Master's thesis

2019

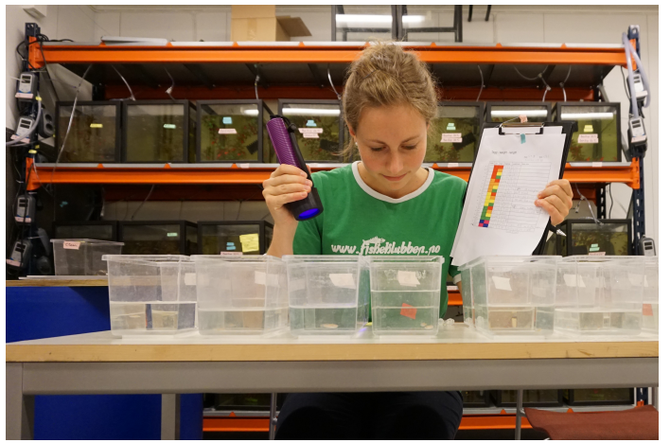
Hanne Margrethe Hildrum

NTNU
Norwegian University of
Science and Technology
Faculty of Natural Sciences
Department of Biology

Hanne Margrethe Hildrum

Selection on high thermal tolerance alters tolerance to cold

May 2019



NTNU
Norwegian University of
Science and Technology

NTNU
Norwegian University of
Science and Technology

NTNU

Norwegian University of
Science and Technology

Selection on high thermal tolerance alters tolerance to cold

Master's thesis in Biology

Submission date: May 2019

Supervisor: Fredrik Jutfelt

Co-supervisor: Rachael Morgan
Mette Helene Finnøen
Jonathan Wright

Norwegian University of Science and Technology
Department of Biology

Acknowledgements

Foremost, I would like to thank the zebrafish group at the Jutfelt Ecophysiology Lab at NTNU. The inclusive culture, weekly meetings, interesting discussions, experiments in the animal facility and social happenings together with great people have brought much joy into the everyday life the last year. Special thanks to my supervisor, Fredrik Jutfelt, for leading and inspiring the group in good science as well as exciting activities. Your constructive feedback on the work and open office door have been very valuable. Thanks to my co-supervisors for your different contributions to the thesis: Rachael Morgan, for using fish, space and equipment from your PhD project as well as all kind support with statistics and writing. Mette Helene Finnøen, for statistical skills and support for Tine and me. Jonathan Wright, for teaching study design and giving start-up advice on the experiments. Thanks to my master partner, Tine, for endless hours of planning and conducting experiments and for your inspiring fascination for the project. It has been a pleasure to drink coffee over frustrations and enjoyments this year. Thanks to Anna and Eirik for being great examples of dedicated master students who also engage in the progress of others. In addition, many great friends in Trondheim have filled the student period with happy moments and memories for life. Thanks to Mom and Dad for always listening and giving personal advice. I hope to bring some of these people's values and knowledge into the future, as I now graduate as a teacher with this master thesis in biology.

Trondheim, May 2019

Hanne Margrethe Hildrum

Abstract

Climate change will lead to increased water temperatures and heat wave intensity. For ectothermal fish, this will challenge their internal physiology and population distribution, depending partly on their thermal tolerance. A commonly used measure of tolerance to extreme temperatures is the critical thermal method (CTM), both a method and a parameter of an individual's critical thermal maximum (CTmax) and minimum (CTmin). Previous studies have tested CTmax and CTmin in non-selected samples of fish, and often measured the effect of acclimation on thermal performance. The present study is the first to investigate whether artificial selection on CTmax over generations has also caused a change in CTmin. This was conducted on fourth generation of zebrafish (*Danio rerio*, n=204), where these thermal limits were compared on an individual level. A negative correlation was found between CTmin and high CTmax, meaning that some individuals had a wide thermal scope while others had narrow. The line previously selected on low CTmax had the narrowest scope, due to low CTmax and high CTmin. Surprisingly, the control line of non-selected, randomly bred zebrafish had the lowest CTmin (9.97 °C). The line selected on high CTmax still got the highest CTmax (41.62 °C) in this generation but were not significantly different from the control line in neither CTmax, CTmin nor scope. The results show that directional selection towards low performance in one end of the thermal tolerance curve (here: CTmax) does not indicate better performance at the other end (here: CTmin).

Sammendrag

Klimaendringer vil føre til økning i vanntemperaturer og intensitet av hetebølger. Dette vil utfordre ektoderme fiskers indre fysiologi og populasjonsutbredelse, delvis avhengig av deres temperatur-toleranse. Et vanlig mål på toleranse mot ekstreme temperaturer er kritisk termisk metode (CTM), som både brukes om en metode og en parameter på et individs kritiske maksimale (CTmax) og minimale (CTmin) temperatur. Tidligere studier har testet CTmax og CTmin på uselekterte utvalg fisker og ofte målt effekten av akklimatisering på evnen til å tåle ekstreme temperaturer. Denne studien er den først til å undersøke om kunstig seleksjon for CTmax over generasjoner også gir endringer i CTmin. Dette ble gjennomført på fjerdegenerasjon av sebrafisker (*Danio rerio*, n=204), hvor disse temperatur-ekstremitetene ble sammenlignet på individnivå. Resultatene viste negativ korrelasjon mellom CTmin og høy CTmax, noe som tyder på at noen individer hadde et bredt temperaturspenn mens andre hadde smalt. Linjen tidligere selektert for lav CTmax hadde det smaleste temperaturspennet grunnet lav CTmax og høy CTmin. Overraskende, kontroll-linjen av ikke-selekterte, tilfeldig avlede sebrafisker hadde lavest CTmin (9.97 °C). Linjen selektert for høy CTmax hadde fortsatt høyest CTmax (41.62 °C) i denne generasjonen, men var ikke signifikant forskjellig fra kontrollgruppen i hverken CTmax, CTmin eller temperaturspenn. Resultatene viser at retningsbestemt seleksjon mot lav prestasjon i én ende av temperaturtoleransekurven (her: CTmax) ikke indikerer bedre prestasjon i den andre enden (her: CTmin).

List of abbreviations and definitions

CTM	critical thermal method
CTmax	critical thermal maximum
CTmin	critical thermal minimum
Thermal scope	temperature range between CTmin and CTmax
HIGH	zebrafish line selected on highest CTmax
LOW	zebrafish line selected on lowest CTmax
RANDOM	control group of non-selected, randomly bred fish
LOE	loss of equilibrium
Line	HIGH/H, LOW/L and RANDOM/R
Replicate	H1 and H2, L1 and L2, R1 and R2
Tank/duplicate	H1.1, H1.2, H2.1, H2.2, L1.1, L1.2, L2.1, L2.2, R1.1, R1.2, R2.1, R2.2
Individual	the colour marked zebrafish
SGR_{first}	specific growth rate for the first measurement; during tagging
SGR_{last}	specific growth rate for the last measurement; after CTmax
$Weight_{first}$	body weight measured during tagging
$Weight_{last}$	body weight measured after CTmax
FCF	Fulton's condition factor; (weight / $length^3$) x 100

Table of contents

1. Introduction

1.1 Temperature changes and thermal tolerance in fish

In the nature, environmental temperature affects ecological and physiological processes for all species (Crawshaw & Podrabsky 2011) and is considered the abiotic master factor for fish (Brett 1971). The expected increase in water temperatures and heat wave intensity at the global level (Seneviratne *et al*. 2014; IPCC 2013) may challenge the thermal performance in ectotherms, whose body temperature is in equilibrium with the surrounding water (Schulte *et al*. 2011). Eurythermal fish are able to thrive in a wide range of temperatures (Cortemeglia & Beitinger 2005) and, in general, warm-water living fish show the biggest thermal scope between minimum and maximum thermal limits (Brett 1956). Diurnal and seasonal fluctuations in water temperatures influence preferred habitat (Crawshaw & Podrabsky 2011). If a fish is not able to migrate to a more suitable environmental temperature, it may be forced to modify its physiology and biochemistry to the new temperature, and thus reduce energy costs and improve fitness (Crawshaw & Podrabsky 2011; Bozinovic & Pörtner 2015).

