CT<sub>max</sub> is repeatable and doesn’t reduce growth in zebrafish

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Critical thermal maximum (CT<sub>max</sub>) is a commonly and increasingly used measure of an animal’s upper thermal tolerance limit. However, it is unknown how consistent CT<sub>max</sub> is within an individual, and how physiologically taxing such experiments are. We addressed this by estimating the repeatability of CT<sub>max</sub> in zebrafish, and measured how growth and survival were affected by multiple trials. The repeatability of CT<sub>max</sub> over four trials was 0.22 (0.07–0.43). However, CT<sub>max</sub> increased from the first to the second trial, likely because of thermal acclimation triggered by the heat shock. After this initial acclimation response individuals became more consistent in their CT<sub>max</sub> reflected in a higher repeatability measure of 0.45 (0.28–0.65) for trials 2–4. We found a high innate thermal tolerance led to a lower acclimation response, whereas a high acclimation response was present in individuals that displayed a low initial CT<sub>max</sub>. This could indicate that different strategies for thermal tolerance (i.e. plasticity vs. high innate tolerance) can co-exist in a population. Additionally, repeated CT<sub>max</sub> trials had no effect on growth, and survival was high (99%). This validates the method and, combined with the relatively high repeatability, highlights the relevance of CT<sub>max</sub> for continued use as a metric for acute thermal tolerance.

Climate change is causing an increase in global water temperatures as well as more frequent and extreme events such as heat waves. Because the body temperature of most aquatic ectotherms conforms to that of the surrounding water, temperature is one of the main environmental factors affecting their performance, fitness and distribution. Climate change will increase the frequency of ectothermic organisms’ exposure to transient warm events and temperatures above their tolerance range. Therefore, understanding how aquatic ectotherms will respond to, and perform under, these conditions is driving research into thermal biology and, in particular, research focusing on organisms’ thermal tolerance. Critical thermal maximum (CT<sub>max</sub>) is a commonly used method for measuring an animal's upper thermal tolerance limit, and can be broadly defined as the temperature at which locomotion becomes disorganised and performance is lost during acute thermal ramping. Through a review of papers on Google Scholar we found that the popularity for using the CT<sub>max</sub> method has rapidly increased in the last decade with five times more publications using it per year between 2010–2017 than from 1990–2000.

Thermal tolerance has been suggested to have a low potential for rapid evolution and, based on biographical distribution patterns, appears relatively conserved. Genetic variation is a requirement for adaptation, and this can be estimated by calculating the heritability of a trait. Heritability (h<sup>2</sup>) of thermal tolerance in fish has only been estimated in a limited number of previous studies; Doyle et al. (h<sup>2</sup> = 0.2), Meffe et al. (h<sup>2</sup> = 0.32) and Baer & Travis (h<sup>2</sup> = 0.15). In addition, technical complications in some of the selection lines make interpretation of Baer and Travis results challenging, limiting the useful number of studies on heritability further. Therefore, more data on the heritability of thermal traits are needed for robust estimates to be made.

Another approach for estimating adaptation potential of thermal tolerance is to assess its repeatability, due to the positive relationship between repeatability and heritability. The repeatability of a trait sets an upper limit on its heritability, as it includes both genetic and environmental variance. By quantifying the proportion of total variation of a trait that is due to differences between individuals, repeatability shows the consistency of an individual’s performance over a longer time scale. If thermal tolerance is inconsistent within an individual over time (i.e., low repeatability), then the adaptive potential of the trait is low. Despite the apparent need to estimate the repeatability of thermal tolerance there has been no study, to our knowledge, that has investigated this in aquatic ectotherms.

