezing of live vertebrates should be avoided). If animals are small, they may be cooled directly in small open-topped arenas submerged in a glycol–water bath (the arenas must be closed because ethylene glycol is toxic to animals; Hymel et al., 2002). However, this limits throughput given the relatively small working area of recirculating water baths. These setups allow good control over thermal ramping by simply changing the setpoint of the water bath. However, there is a risk of contamination of the CT_{min} arena water with the toxic glycol-water mixture, and they are prone to freezing the water without sufficient stirring.

To avoid contamination, water from the test arena can be circulated through a heat exchange coil within an external glycol bath (Fangue et al., 2006). The flow rate should be high enough to avoid water freezing within the heat exchange coil, and slow enough to ensure sufficient heat exchange (i.e. water returning to the test arena should be as close to freezing as possible). Note that switching on and off the recirculating pump is problematic because water inside the coil will freeze without flow; control over cooling rate can instead be achieved by using a pump with adjustable flow. Decreasing the setpoint on the recirculating water bath chiller is also possible, but it may not cool quickly enough to match the required thermal ramp. Pre-frozen ice blocks (or crushed ice made using non-chlorinated water) can be added to the CT_{min} equipment section to keep cooling rates more stable as temperatures approach freezing. For saltwater species, freshwater ice would have to be kept physically separated (e.g. in sealed bags) from the arena water to avoid changing the arena's salinity.

13 | REPORTING

To improve reproducibility and transparency, and to facilitate evidence synthesis, researchers should strive for detailed and consistent reporting of their methods (Percie du Sert et al., 2020). Inspired by Killen et al. (2021), who provided a detailed guide on reporting methods for aquatic respirometry, here we provide a checklist for reporting on CT_{max} experiments, along with a downloadable (fillable) version that authors can use (Table 1; see Supporting Information S1).

14 | SUMMARY AND KNOWLEDGE GAPS

Here we have provided methodological advice for designing, carrying out, and reporting on CT_{max} (and CT_{min}) experiments. A common theme is that researchers should always conduct careful pilot experiments after thinking through recommendations in Sections 2–13 (above). Pilot experiments are especially important whenever using a new CT_{max} arena, new species, new life stage, or new warming rate. Limited access to animals (e.g. for rare or threatened species) could preclude pilot experiments in some cases, but pilot experiments without animals are nevertheless useful (e.g. to ensure consistent warming rates, thermal homogeneity). Our hope is that the guidance will be particularly useful for researchers developing a new interest in CT_{max}.

While there is ample literature on thermal biology to provide evidence-based recommendations for CT_{max} experiments, knowledge gaps remain that we hope to see addressed. These include, but are not limited to:

a. The tissue-specific physiological mechanism(s) that cause loss of motor function at extreme temperatures at fast (e.g. 0.3°C min^{-1}) and slow (e.g. 1°C h^{-1}) rates of warming.
b. The confounding roles of biotic factors including stress caused by capture and handling, confinement, or agonistic social interactions, as well as the impacts of digestion and reproduction—these factors could have implications for interpreting CT_{max} data in an ecological context.
c. The effect of body mass on the temperature lag of different tissues (i.e. heat transfer time). A robust dataset collected across a range of body sizes and species is lacking. Such data could be used by researchers to make decisions on what warming rates are appropriate for the range of body sizes in their study.
d. The effects of varying warming rates on CT_{max} (and CT_{min}). Thermal biologists would benefit from more empirical data (across species) from experiments focused on this question. This could include interactions between warming rate, body mass, and acclimation temperature.
e. Acclimation dynamics. Surprisingly few studies have focused on the dynamics of thermal acclimation such as quantifying the time needed for CT_{max} to exhibit 'full' acclimation to a new temperature or the duration after which CT_{max} starts changing (but see Bennett et al., 1998; De Bonville et al., 2025; Fangue et al., 2014; Fu et al., 2018; Stewart et al., 2023), and it seems likely that acclimation is quicker in some species than in others (Burton & Einum, 2025). For designing lab experiments and quantifying acclimation in field studies, more data about acclimation dynamics would be useful.
f. The physiological difference and relative ecological meaning of 'typical' CT_{max} and CT_{min} experiments when compared against alternative approaches in which animals are forced to exercise during thermal ramping, such as the critical thermal maximum for swimming (CTS_{max}; Blasco et al., 2020).
g. More controlled studies that assess the difference in CT_{max}

TABLE 1 Considerations when preparing for, conducting, and reporting on critical thermal limit experiments. In most cases, one or more of the 12 sections of the paper are relevant to each methodological detail; we have indicated under 'rationale' which sections are relevant (where applicable). A fillable version of this table has been included as a downloadable, Supporting Information S1. Table S5 provides an example of this table filled out with details from a case study.