Thermal tolerance is affected by genetics, developmental plasticity and acclimation processes (López-Olmeda & Sánchez-Vázquez 2011). If the innate physiological mechanisms cannot satisfy the individual's needs, the inherent range of genes could eventually express characteristics suitable for the present environment. This evolutionary capacity could make the fish tolerate more extreme temperatures in the future (Bozinovic & Pörtner 2015; Sandblom *et al*. 2016). The accepted parameters of thermal tolerance in ectotherm vertebrates are the critical thermal maximum (CTmax) and minimum (CTmin) (Becker & Genoway 1979), that is, the temperatures at which the individuals display predefined endpoint criteria when driven through acute temperature increase or decrease, respectively. Since the first established procedures of these critical thermal methods (CTM) by Cowles & Bogert (1944), the recommended routines and endpoint criteria (Cox 1974; Becker & Genoway 1979) are still used (among others in Cortemeglia & Beitinger 2005; Sidhu *et al*. 2015; Moyano *et al*. 2017; Morgan et al. 2018). Loss of equilibrium (LOE) defines the end of the test, when the fish shows abnormal swimming patterns and reduced dorsoventral righting reflex (Cox 1974). The body of zebrafish has just a slight (<1 °C) temperature difference to the water temperature (Morgan *et al*. 2018), so this is a reliable measure of its actual thermal tolerance. CTmax and CTmin can describe both a parameter and a method where time, temperature

and body characteristics of the fish are important variables (Becker & Genoway 1979). The thermal scope is the temperature range between CTmin and CTmax (Bennet & Beitinger 1997). Many studies have documented the effect of acclimation on thermal tolerance (Becker & Genoway 1979; Currie *et al*. 1998; Beitinger *et al*. 2000; Cortemeglia & Beitinger 2005), and variation in both CTmin and CTmax with different acclimation temperatures (Currie *et al*. 1998; Fangue *et al*. 2006; Tongnunui & Beamish 2017).

1.2 The study species

Zebrafish (*Danio rerio*) is a tropical, freshwater fish naturally inhabiting shallow lakes and slowly flowing waters with temperature range from 24 °C to 34 °C. The species belong to Cyprinidae and is mainly distributed in South-Eastern Asia (Engeszer *et al*. 2007; Spence *et al*. 2008; Rey *et al*. 2015). Zebrafish are used in a wide range of research fields in laboratories around the world. About 40 years of studies indicate established knowledge about their properties and environmental requirements for housing facilities (Research Council of Norway 2009). In its native areas, especially India, zebrafish has been used to cover research within functional genomics, embryonic development, human diseases and biotechnology (reviewed by Sarasamma *et al*. 2017). This aquaria fish is favoured by its high fecundity, short generation time, and fast external embryogenesis where it develops organ system ready for activity and feeding within a week (Spence *et al*. 2007; Sarasamma *et al*. 2017). Its genome is largely surveyed, making it a usable organism for studies on genetic basis of both physiological mechanisms and social behaviour (Spence *et al*. 2008). Analogy to vertebrate genomics and physiology make zebrafish a suitable organism to study several biological questions, among them thermal tolerance.

In recent years, zebrafish and other eurythermal fish species have been used to investigate possible connections between thermal preference, behaviour, and optimal temperature ranges for survival (Rey *et al*. 2015). Many studies have focused on how CTmin and CTmax are affected by acclimation temperature (Beitinger *et al*. 2000; Cortemeglia & Beitinger 2005; López-Olmeda & Sánchez-Vázquez 2011), and also different thermal regimes (stable, stochastic or cyclic) before CTmax tests (Schaefer & Ryan 2006). Most thermal tolerance experiments use wild-caught fish (Schaefer & Ryan 2006), and little is known about how the thermal history of ancestors eventually alters CTmin or CTmax over generations. In a climate perspective, it would be highly interesting

to investigate whether selection on thermal performance at one end of the tolerance scope would also alter the performance in the opposite end. No previous studies have tested the effect of selection on CTmax over generations on performance in CTmin tests. To compare with other physiological characteristics, the possible correlation between critical thermal tolerances would be advantageous to study on a model organism like the zebrafish.

1.3 Study aim, procedure and hypothesis

The aim of this study is to compare upper and lower acute thermal tolerance in selected lines of zebrafish (the study population is detailed in part 2.1.2). The main question is how artificial selection on tolerance in CTmax tests will affect lower thermal tolerance in CTmin tests, and thus thermal scope between CTmax and CTmin. To investigate this, we used wild-caught zebrafish which originated from India, separately bread and housed as selected lines in Jutfelt Ecophysiology lab at NTNU based on each generation's performance in CTmax tests. The lines included fish with highest CTmax (termed HIGH), lowest CTmax (LOW) and a control group of randomly bred fish (RANDOM). In this study, we bred and tested the fourth selected generation (F_4), all acclimated to 28 °C. We first developed a method for measuring CTmin which involved a flow-through system for cooling the water at the same rate as recommended for increasing water temperature in CTmax tests; 0.3 °C min^{-1}. The same endpoint criteria, loss of body equilibrium, was used for CTmin and CTmax. We tested CTmin and CTmax in individually tagged fish from each of the three selected lines (HIGH, LOW, RANDOM).

Theoretically, there are several possible outcomes for each of the three lines (HIGH, LOW, RANDOM):

1. Wider thermal scope, due to higher CTmax combined with lower CTmin (Fig. 1A).
2. Narrower thermal scope, due to lower CTmax combined with higher CTmin (Fig. 1A).
3. Shifted scope, due to higher CTmax combined with higher CTmin, or lower CTmax combined with lower CTmin (Fig. 1B).
4. Combinations of 1, 2 and 3.

In addition, a possible outcome is no difference between the three lines in the tolerance tests.

Based on the expected effect of selection (CTmax for HIGH > RANDOM > LOW), a combination of the outcomes was chosen as the study hypothesis (sketched in Fig. 1C): Compared to RANDOM, HIGH was expected to get a shift in scope towards higher CTmax while LOW would get a shift towards CTmin. Correlation between CTmax and CTmin would then be positive. The idea behind these expectations is that the individuals selected on high CTmax would presumably be less tolerant to cold temperatures. The individuals selected on low tolerance to warm might have retained physiological traits that make them better in CTmin than the line of fish selected on high CTmax. However, as the LOW line has previously shown generally poor conditions (e.g. poor fecundity) in the lab, selection on genes involved in tolerance to warm may also be involved in other traits like tolerance to cold. A somewhat wider scope was presumed for HIGH than LOW. The RANDOM group was expected to get intermediate results as they are neither selected *for* nor *against* critical thermal tolerance. The RANDOM line would act as the control group for other potential effects of the artificial life in the lab that may affect thermal tolerance. The performance in CTmin tests were uncertain in all three lines and would be interesting to study.