Although there are many studies that use CT<sub>max</sub> very few report survival after a CT<sub>max</sub> test. Of the studies that do, survival is claimed to be above 90%. In zebrafish however, only one study has looked at survival after CT<sub>max</sub> and although they had high survival in fish acclimated to 20°C, a low survival rate was seen in fish acclimated to 28°C, likely because of thermal acclimation triggered by the heat shock. After this initial acclimation response individuals became more consistent in their CT<sub>max</sub> reflected in a higher repeatability measure of 0.45 (0.28–0.65) for trials 2–4. We found a high innate thermal tolerance led to a lower acclimation response, whereas a high acclimation response was present in individuals that displayed a low initial CT<sub>max</sub>. This could indicate that different strategies for thermal tolerance (i.e. plasticity vs. high innate tolerance) can co-exist in a population. Additionally, repeated CT<sub>max</sub> trials had no effect on growth, and survival was high (99%). This validates the method and, combined with the relatively high repeatability, highlights the relevance of CT<sub>max</sub> for continued use as a metric for acute thermal tolerance.
acclimated to 30 °C. This suggests that CT max tests can have negative physiological consequences for fish, which may be under-reported in the literature.

The aim of this study was to determine whether CT max is a repeatable trait within an aquatic ectotherm, the zebrafish (Danio rerio), and thereby establish its potential applicability as a metric for thermal tolerance. In addition, we aimed to validate the CT max method, which is widely used in the field of thermal biology, by testing whether repeated CT max tests affect zebrafish growth and survival. We hypothesized that CT max is a repeatable trait, that multiple CT max tests negatively affect zebrafish growth, and that survival after CT max tests would be high.

Results

Repeatability of CT max. The repeatability of CT max was estimated as 0.216 (95% C.I.: 0.066–0.434) when all CT max trials were included and increased to 0.447 (95% C.I.: 0.284–0.645) when the first CT max trial was omitted from the model. CT max also increased from the first (40.97 ± 0.10 °C) to the second trial (+0.30 ± 0.10 °C, F 4,125 = 10.98, p < 0.01, Fig. 1). In the third trial CT max increased further, (+0.19 ± 0.08 °C, p = 0.03) before declining in the final trial (–0.26 ± 0.08 °C, p = 0.003) to the level of trial 2 (F 4,125 = 10.98, Fig. 1).

Acclimation response. A negative relationship was found between an individual's initial CT max and their acclimation response; the change in CT max from trials 1 to 2 (F = 53.92, p < 0.01, Fig. 2). Therefore, individuals with a low CT max in trial 1 had a larger increase in their score from trial 1 to trial 2 than individuals with a high initial CT max (Fig. 2). This relationship appeared more pronounced in males than in females (Sex × CT max Trial1,
Effect of CT\text{ max} on growth. There was no difference in growth between the sham and repeatability groups ($F_{1,104} = 1.03, p = 0.31$, Fig. 3), and growth did not differ between the sexes ($F_{1,104} = 0.58, p = 0.45$, Fig. 3).

Size and CT\text{ max}. There was no relationship between CT\text{ max} and length, weight and condition (Fig. 4) and CT\text{ max} did not differ between the sexes ($F_{1,107} = 1.31, p = 0.26$, Fig. 4).

Discussion
We show that after an initial heat shock CT\text{ max} is repeatable over three trials within individuals, meaning that consistent individual differences exist over medium timescales. The repeatability when all four trials were included was significant but not high. However, the repeatability increased when the first trial was excluded. This increase in repeatability could be caused by the increase in CT\text{ max} from the first to the second trial, where an acclimation response altered the thermal tolerance differently depending on the initial CT\text{ max} response.

When fish undergo a heat shock such as a CT\text{ max} trial, physiological mechanisms associated with warm acclimation are activated. Such mechanisms can include increased heat shock protein production\textsuperscript{35,36}, changes in membrane fluidity\textsuperscript{37,38}, protein isoforms\textsuperscript{39,40}, and altered mitochondrial density\textsuperscript{41}. Such adjustments are considered beneficial for coping with future thermal challenges\textsuperscript{36}. Similar increases in CT\text{ max} have been shown in earlier
studies\textsuperscript{15,42,43}, which named the effect “heat hardening”. Due to these acclimation effects, individuals were physiologically different in the second trial than they were in the first trial. Whilst $CT_{\text{max}}$ increased further in the third trial, it was to a lesser extent. This suggests that the biggest physiological responses occurred between the first and the second trial.