	Description	Rationale
Pre-trial		
1	Information on feeding history of animals (fasted or not, duration of fasting)	Whether or not animals were fasted prior to testing should be explicit. For field CT_{max} where the satiation states of animals are unknown and likely to vary, that caveat should be noted. Most laboratory studies fast animals for *ca.* 24 h prior to CT_{max}. [see Section 9]
2	Habituation time to the laboratory (or time since capture for field studies)	A change of environment could cause endocrine responses with unknown consequences for CT_{max}. Behavioural changes with lab habituation (e.g. establishment of social hierarchies) could conceivably affect CT_{max}. Report whether animals were raised in captivity or fully domesticated. [see Sections 9 and 11]
3	Thermal acclimation (temperature and duration)	Thermal acclimation has strong effects on CT_{max} (Chrétien & Chapman, 2016; Fangue et al., 2014), so authors should clearly report the temperature (and duration) of acclimation, including whether temperatures were stable or fluctuating. Additional information about long-term prior thermal exposures is also valuable. If known, provide basic context about the thermal biology of the species or population (e.g. optimal temperature). [see Section 11]
4	CT_{max} arena dimensions and water volume	Arena size and water volume can influence ramping rate, accumulation of CO_2 and nitrogenous waste, and subject behaviour. [see Sections 2–5, 9 and 10]
5	Body mass of animals at time of CT_{max}	CT_{max} is correlated with body mass in some animals (Barlett et al., 2022; Ospina & Mora, 2004). Studies should measure body mass of each animal immediately before or after CT_{max} (mass values linked to each CT_{max} value) and not simply report a mean mass for the whole sample. [see Section 9]
6	Total animal biomass: water volume ratio	The ratio of animal biomass to water volume will influence the accumulation of CO_2 and nitrogenous waste in the arena. Low animal biomass: volume ratios are especially prone to increases in DO at fast ramping rates. [see Sections 2 and 10]
7	Details on source water	Studies should indicate whether the water used was filtered or treated in any way, as well as the source (i.e. natural lake, river or seawater, or dechlorinated municipal tap water). For field-based CT_{max}, indicate whether the water was collected directly from the sampling site. [see Sections 10 and 11]
8	Type of temperature probe and details on data logging	Different probe types have different levels of precision and accuracy (provide these values). Indicate the equipment used for scoring CT_{max} and the equipment used for logging arena temperatures (if these were separate devices). [see Sections 2 and 4]
9	Type, number, and wattage of heaters	Different heater types have different efficacies based on their design. Indicate if and how water was circulated through/around the heating element(s). [see Sections 2 and 4]
10	Life stage and sex (if known) of the test animals	CT_{max} may differ across life stages and/or between the sexes (Cowan et al., 2023; Wheeler et al., 2022). [see Section 9]
11	Information on treatments/health metrics of wild animals	Parasites and pathogens can influence thermal tolerance and should be considered (Chrétien et al., 2023; De Bonville et al., 2024). Studies should mention whether animals are treated, if infected/sick individuals are excluded or if parasite load was quantified. If health metrics are unknown, that should be stated. [see Section 9]
12	Time of the day that trials were run, and photoperiod	Diurnal cycles could potentially influence thermal tolerance (Healy & Schulte, 2012). [see Section 9]
13	Habituation time in the CT_{max} arena before trial start	Times can vary between studies and could affect stress and behaviour of the animals depending on density. [see Sections 9 and 11]
14	Temperature at which the trials were started	Variations in starting temperature can affect the accumulation of thermal injury over the course of a CT_{max} trial (Cicchino et al., 2024). [see Section 9]
15	Details of pilot experiments used to refine main trials	Indicate whether pilot trials performed on subsets of animals to refine thermal ramping profiles, whether intratissue thermocouples were used determine thermal inertia and appropriate thermal ramping rates. [see Sections 2–6]
During trial		
16	Warming rate with measures of variation (e.g. standard deviation or range)	Warming rates should be monitored and calculated throughout the trial. Best practice is to use temperature loggers in the test arena that can be compared with digital thermometer recordings during trial runs (e.g. De Bonville et al., 2024). The logger outputs can be plotted (Figure 6) and included (for each trial) as supplemental material (with R^2 values for warming rates). [see Sections 4 and 5]