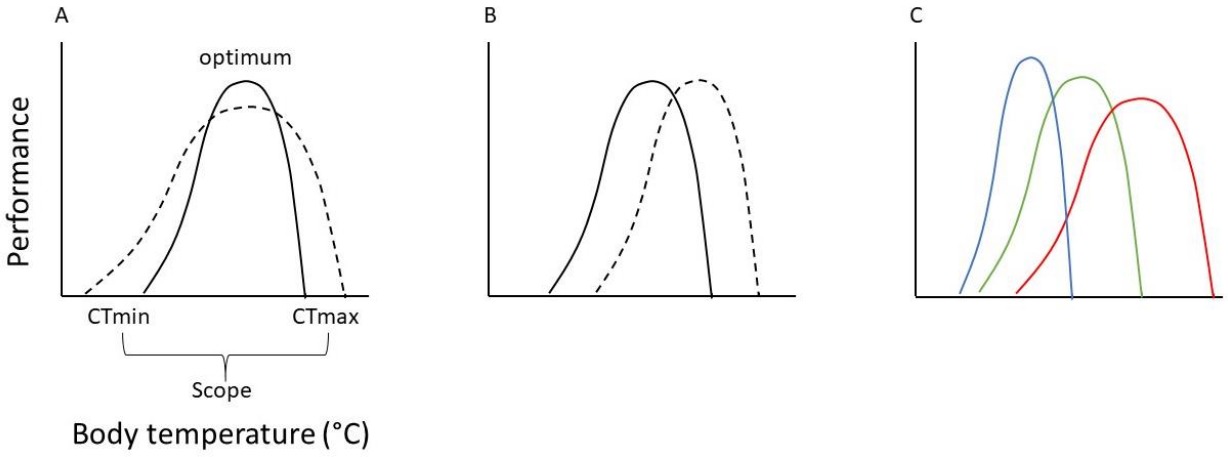


Figure 1. Expected thermal tolerance curve of different lines of F_4 generation zebrafish selected on performance in critical thermal maximum tests. Lower and upper ends of the curves represent the temperature at which the fish lose their body equilibrium, that is, reaching CTmin and CTmax, respectively. Black, solid lines represent unselected trends, dotted and coloured lines show expectations after selection. (**A**) Outcome 1: Wider scope between CTmin and CTmax. (**B**) Outcome 3: Shift in thermal scope towards higher temperatures. (**C**) The study hypothesis: slightly wider scope shifted against CTmax for HIGH (red) and slightly narrower scope shifted against CTmin for LOW (blue), compared to RANDOM (green). The non-selected group of RANDOM control fish is assumed to represent the natural temperature tolerance unaffected by selection. The figure is adapted from Huey & Kingsolver (1993).

2. Methods

2.1 Project and study species

2.1.1 Experiments and ethics

All procedures and experiments were conducted from June to October 2018 in the Jutfelt Ecophysiology lab at Norwegian University of Science and Technology, Trondheim. Experimental design and all practical work were done together with another master student, Tine, with a shared group of zebrafish. Briefly, our projects involved reproduction (six days), tagging and individual measurements (two days), her behaviour experiments (eight days), my CTmin tests (four days) and CTmax tests (three days), as well as pilots and housing. Keeping and experimental procedures of the zebrafish in the NTNU Animal facility were approved by the Norwegian Animal Research Authority (Permit Number: 8578). Handling of the animals were done in compliance with the Regulation on use of animal in research and Norwegian Animal Welfare Act No. 97 2009. Decisions during planning, training and pilots were based on recommendations from trained personnel in the lab and literature related to our techniques and study species, like Lawrence (2007). Risk assessment were drawn in advance. Welfare indicators and score sheets for the fish were defined and considered in the daily log. Experimental procedures are described in part 2.2.

2.1.2 Study population

The study population of zebrafish (*Danio rerio*) was reproduced in the animal facility at NTNU in July 2018 and became the fourth generation of selected lines according to the long-lasting selection experiment in the facility. Their origin is wild-caught zebrafish from India and was brought to Norway in 2016. For every selection event of the three former generations, 33 % of the individuals with highest (termed HIGH) and 33 % of the individuals with lowest (LOW) tolerance in CTmax tests were selected and separately bred and housed. A control line was formed of randomly bred zebrafish (RANDOM). Each line had two replicates: HIGH: H1 and H2, LOW: L1 and L2, RANDOM: R1 and R2. Overview of the selected lines are given in appendix Figure A1.

In our project, we considered 32 fish from each replicate a sufficient number with respect to statistical power. Each replicate was split into two tanks, giving a total of 12 tanks. Two extra fish per tank were included as reserves if any would die during the experiments. This would reduce the effect of eventual incidents or confounding factors. Twelve tanks of 18 fish (36 per line) would

provide a number divisible by eight, a practical consideration as these fish were also used in behaviour assays with a limitation of eight fish per trial.

In the reproduction, a total of 252 fish of the F3 generation served as the parental stock. We grouped three females and three males in each of seven boxes (2.5 L) per replicate. This estimate of 21 fish per sex per line was based on previous experience with reproductive success in the facility. The expected mortality from egg to adult fish were 40 %, so to reach the goal of 216 fish in total, we calculated 60 as a necessary minimum number of individuals per replicate. We wanted to get valid data with as few fish as possible, in accordance with the 3Rs (Replacement, Reduction and Refinement) and animal welfare (Animal Welfare Act 2009). This reproduction was highly successful, with 140 as the lowest number of eggs per replicate.

2.1.3 Housing conditions

The 12 tanks were randomly arranged in the acclimation room (Fig. 2). This tank setup was used to control for potential influencing environmental factors/sources of error such as temperature and light differences at various levels from the floor, distance to heating wall and the door, biased system in the feeding regime and disturbance from noise and movements of personnel.

All fish were maintained at 28.0 ± 0.5 °C with conductivity between 600-1000 S/L on a 14/10 h light/dark cycle. Housing tanks (50x30x30 cm) were filled with 35 litres of water and aerated with filters. Regular cleaning of tanks and filters were done when necessary, and half of the water was replaced per week. The carbon-filtered incoming water was pre-treated with salt and AquaSafe (Tetra®, Blackburg, VA, USA), both of approximate 0.5 dl per 100 L water, and pre-heated in barrels (200 L) with Titanium heaters (TH-100, Aqua Medic, Bissendorf, Germany). Samples of water were regularly tested with JBL Ammonium Test NH4 to ensure that nitrogen levels were safe. Fish were fed with ground flakes of TetraPro Energy (Tetra®, Blacksburg, VA, USA) twice a day and Artemia once per day. Dead fish were removed, and signs of sickness would be considered according to the score sheet. Good water quality and the replicated tank setup would reduce the probability for diseases or accidents that could otherwise make potential harm both fish and experimental results. Number of fish per tank was about 150 from larva stage to the time we randomly grouped and tagged the study population.

Figure 2. Tank setup in the acclimation room. Twelve of the tanks were used for our experimental fish; the six rightmost on the two upper shelves. Two filters in each tank and glass covers on top were adjusted to keep the water aerated and 28.0 ± 0.5 °C. Close-up photo of the front of one tank with updated labels after each treatment or test.

2.1.4 Tagging procedure and individual measurements

All test fish were injected with visible implant elastomer tags (VIE, Nortwest Marine Technologies, Shaw Island, WA, USA) on two spots: left and right side of the dorsal fin. Tagging allowed fish to be individually identified and multiple measurements to be taken on the same individual. Four colours (red, orange, yellow, and green) were matched to get 16 combinations per tank, and the two extra fish got separate colours (blue and pink). Prior to tagging, individual fish were anaesthetised in buffered tricaine methanesulfonate (MS-222) in water (3 ml : 90 ml). Concentration and induction time were adjusted to suit the duration of tagging. At this time, body weight (to the nearest 0.001 g) and standard length (to the nearest 0.01 mm) were measured in all the fish (Fig. A2 in appendix). Survival after tagging was high and fish recovered rapidly. In the very end of the project, length and weight measurements were repeated (Fig. A3 in appendix).