Whilst thermal tolerance increased from the first to the second $CT_{\text{max}}$ trial at the group level, there was large individual variation. Some individuals had high innate thermal tolerance and were therefore top performers in the first trial, however, the same individuals appeared less plastic in their acclimation response and were thus not top performers in subsequent trials. Indeed, their change in $CT_{\text{max}}$ in the second trial was minimal or negative. Conversely, individuals with a low innate thermal tolerance (i.e. the poor performers in trial one) had a large capacity for acclimation, and increased their thermal tolerance, becoming top performers in the second trial. This effect is not due to the fish reaching their maximum post-acclimation thermal tolerance, as we have measured $CT_{\text{max}}$ of above 43°C after longer acclimation (Morgan et al. unpublished data). This shows that zebrafish have varying levels of thermal plasticity and capacity for acclimation between individuals, and it can explain the increase in repeatability scores as acclimation allows the group to become more heat tolerant as a whole. These individual differences in acclimation response suggest two different tolerance strategies: (1) having a high innate thermal tolerance and a low level of thermal plasticity, or (2) having a low innate thermal tolerance and a high level of thermal plasticity.

Acclimation, or “heat hardening”, inevitably occurs between the first and second $CT_{\text{max}}$ challenges. The first trial therefore represents the innate thermal tolerance while the second trial gives a measure of the acclimated thermal tolerance. It may be impossible to get a true estimate of the repeatability of the innate thermal tolerance, as a thermal challenge such as $CT_{\text{max}}$ can only be experienced as novel once. An estimate of repeatability that includes the first $CT_{\text{max}}$ may therefore not be optimal as it includes both the innate and acclimated thermal tolerance, which from this study appear to represent two separate biological traits. After the first trial, and the resulting increase in thermal tolerance due to acclimation, the subsequent individual $CT_{\text{max}}$ temperatures (trials two to four) were more consistent, which can be seen by the increase in repeatability. The second estimate of repeatability is therefore a more accurate representation of the repeatability of $CT_{\text{max}}$ after acclimation. It should be noted however that the sustained “heat hardening” effect observed here may be specific to the species and protocol we used (heating rate of 0.3 min\textsuperscript{-1} & 1 week between trials) as previous experiments have found that heat-shock benefits to thermal tolerance diminish to “pre-hardened” levels after only 24–32 hours\textsuperscript{42,44}.

The relatively high level of repeatability in $CT_{\text{max}}$ we found is greater than the heritability of thermal tolerance reported in other studies\textsuperscript{24–26}, and indeed, the repeatability sets an upper limit for heritability. While it is unclear in the present study how much of the repeatability stems from environmental factors and how much is caused by genetic differences, it does suggest a degree of genetic variation is present in thermal tolerance. Such variation may allow populations to evolve their thermal tolerance, aiding in range expansion and coping with climate change.

The lack of difference in growth between the sham and repeatability groups shows that multiple $CT_{\text{max}}$ tests do not impose a growth penalty on the fish. Decreased growth was expected in the repeatability fish compared to the sham fish as the heat stress the fish experience during a $CT_{\text{max}}$ trial could trigger an energetically costly stress response, hence diverting energy away from processes such as growth\textsuperscript{45}. It is also conceivable that cell and tissue damage could occur during heat shocks that could require costly repair processes. This was not the case however, perhaps because of the short exposure to high temperatures (the duration of the $CT_{\text{max}}$ trial was approximately 40 minutes, and the temperature was only high enough to cause agitated behaviour for the final minutes), or because zebrafish are a robust and tolerant species\textsuperscript{46–48}. Indeed, the fish regained equilibrium within seconds of returning to 28°C, and would resume feeding within minutes when presented with food. Similar growth rates to what we observed in both the sham and repeatability fish have been shown for adult zebrafish\textsuperscript{39,50} suggesting that additional experimental procedures (e.g. tagging and anaesthesia) had no major negative impact on the growth rates we observed here.