(Continues)

TABLE 1 (Continued)

	Description	Rationale
17	Details on how consistent ramping rates were achieved	In some cases, heaters are simply switched on and left on for the entire trial, in others, they are manually switched on/off throughout the trial (Ern & Jutfelt, 2024; Morgan et al., 2018; Stewart et al., 2023). In either case, supplementary plots of warming rates (i.e. raw data; Figure 6) will allow readers to assess the consistency in warming rates. [see Section 4]
18	Indicate whether CT_{max} arena was visually shielded from external disturbance	External disturbance may cause agitation and stress, potentially altering CT_{max} (McDonnell & Chapman, 2015). [see Section 9]
19	End points	Critical thermal end points assessed behaviourally can vary qualitatively among study organisms (Morgan et al., 2018). Providing a clear description of the end point used in the experiment in terms of behaviours observed (i.e. loss of equilibrium (LOE), erratic swimming, loss of righting response [LRR]), as well as the duration of this behaviour prior to removal of the individual (i.e. LOE for >5 s) can facilitate replication. [see Sections 6 and 7]
20	Water quality monitoring	Indicate whether water in the test arena was aerated during the trials, and whether dissolved oxygen or other water parameters (e.g. salinity, conductivity, pH, ammonia) were measured. [see Sections 2 and 10]
21	Numbers of animals and replicate trials	State the number of animals per CT_{max} trial, the number of replicate trials per treatment, and the resulting total n per treatment. If animals were kept in separate tanks for treatments prior to the trial, state which tank(s) they were kept in. [see Section 8]
After trial		
22	Duration of all trials combined	Should ideally be reported as the start and end dates of data collection for the study. Animals may habituate or acclimate to the laboratory setting, which could affect behaviour and CT_{max}. Body mass or life stage may also change over time. [see Sections 9 and 11]
23	Recovery duration and temperature	Survival is typically monitored for 30 min—1 h following the trial, but can be reported over longer durations as well (24 h or more). Recovery temperatures could potentially affect survival if they are either too low (cold shock) or too high (continued thermal stress).
24	Rates of survival	CT_{max} is defined as a non-lethal procedure. The per cent recovery of all individuals in a trial following their return to cooler temperatures should be verified and reported.
25	Arena cleaning and maintenance	CT_{max} arenas should be fully drained and refilled between trials. Indicate if any other cleaning or maintenance tasks took place between trials. [see Section 2]
Data handling and statistics		
26	Assignment of a CT_{max} value	The source of the CT_{max} value chosen must be specified, particularly when multiple pieces of equipment are used to record temperature, such as a handheld thermometer and logger(s) in the water bath. [see Sections 2, 4–7]
27	Observer bias	Report whether any measures were taken to minimise observer bias (e.g. any form of masking/blinding and if so how that was achieved and if it applied to masking/blinding of both treatment and/or temperature and ramping), and whether CT_{max} trials were video recorded (and if videos are uploaded to a repository). Blinding increases the reliability of the experiment (Holman et al., 2015). [see Section 7]
28	Approach to ensuring scoring consistency	Ideally a single observer is used across treatments, after having gone through one or two pilot experiments. Otherwise, multiple observers need to agree on end point criteria that are as objective as possible, and should be trained together during pilot trials. Video footage can accompany publications to enable replication. [see Sections 6 and 7]

between animals measured in situ (field) vs. in animals acclimatised to the laboratory for varying durations.

Ultimately, addressing these knowledge gaps will help improve future CT_{max} studies and help interpret the vast literature on CT_{max}. CT_{max} will likely continue to be a popular metric in animal biology (Figure 1), providing value for comparative ecophysiology, evolutionary biology, risk assessments, and species distribution modelling. Ensuring CT_{max} data are as precise and reliable as possible, through sound experimental approaches and reporting, will help optimise their value.

AUTHOR CONTRIBUTIONS

Graham D. Raby conceived of the idea for this paper; Erin M. C. Stewart conducted the analysis of inter-trial variability; all authors contributed to drafting and revising the manuscript.