2.2 Experiments

To examine CTmin and CTmax of the zebrafish, the fish were exposed to water temperatures with a constant decrease or increase, respectively, from the acclimation temperature to the endpoint criteria were reached. Based on how core temperatures track the surrounding temperature, 0.3 °C min^{-1} have been used as a suitable rate for critical temperature studies on small fish (Becker & Genoway 1979; Beitinger *et al*. 2000; Recsetar *et al*. 2012; Pang *et al*. 2017; Morgan *et al*. 2018). To keep the method similar to CTmax and thus making it more comparable, 0.3 °C min^{-1} was also used for cooling the fish. This rate has also been used for CTmax and CTmin in, among others, Cortemeglia & Beitinger (2005). As recommended for heating rate (Schulte *et al*. 2011), pilots and test protocols were done on the actual study species, here groups of excess zebrafish. The rate was carefully controlled using a high precision thermometer of accuracy of ±0.1 °C (testo-112, Testo, Lenzkirch, Germany).

The order of the two critical thermal tests were based on results from a pilot. In the pilot experiment, two groups of excess zebrafish were compared: group 1 (n=16) were tested for CTmin before CTmax and group 2 (n=16) in CTmax before CTmin. Another two control groups (n=16 each) were tested in only one of the tests. CTmax was not affected by prior testing of CTmin whereas CTmin was affected by prior testing of CTmax. Therefore, CTmin was chosen to be the first test performed. Quick recovery was observed after CTmin.

The fish was recommended to be fasting 24 hours prior to the tests, as all fish would then be stated as “hungry”. One half of the tank (8 or 9 fish) were run per trial. The observer removed fish as soon as they reached loss of equilibrium criteria, without looking at the thermometer. Another person noted the temperature for each fish’s end point. This removed any observer bias. We used LOE criteria as defined in Cox (1974) and Beitinger *at al*. (2000); disorganized locomotion movements and loss of dorso-ventral righting reflex, and little reaction to potential harmful stimuli. If necessary, the observer could confirm whether the fish had reached LOE criteria or not by touching it with a plastic tube, but a limitation of three pokes per fish were set because this could stress the remaining fish. After each trial, the fish were returned to their housing tanks with updated labels (as in Fig. 2). We tried to minimize stress and biasing environmental factors, which might otherwise disturb the results (Ruxton & Colegrave 2016). Any sick or dead fish were identified.

2.2.1 Critical thermal minimum (CTmin)

The experimental setup for the CTmin tests was developed and improved during a week of pilots in June 2018. From a cooling machine (julabro F18) on the floor, the water was circulating through a water pump (Eheim Universal 300, Deizisau, Germany) up to a plastic tank (27x17x16 cm, 4 L) on the table and drained back down through a plastic pipe (Fig. 3 and 4). The volume of water to reach a declining temperature rate of 0.3 °C min^{-1} were in the pilot found to be approximately 14 litres. If necessary, the observer gently added or removed 1-2 dl of water to the water cooler to stabilize the decline of 0.3 °C min^{-1}. The water was replaced between trials and new fish were introduced once the temperature stabilised at 28 °C. In the water cooler, an air stone was added to promote oxygen saturation, and an interior box of stones kept the surrounding surface above the cooling radiators in the machine. In the experimental box, the fish would be exposed to a constant decreasing water temperature. An expanded end of the tube ensured a smooth flow in the tank and a more homogenous water temperature, with a difference of <0.1 °C measured between incoming and out-streaming water. The thermometer was attached to the wall. The drain, 9 cm from the bottom, was covered with a net and glass plates were placed upon the tank when the fish were inside. To avoid unnecessary stress for the fish, the box was covered with non-transparent painting and a wall separated the experimental tank from the door and barrels of water in the room. After LOE was reached, the fish were quickly transferred into individual selection boxes (16x9x10 cm, 1L) of 28 °C for recovery and identification.

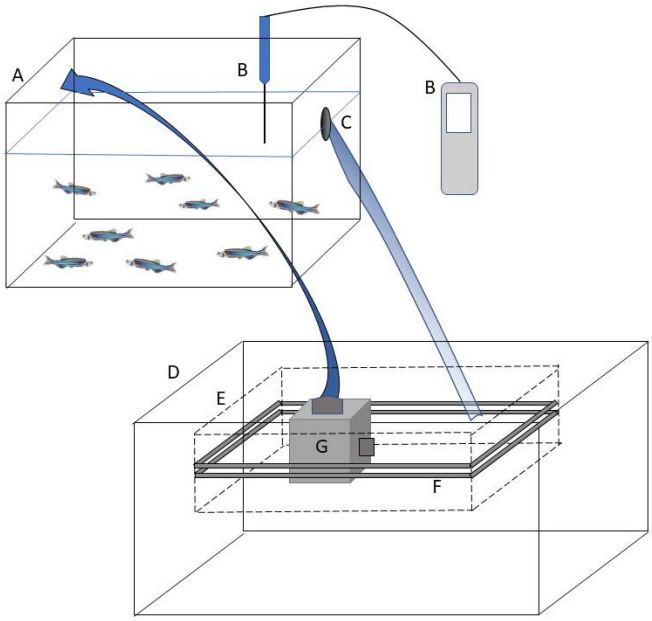


Figure 3. Experimental setup for testing CTmin. (**A**) Experimental box, (**B**) thermometer, (**C**) drain from experimental box to cooler, (**D**) cooler, (**E**) inner container of the cooler filled with water, (**F**) cooling radiators, (**G**) water pump flushing towards the experimental box.

Figure 4. Photo of the experimental setup for testing CTmin, described in figure 3.

2.2.2 Critical thermal maximum (CTmax)

The experimental protocol for CTmax followed that of Morgan *et al*. (2018) using the same lab equipment. Briefly, fish were placed in two heating tanks (25x22x18 cm) filled with 9 L water (Fig. 5 and 6). A mesh separated the fish from the heating system; a 300 W coil heater inside a steel cylinder connected to a water pump (Eheim Universal 300, Deizisau, Germany). Similar to the CTmin tests, the heating rate of 0.3 °C min^{-1} was controlled and eventually adjusted by addition or removal of a 1-2 dl throughout the trial. In contrast to CTmin test, it was possible to run parallel trials of CTmax tests since we used two setups, including sufficient delay in starting time. Per trial, each tank of fish was randomly divided in two (8-9 fish in each) and transferred to the two heating tanks with start water temperature of 28 °C.

Previous experiments have shown that *"*recovery of equilibrium generally occurred within two minutes after CTmax test, and that normal behaviour was regained after approximately five minutes" (Morgan *et al*. 2018). In this experiment, since this was the last test, the fish were put in selection boxes (16x9x10 cm, 1L) filled with ice water to make a humane endpoint. To ensure death had occurred, the fish were kept in ice water in a minimum of 20 minutes before further measurements. Fish identity, weight (to the nearest 0.001 g) and length (to the nearest 0.01 mm) was recorded.

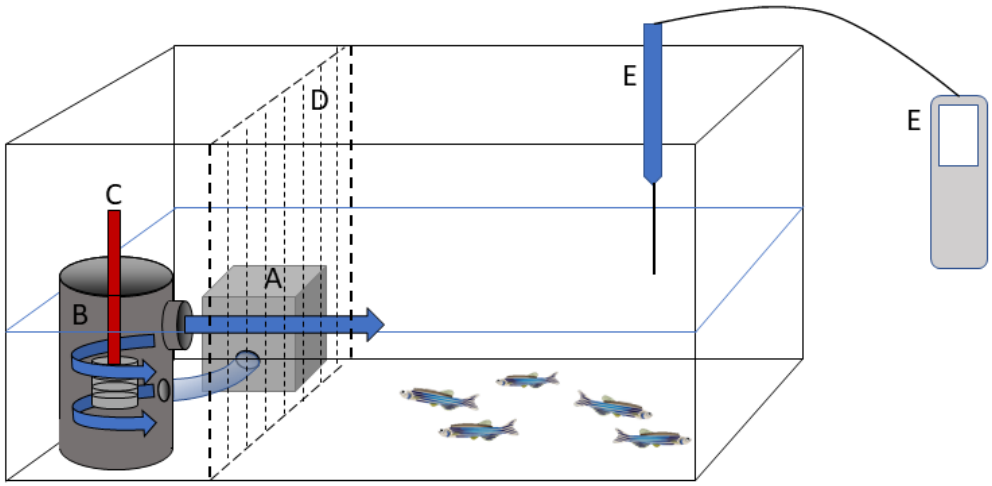


Figure 5. Experimental setup for testing CTmin. (**A**) Water pump, (**B**) steel heating case, (**C**) 300 W coil heater, (**D**) mesh separating the test fish and the heater and pump, (**E**) thermometer. Sketch adapted from figure published in Morgan *et al*. 2018.