Contrary to the general perception that larger individuals have a lower thermal tolerance than smaller individuals\textsuperscript{18,51,52}, we found no relationship between $CT_{\text{max}}$ and length, weight or condition. Similarly, no effect of length\textsuperscript{30} or weight\textsuperscript{34} on $CT_{\text{max}}$ has been reported in other species, suggesting a species-specific effect. A minor effect of size on thermal tolerance in these zebrafish may have gone undetected due to a limited size range in the current experiment.

Additionally, $CT_{\text{max}}$ did not differ between the sexes, which might be important in order to maintain a balanced sex ratio in populations facing heat spell challenges.

In summary, we have shown that $CT_{\text{max}}$ increases after an initial heat shock, which, in turn increases the repeatability of the trait within individuals in subsequent trials. In addition, individuals with a low innate thermal tolerance have a greater acclimation response after heat shock than individuals with a higher innate thermal tolerance. A repeatability estimate of 0.45 (0.28–0.65) after acclimation shows that $CT_{\text{max}}$ is a repeatable, and therefore useful measure of thermal tolerance. However, a reliable estimate of repeatability of innate $CT_{\text{max}}$ was not possible to achieve, as fish are only naïve to heat shock at the first trial. Furthermore, no growth penalty was imposed on zebrafish after repeated $CT_{\text{max}}$ measurements. This suggests the method used does not have major negative physiological impacts on zebrafish, further validating it as a method and valuable metric for continued use within thermal biology.

**Materials and Methods**

The experiments were conducted in July 2016 using ornamental zebrafish (Tropehagen Zoo, Trondheim, Norway), which were housed in the animal facility at the Norwegian University of Science and Technology for four months under controlled conditions prior to the experiment. At the start of the experiment, 78 adult zebrafish were tagged (see below) and distributed randomly into four 63-L glass aquaria housing tanks: two sham tanks...
and two repeatability tanks, all with a maximum density of 4 fish/10 L. The temperature of the housing tanks was kept at 28 °C and the water was well aerated. Each tank was fed 0.1 g of TetraMin dry flakes four times a day, and live Artemia was provided once every two days, replacing one of the TetraMin feeds. The fish were fasted for 20–28 hours prior to critical thermal maxima tests.

The experiments were approved by the Norwegian Animal Research Authority (Permit Number: 8578) and all methods were performed in accordance with the relevant guidelines and regulations.

**Experimental design.** To estimate the repeatability of CT_{max}, 78 fish were randomly assigned to the control group or the repeatability group. In the repeatability group, each individual fish underwent a CT_{max} test four times with a week between each trial: 7th, 14th, 21st and 28th of July 2016. To control for any physiological consequences of carrying out CT max experiments 38 fish were assigned to the sham group. These fish underwent a sham CT_{max} test that was identical to the real CT_{max} tests but with water kept constant at 28 °C throughout the first three trials before undergoing an actual CT_{max} test in the fourth trial. Length (to the nearest 0.1 mm) and weight (to the nearest 0.01 g) were measured for all fish after each trial, which allowed for growth comparisons between the sham and repeatability groups. By undergoing an actual CT_{max} test during the fourth trial, handling stress and measurement error were controlled for by allowing comparisons to be made between the CT_{max} of the sham fish with the first CT_{max} trial of the repeatability group. Trials were carried out on the same days for both the sham and the repeatability fish.