ACKNOWLEDGEMENTS

We thank the 2024 FIN club for the opportunity to write this paper. This work was funded primarily by an ERC consolidator grant (CLIMEVOLVE) to Jutfelt. Raby, Blewett, Binning and Speers-Roesch are supported by Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grants. Stewart and

Reid were supported by NSERC doctoral scholarships. Hoots and Kuchenmüller were supported by PhD scholarships through Deakin University, Australia. Ern was supported by Marie Skłodowska-Curie Actions (893895). Clark was supported by an Australian Research Council Future Fellowship (FT180100154) funded by the Australian Government.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/2041-210X.70103.

DATA AVAILABILITY STATEMENT

Data and analysis code available via the figshare repository: https://doi.org/10.6084/m9.figshare.28319774.v1 (Raby & Stewart, 2025).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. Example of generator-powered field-based CT_{max} experiment (CT_{max} arena shown in Figure 3c; from Stewart et al. 2024, *Cons. Physiol.* https://doi.org/10.1093/conphys/coae086). Note that a tent was used (at all field sites) to ensure the CT_{max} arena was shaded from direct sunlight to help control warming rates. In both cases, individual recovery containers (buckets) were prepared in advance, with water set to an intermediate temperature (above ambient but well below CT_{max}) for recovery. Once animals were given ca. 10–20 min to recover (and confirm survival), they could be individually weighed and measured to link mass and length data to CT_{max} at the individual animal level (the fish were then released back to the wild).

Table S1. Overall warming rates and R^2 achieved in CT_{max} trials in Figure 6. The achieved rate (slope, *b*) and R^2 were calculated from the linear regression of each trial. Classifications were based on the consistency of the warming rate throughout the trial (in Figure 6: blue = optimal, orange = non-optimal). The warming rates correspond to the same panel(s) in Figure 6, where optimal = the blue lines, non-optimal = the orange lines.

Table S2. Warming rates and R^2 achieved in the initial and final stages of the CT_{max} trials in Figure 6. Final versus initial sections were classified on a trial-by-trial basis by visual inspection of curves in Figure 6 (Section). The achieved rate (slope, *b*) and R^2 were calculated from a linear regression of each section of each trial. Classifications were based on the consistency of the warming rate throughout the trial (in Figure 6: blue = optimal, orange = non-optimal). The warming rates correspond to the same panel(s) in Figure 6.

Table S3. Multi-species CT_{max} Bayesian regression coefficients (Estimate) and 95% credible intervals (l-95% CI, u-95% CI) of all four trial replicates for each of 12 species. Trials were modelled as covariates to test for trial effects (i.e., differences in mean CT_{max} from the initial trial [Intercept] for each replicate trial). Where the credible interval does not include 0, evidence of a trial effect exists. R-hat, Bulk ESS (estimated sample size), and Tail ESS are diagnostic criteria for model convergence and efficiency where R-hat should = 1, and Bulk and Tail ESS should be ≥100 per Markov Chain.

Table S4. Zebrafish CT_{max} Bayesian regression coefficients (Estimate) and 95% credible intervals (l-95% CI, u-95% CI) for all 12 trials in each of eight treatments. Trials were modelled as covariates to test for trial effects (i.e., differences in mean CT_{max} from the initial trial [Intercept] for each replicate trial). Where the credible interval does not include 0, evidence of a trial effect

exists. R-hat, Bulk ESS (estimated sample size), and Tail ESS are diagnostic criteria for model convergence and efficiency where R-hat should = 1, and Bulk and Tail ESS should be ≥100 per Markov Chain.

Table S5. Example of how to fill out Table 1 (from the manuscript), using a previously published CT_{max} study (Stewart et al. 2024, *Cons. Physiol*. https://doi.org/10.1093/conphys/coae086) as an example. Another example is available as a supplementary file for Vallin et al. 2025, *J Thermal Biol* 130:104155: https://doi.org/10.1016/j.jtherbio.2025.104155.

How to cite this article: Raby, G. D., Morgan, R., Andreassen, A. H., Stewart, E. M. C., De Bonville, J., Hoots, E. C., Kuchenmüller, L., Metz, M., Rowsey, L. E., Green, L., Griffin, R. A., Martin, S., Reid, H. B., Ern, R., Åsheim, E. R., Cowan, Z.-L., Leeuwis, R. H. J., Blewett, T. A., Speers-Roesch, B., … Jutfelt, F. (2025). Measuring critical thermal maximum in aquatic ectotherms: A practical guide. *Methods in Ecology and Evolution*, *16*, 2208–2228. https://doi.org/10.1111/2041-210X.70103