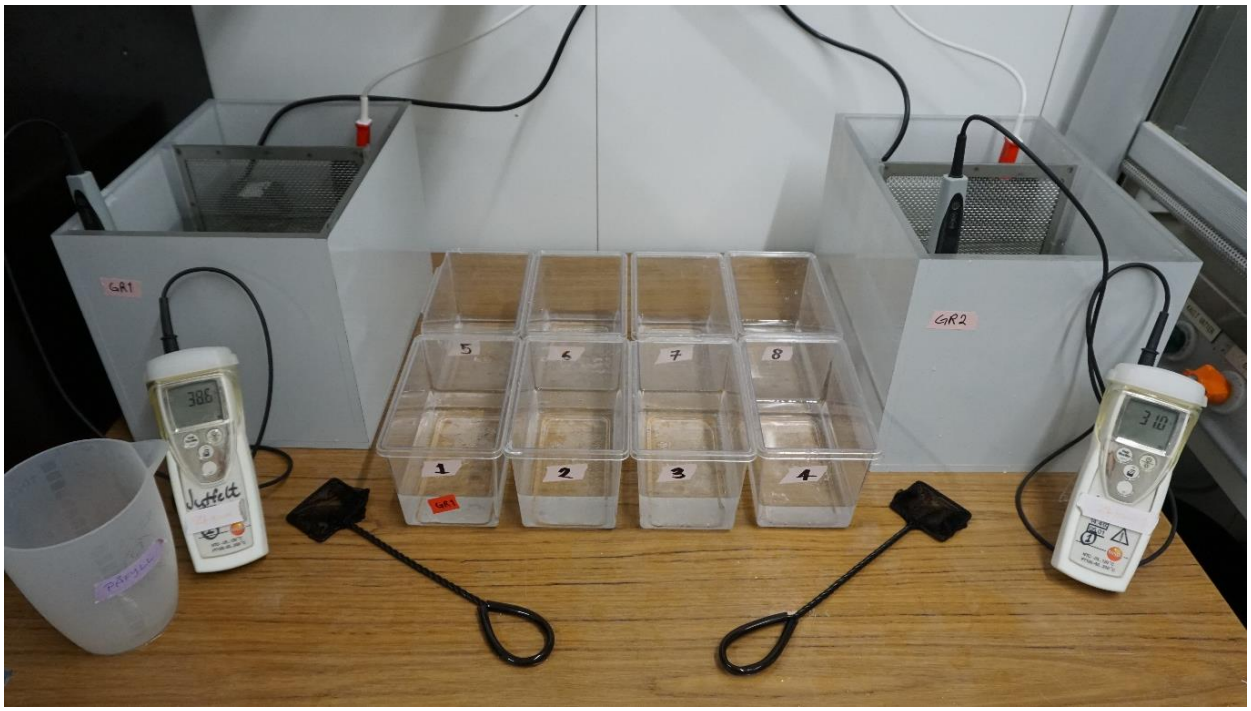


Figure 6. Experimental setup for testing CTmax, described in figure 5. Parallel trials with two setups for CTmax; two set of numbered selection boxes (1 L) with ice water, nets, and thermometers and a water mug.

2.3 Data and statistical analysis

All statistics were conducted in R 3.5.2 (R Core Team, 2018), with $p < 0.05$ as level of significance for all tests. For each fish in the tanks, we got data on CTmax and CTmin, weight (to the nearest 0.001 g) and length (to the nearest 0.01 mm) from measurements during tagging (termed "first") and after humane killing (termed "last"). The CTmin and CTmax tests were conducted in the period 84 to 95 days post fertilization. We included test order of the day from the morning (termed "1") to evening (number "6" or "8" depending on the test) in the dataset. If weight and length were not correlating, the model would include Fulton's condition factor (FCF, equation 1), calculated for the first and the last measurement as FCF_{first} and FCF_{last}, respectively. Specific growth rate (SGR, equation 2) for weight were calculated by filtering number of days from first to last measures (time intervals between 34 and 38 days).

$$FCF = (weight / length^3) \times 100 \quad (1)$$

$$SGR_{weight} = (\ln(weight_{last}) - \ln(weight_{first})) \times 100 / \text{time interval} \quad (2)$$

Strong correlation was observed between length and weight for both first (R=0.94) and last (R=0.87, Fig. A4 in appendix) measurement. Thus, we were free to choose weight or length as indicator of growth. We saw the same pattern in SGR for weight (SGR_{weight}) against $length_{last}$ as in SGR for length (SGR_{length}) against $weight_{last}$ and decided to use SGR based on weight (SGR_{weight}). Regarding weight, the last measured would be closest to the actual weight during the CT tests, and weight was also assumed to be more correctly measured compared to standard length. The weak relationship observed between SGR_{weight} and the last weight measured (Fig. A5 in appendix) validated the use of both these factors in the linear model.

Correlation between CTmax and CTmin for the different lines were tested with Pearson correlation method using the function ggscatter() within the ggpubr package (Kassambara 2017). Distribution of the LOE temperatures in CTmin and CTmax, and thus thermal scope, for each replicate were presented as violin plots using the functions ggproto() and ggplot()+geom_split_violin() from the package ggplot2 (Wickham 2016).

To investigate whether other variables explained or influenced the association between fish lines and tolerance for CTmax and CTmin, linear mixed-effects models (LMER) were fitted using the function lmer() within the packages lmeTest (Kuznetsova *et al*. 2017) and car (Fox *et al*. 2012). Three models were created with CTmin, CTmax and scope as the response variables, respectively. Based on the study question, the selected lines (HIGH, LOW, RANDOM) were the main predictor variables. Five other predictor variables were included in the models: the interaction between line and replicates, SGR_{weight}, $weight_{last}$, and test order of the day, and tank was included as a random effect. In the models, SGR_{weight} and $weight_{last}$ were mean centred by subtracting the means of SGR_{weight} and $weight_{last}$, respectively, from each value. The models were tested using analysis of variance (ANOVA) using Type III Wald chi-square test. Model selection was based upon the AIC value ($\Delta AIC>2$). None of the response variables got pivotally improved AIC values when non-significant predictor variables were excluded, and the full model was used for both CTmax, CTmin and scope to simplify the comparison. Each model was visually checked for normality and homogeneity in the residuals by fitting a residual plot and a QQ-plot using the functions resid(), qqnorm() and qqline().

3. Results

3.1 Correlations between CTmin and CTmax

CTmin negatively correlated with CTmax in all three fish lines (Fig. 7), meaning individuals with a low CTmin had a high CTmax and vice versa. The strongest correlation was observed for line HIGH (R = -0.44, Fig.7A). The line selected on LOW tolerance to CTmax had weak relationship between CTmax and CTmin and many individuals had low CTmax and high CTmin compared to the control group. More of the individuals in the RANDOM control line were located in the upper left part of the plot, meaning they had good performance in both CTmax and CTmin tests.