**Tagging.** All fish were tagged with visible implant elastomer (VIE) tags (Northwest Marine Technology, Shaw Island, WA, USA) allowing for individual identification throughout the experiment. Prior to tagging, the fish were anaesthetized in 110 mg/L buffered tricaine methane sulfonate (MS222). Tags were injected at two of three locations: the base of the dorsal fin, anal fin and caudal peduncle according to55. After tagging the fish were weighed to the nearest 0.01 g and photographed using a standardised setup on millimetre paper for measurement of total length to the nearest 0.1 mm, which was quantified using the line function in the program ImageJ (https://imagej.nih.gov/ij/).

**Critical thermal maxima (CT_{max}) test.** For the CT_{max} test, a heating tank (25 × 22 × 18 cm) was filled with 9 L of 28 °C water. A water pump (Eheim Universal 300, Deizisau, Germany) was attached to a custom-made cylindrical steel heating case consisting of an inflow nipple, a wide outflow and a 300 W coil heater (Fig. 5). The pump pushed water through the heating cylinder and into the fish arena creating stirring, and the heating system was separated from the fish arena by a mesh. This setup ensured a homogenous temperature in the entire arena (<0.1 °C), whilst minimising the water current within the tank. A recently factory calibrated high precision digital thermometer with a ±0.1 °C accuracy (testo-112, Testo, Lenzkirch, Germany) continuously measured the water temperature in the fish compartment.

A group of randomly selected individuals (3–6) were caught from their holding tank and transferred to the heating tank. The water was then heated at a steady rate of 0.3 °C per minute11, in accordance with17. A pilot experiment with zebrafish instrumented with small thermocouples showed that this heating rate caused a lag of heating of less than 0.2 °C between the ambient water temperature and the deep dorsal muscle (see below). In addition, oxygen concentrations at or above 100% saturation were retained throughout the test. Loss of equilibrium (LOE), defined as uncontrolled and disorganised swimming for two seconds, was chosen as the CT_{max} endpoint56. Once LOE occurred in an individual, the water temperature was recorded with an accuracy of 0.1 °C, and the fish was immediately transferred into an individual tank of 28 °C water for recovery. Once the fish had recovered, it was anaesthetised, identified, weighed and photographed before being returned to its holding tank. Recovery of equilibrium generally occurred within two minutes, and normal behaviour was restored after approximately five minutes. During pilot experiments fish commenced feeding within fifteen minutes of a completed CT_{max} test, indicating that the thermal challenge didn’t cause major trauma. All but one of the fish recovered after

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**Figure 5.** CT_{max} experimental setup, ensuring homogeneous water temperature and consistent heating rate for all trials. (A) Water pump; (B) custom-made cylindrical steel heating case; (C) 300 W coil heater; (D) mesh (preventing fish swimming into the heating compartment); (E) fish compartment with 9 L of 28 °C water; (F) Photograph of the CT_{max} box.
the CT<sub>max</sub> tests in the experiment (99% survival). Additionally, two treatment fish do not have a fourth CT<sub>max</sub> measurement as they jumped out of the heating tank during the test.

The sham CT<sub>max</sub> test consisted of the fish being put into the heating tank for the same duration (~40 minutes) as the treatment fish but the water was kept at 28 °C throughout. At the end, the fish were individually removed, anaesthetised, weighed, photographed and returned to their holding tank.

A condition index (K, equation 1) was calculated for each fish using the total length and weight measurements. Specific growth rate (SGR, equation 2) was calculated for four growth intervals, the first one from the date of first tagging until the first CT<sub>max</sub> test, and weekly thereafter.

\[ K = \frac{\text{Weight}}{\text{Length}^3} \times 100 \]  

\[ SGR = \frac{\ln(W_f) - \ln(W_i)}{\text{time interval}} \times 100 \]

**Thermal ramping rate and muscle temperature.** To investigate whether there was a lag between body temperature and water temperature at a thermal ramping rate of 0.3 °C min<sup>-1</sup> a pilot experiment was carried out. Two zebrafish were anaesthetised in 110 mg/L buffered tricane methane sulfonate (MS-222) before a small thermocouple was inserted into the dorsal muscle to a depth of approximately 2 mm on each fish so the tip of the thermocouple was not visible under the skin and deep enough so the thermocouple held in place. The fish were then carefully placed in the CT<sub>max</sub> heating tank with MS-222 at a concentration of 55 mg/L in the water to keep the fish anaesthetised throughout the procedure. Another thermocouple was placed in the water to measure ambient water temperature. All thermocouples were calibrated before the experiment.