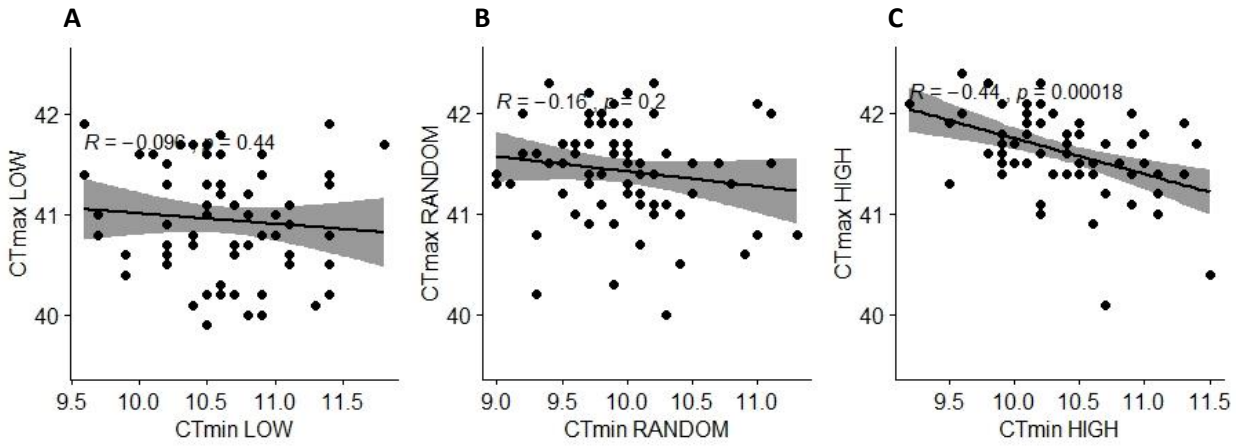


Figure 7. Correlation plots for CTmax against CTmin for the line (**A**) LOW (n=67, R = -0.096), (**B**) RANDOM (n=70, R = -0.16), (**C**) HIGH (n=67, R = -0.44). Regression line fitted by Pearson correlation with 95 % C.I. All test fish were fourth generation zebrafish acclimated to 28 °C. The lines were selected on highest or lowest in CTmax, or randomly selected.

3.2 Effect of selection on thermal tolerance

The distributions of thermal tolerance for all replicates are displayed in the violin plots for CTmax and CTmin (Fig. 8) and for scope (Fig. 9). Means of both replicates of line LOW had lower CTmax, higher CTmin and smaller scope than means of both replicates of HIGH and RANDOM. In addition, CTmax for LOW was more spread than for RANDOM while HIGH was tighter clustered. Same trends were observed among other F_4 generation zebrafish in the lab.

Table 1 shows χ^2- and p-values for the five predictor variables included in the linear mixed-effects models of CTmin, CTmax and scope. As the AIC values of the three models did not improve when the line x replicate interaction was removed, it was included and would count for possible effect of replicate. Table 2 shows mean values for CTmin, CTmax and scope in all replicates after adjusted for predictor variables in the models. There was significant difference between lines in all response variables (Table 1). Line LOW was significantly different from HIGH and RANDOM, but HIGH and RANDOM did not differ significantly (Table 2). There was no significant difference between replicates (Table 2; p-values of replicate 2 compared to replicate 1 within the same line) in CTmin, CTmax or scope, except between R1 and R2 in CTmax. The overall trend for each line, included SE, were: RANDOM had lowest CTmin, intermediate CTmax and widest scope, HIGH had intermediate CTmin, highest CTmax and intermediate scope, and LOW had highest CTmin, lowest CTmax and narrowest scope.

Among the five predictor variables included in the models (in addition to the fish lines), $weight_{last}$ was the only variable with statistically significant coefficients in all three models (Table 1). These coefficients were also greater than those for the other four predictor variables. In estimate of CTmin, the coefficient for $weight_{last}$ was negative, compared to positive in estimates of both CTmax and of scope. The line x replicate interaction and order had no significant effect on none of the response variables. SGR_{weight} had a significant effect on CTmin, but not on CTmax or scope. Compared to observed values, all replicates got scarcely changed estimated values in the model.

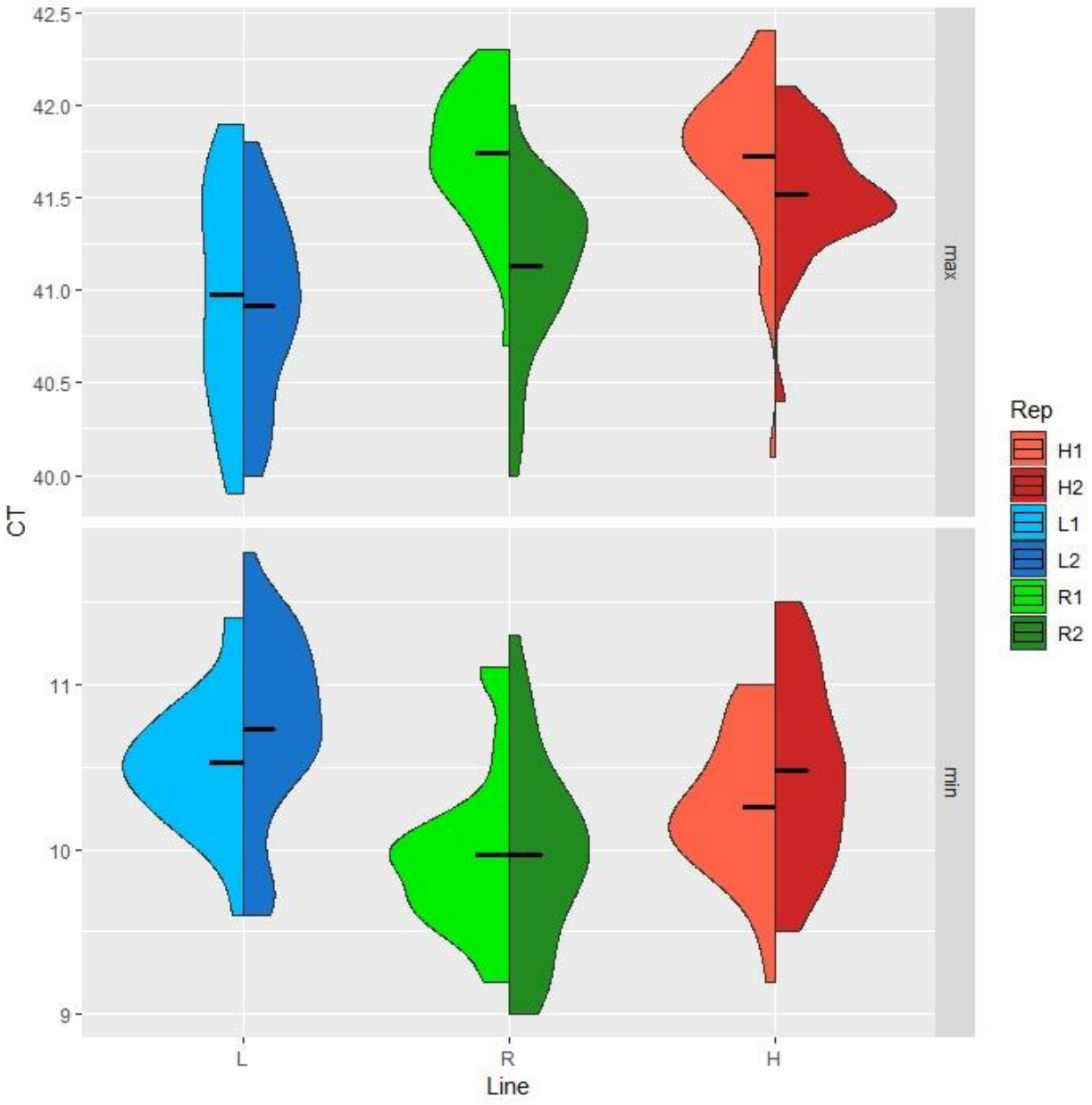


Figure 8. Distribution of critical thermal limits (°C) for zebrafish (n=204) in CTmax and CTmin tests. The lines LOW, RANDOM and HIGH are separated into replicates: L1 (light blue, n=34) and L2 (dark blue, n=33), R1 (light green, n=34) and R2 (dark green, n=36), and H1 (light red, n=35) and H2 (dark red, n=32). Black lines represent the mean of each replicate.