The CT<sub>max</sub> method was then carried out using the same heating rate described above and as shown in the Fig. 5. Temperatures were recorded every 10 seconds from both the thermocouples in the fish and the ambient water and the temperature was ramped until reaching 43 °C. The operculum movement of the fish was monitored during the process and the temperature at which the fish ceased breathing was recorded. There was a lag of less than 0.2 °C between the body temperature of the fish and that of the water temperature and no obvious difference in temperature lag was observed when the fish were alive and after they died (Fig. 6).

**Statistical analyses.** All statistical analyses were conducted in R 3.3.0 (R Core Team, 2016) with effect sizes with p-values less than 0.05 considered statistically significant. The repeatability of CT<sub>max</sub> and the corresponding 95% confidence intervals were estimated with generalised linear mixed effect model's (GLMM's) and a Bayesian approach using the function MCMCglmm()<sup>57</sup> and codas HDPinterval() function<sup>58</sup> based on the method recommended by Dingemanse & Dochtermann<sup>59</sup>. Individual identity was included as a random factor, and week number was included as a fixed effect in the model to account for any variation caused by the order of the measurements. Two repeatability measures were estimated, the first using all trials and the second omitting trial 1. The latter was estimated to determine whether the effect of the first thermal challenge had a long-lasting effect on the fish's CT<sub>max</sub> in subsequent trials.

A linear mixed effect (LME) model was used to test whether CT<sub>max</sub> changed between trials, using the lmer() function within the lmerTest package<sup>60</sup>. Individual identity was included as a random factor to account for multiple measures of the same individuals.

An individual’s acclimation response was calculated by subtracting their CT<sub>max</sub> in trial 1 from their CT<sub>max</sub> in trial 2 and a linear regression model was used to test the relationship between the first CT<sub>max</sub> (trial 1) and this acclimation response. Sex was also included in the model, as well as the interaction between sex and the first CT<sub>max</sub>. The correlation between CT<sub>max</sub> in trial 1 and trial 2 was also tested using Pearson's product-moment correlation coefficient.

Growth (SGR), sex and treatment (repeated or sham) were analysed using a two-way analysis of variance (ANOVA). SGR is given as the mean for each sex ± standard error of mean (s.e.m.).

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*Figure 6.* Body temperature of deep dorsal muscle of two zebrafish (Fish 1, weight = 0.4 g, length = 28 mm: red line; Fish 2, weight = 0.4 g, length = 26 mm: yellow line) plotted against ambient water temperature (light blue line) shows a lag in temperatures of less than 0.2 °C. The desired thermal ramping rate of 0.3 °C min<sup>-1</sup> (dark blue line) is also plotted to show that the ambient water temperature closely follows the desired water temperature.
Linear regressions were used to test the effect(s) of length, weight and condition (K) on CT_{max}. Length, weight and condition were all tested in separate models due to the significant positive correlation between them (Length & Weight, r = 0.76, p < 0.001; Length & Condition, r = 0.28, p < 0.001; Weight & Condition, r = 0.83, p < 0.001). Due to these correlations, weight was chosen as an appropriate proxy for size. A linear mixed effect (LME) model was used to test whether CT_{max} differed between the sexes, or changed with weight (covariate). As weight could have differed between sexes, an interaction was included for weight and sex and the model accounted for repeated measures with individual identity as a random factor.

**Data availability.** The dataset generated during the current study is available in the figshare data repository (doi:10.6084/m9.figshare.6148373).

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