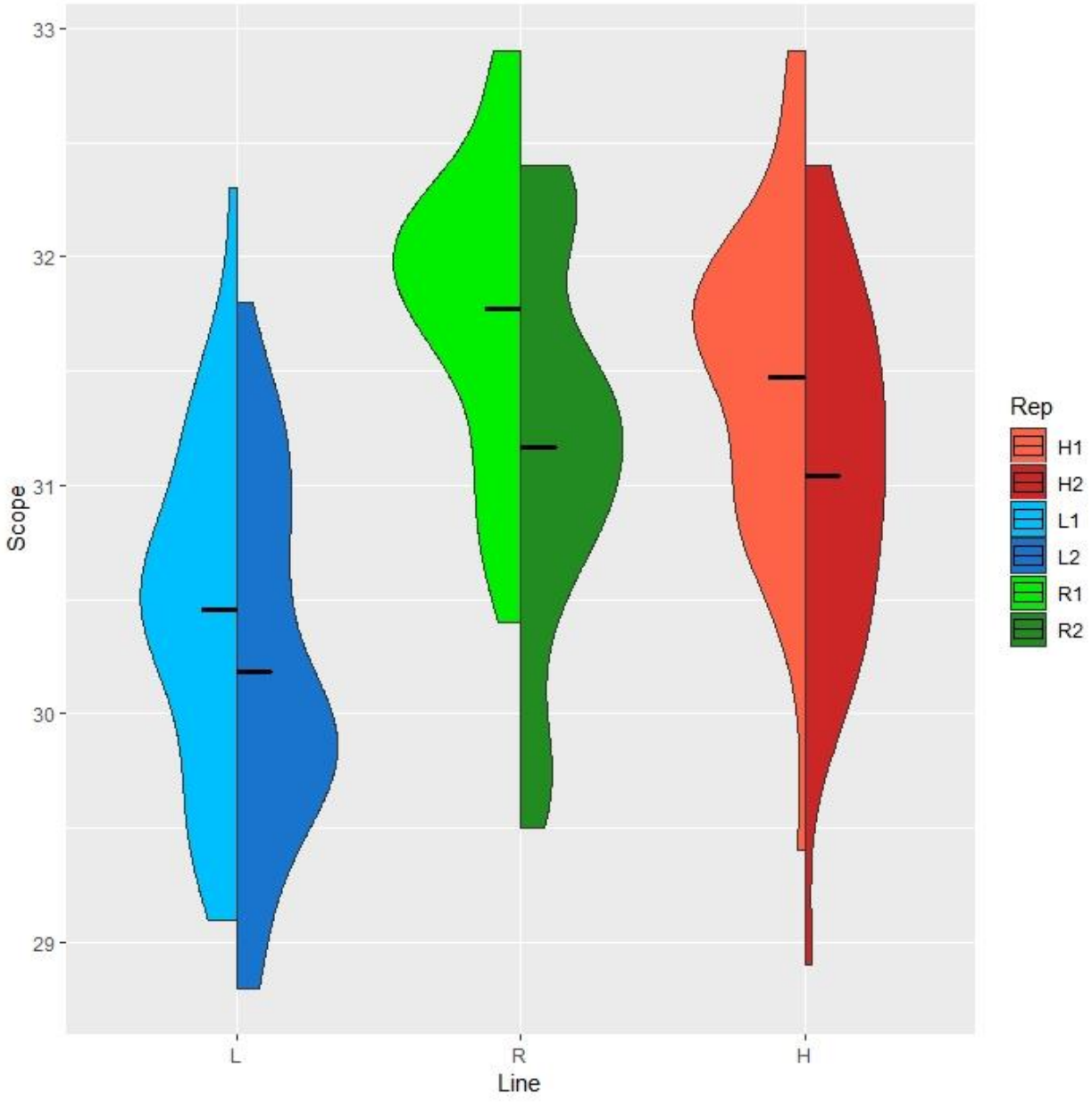


Figure 9. Distribution of scope (°C) between CTmax and CTmin for zebrafish (n=204). The lines LOW, RANDOM and HIGH are separated into replicates: L1 (light blue, n=34) and L2 (dark blue, n=33), R1 (light green, n=34) and R2 (dark green, n=36), and H1 (light red, n=35) and H2 (dark red, n=32). Black lines represent the mean of each replicate.

Table 1: Summary of the predictor variables in the selected models of CTmin, CTmax and scope.

	CTmin		CTmax		Scope	
	χ^2	p	χ^2	p	χ^2	p
Line	3.31	<0.001	38.75	<0.0001	30.95	<0.0001
Order	10.54	0.069	1.66	0.198	0.24	0.627
$Weight_{last}$	7.65	0.001	11.02	<0.001	20.03	<0.0001
SGR_{weight}	1.00	0.006	0.36	0.549	3.04	0.081
Line x rep	1.00	0.606	5.71	0.057	1.04	0.596

Table 2: Acute thermal tolerance (°C) in CTmin and CTmax tests, and thus thermal scope between CTmax and CTmin, in replicates of zebrafish selected on performance in CTmax tests.

	CTmin			**CTmax**			**Scope**		
	Estimated[1]	SE	p	Estimated[1]	SE	p	Estimated[1]	SE	p
R1	9.65	0.195		41.81	0.098		31.89	0.204	
R2	9.74	0.192	0.647	41.29*	0.141	0.022	31.38	0.294	0.145
H1	10.04	0.193	0.108	41.81	0.128	1.000	31.62	0.267	0.359
H2	10.28	0.285	0.438	42.15	0.193	0.152	31.53	0.399	0.830
L1	10.40*	0.188	0.012	41.08**	0.134	0.004	30.44**	0.277	0.003
L2	10.40	0.274	0.992	41.52	0.189	0.083	30.73	0.394	0.504

[1]Estimated in linear mixed-effects models, adjusted for the interaction lines x replicates, SGR_{weight}, $weight_{last}$, and test order of the day. P-values of replicate 2 (e.g. L2) are compared to replicate 1 (e.g. L1) and p-values of replicate 1 (e.g. L1) are compared to R1. Significance codes: * = p<0.05, ** = p<0.01.

3.3 Additional result: the relation between weight and scope

As $weight_{last}$ was the strongest adjusting variable in the analyses of the relation between fish lines and CTmin, CTmax and scope, the relationship between $weight_{last}$ against scope ($p < 0.001$) was plotted (Fig. 10). From the smallest to the largest fish, the scope increased with 1.25 °C per 0.1 g body weight.

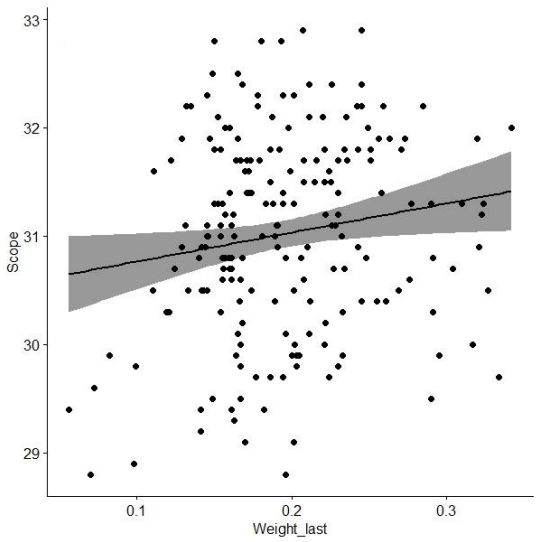


Figure 10. Thermal scope as a function of body weight for all fish (n=204) measured after the final test; CTmax. Scope equals temperature difference between CTmin and CTmax.

4. Discussion

The aim of this study was to examine whether artificial selection on upper thermal tolerance in CTmax tests affects lower thermal tolerance in CTmin tests, and if so, how the thermal scope between CTmin and CTmax would change. Critical thermal tolerance was compared between fourth generation zebrafish which were selected through CTmax tests becoming the lines HIGH, LOW and RANDOM. The most noticeable result was the narrower scope in LOW. LOW had significantly lower CTmax and higher CTmin than RANDOM, whilst HIGH did not differ significantly from RANDOM in any of the tests. Although this generation of selected fish could not confirm the hypothesis that HIGH would show a shift towards a higher thermal window combined with the highest CTmax, a tendency for higher CTmax for HIGH was observable. Moreover, there might be more pronounced differences in CTmax (and in turn CTmin) between the three treatments in later generations of selection. Surprisingly, there was a negative correlation between CTmin and CTmax, meaning that some individuals had wide thermal scope while others had narrow. The following discussion of the observed thermal scopes and correlations between CTmin and CTmax for the three lines will include aspects of thermal history and effect of selection. In addition, genetics and acclimation are major explanatory factors for thermal tolerance (Beitinger *et al*. 2000).

The negative correlation between CTmin and CTmax in the scatter plot and the scope widths in the violin plot present the same trends. They indicate that mechanisms regulating thermal tolerance to extremely cold water are not genetically independent of CTmax. Opposite of what was expected, these results suggest that some fish are high performers while others are low performers in both ends of the thermal tolerance curve. If this is true, it is not surprising that HIGH had the strongest correlation, because this line is grouped by good tolerance in one end of the thermal scope. However, as there was no significant difference on average in scope width between HIGH and RANDOM, it indicates that selection on high performance in CTmax tests does not give an overall advantage regarding critical temperatures. The lack of significant difference between HIGH and RANDOM in both CTmin and CTmax tests, might be because HIGH still have similar “stress-resistant” genotypes as the control group (representing the thermal tolerance in the nature). Huey & Kingsolver (1993) propose two alternative links between genetic variation and thermal scope. If wide scope means large genetic variation, then individuals with broad thermal range would be

best suited to survive environmental changes. In this case, HIGH is better suited than LOW. If there is a trade-off between scope and CTmax, then individuals that tolerate extreme temperatures will overcome thermal generalists if heat waves occur. Thus, because LOW had the narrowest scope and was significantly different from HIGH in CTmax, LOW might have less genetic variation and lower ability to tolerate acute warming compared to the two other lines.

Selection on warm or cold tolerance might create lines with shifted thermal scopes along the temperature scale (Huey & Kingsolver 1993). It is important to remember that fish in the LOW line were not selected on cold tolerance, but on low performance in upper thermal tolerance tests. The narrow scope for LOW is an informative representation of the effect of artificial selection in this lab. The results speak against the hypothesis that LOW has genes advantageous for cold tolerance as a compensation for missing tolerance to warm. It is more likely that some genes affect both CTmax and CTmin. LOW showed other poor characteristics as well, like poor fecundity. Selection on one trait can be followed by changes in expression of other traits (Kern *et al*. 2016). In nature, poor characteristics could be selected against and decrease in frequency over generations for the benefit of genes involved in higher performance. This could be why RANDOM had a high performance and equal thermal tolerance as HIGH; any low performers have been selected against. The artificial selection of the zebrafish might have created shifts in thermal scope that would not occur over four generations in the wild.

Most studies of thermal tolerance use one generation wild-caught fish with some pre-experimental time in the laboratory for acclimation or thermal regimes (Schaefer & Ryan 2006). In contrast to these, an important advantage of the present study is more information about the fishes' thermal history. Among a limited number of studies investigating the effect of artificial selection on thermal stress in ectotherms, Baer & Travis (2000) tested eight generations of least killifish (*Heterandria formosa*). Surprisingly, they observed no increase in CTmax despite the strong directional selection on lines of heat and cold tolerance in this freshwater fish. Similarly, the F_4 generation of the line specifically selected on high CTmax were not significantly different from the control line but could eventually diverge from the two other lines in both CTmax and CTmin tests in the following selected generations. Studies of *Drosophila* (Huey & Kingsolver 1993) found rapid response in thermal sensitivity to selection. Based on the trends observed in the zebrafish

tested in this study, it is likely that the line of low performers would give the most pronounced response to selection.

In the present study, weight (strongly correlated with standard length, Fig. A4) had a strong effect on CTmin, CTmax and scope, and had a positive relation when plotted against scope. A positive effect of body length on CTmax was also found in Moyano *et al*. (2017) testing seabass larvae. Opsina & Mora (2004), however, found no relationship between body size and thermal tolerance when testing a mix of juveniles and adults of different reef fish species. Morgan *et al*. (2018) tested adult zebrafish in CTmax and saw no effect of weight. Sexual maturation, which happens approximately at three months age when housed in 28.5 °C (Singleman & Holtzman 2014), could possibly explain the difference between their results and what is presented in this study. These F_4 zebrafish were still immature. Ho & Burggren (2012) ran CTmin and CTmax tests on zebrafish and showed increased thermal scope from 20 to 60 days post fertilization when they presented the effects of hypoxia. This suggests that thermal scope depends partly on the body size, which increases with age. The positive relation between weight and scope found in F_4 could disappear after maturation. Still, ss weight was a strong adjusting variable in the mixed-effects models, it should definitely be considered a potential factor affecting thermal tolerance.

Studies on different species have shown variation in both CTmin and CTmax with different acclimation temperatures (Currie *et al*. 1998; Fangue *et al*. 2006; Tongnunui *et al*. 2017). Increased acclimation temperature is found to increase CTmax (Beitinger *et al*. 2000; López-Olmeda & Sánchez-Vázquez 2011) but is often disadvantageous for the thermal scope. In this study the fish were acclimated to 28 °C which is closer to the upper end of their thermal scope (42 °C) than their lower end (10 °C). The results show changes in LOW selected fish but not in HIGH selected fish compared to RANDOM. A possible explanation could be that the HIGH and RANDOM lines had reached a so-called 'physiological ceiling' and the only possible flexibility was downwards. An idea of 'plastic floor, concrete ceiling' was raised by Sandblom *et al*. (2016) testing European perch (*Perca fluviatilis*). They showed a natural tendency of decreased ranges between resting and maximum levels of physiological parameters (e.g. oxygen consumption, cardiac output and heart rate) when exposed to acute heating. During long-term warming, however, the fish tended to acclimate to the environment by lowering the basal energy requirements. This suggests

eurythermal fish can increase their physiological range, although lethal upper temperatures may scarcely be elevated. Cortemeglia & Beitinger (2005) suggested that zebrafish can get even lower CTmin when acclimated to cold water than high CTmax when acclimated to warm water. Selection for high performance in CTmin tests could hypothetically be more distinct from the control group than HIGH was distinct from RANDOM in this study.

For the present study population, housing temperatures were kept at 28 ± 0.1 °C, categorized as constant. However, the zebrafish could have experienced small fluctuations in water temperatures during transport between rooms and waiting period in transport tanks. In addition, the fact that the CTmax tests were conducted in the very end of a series of experiments might have influenced the critical thermal values. In two behaviour experiments, the fish were kept in assay tanks of 26 and 30 °C, each of 40 minutes. Episodes of acute thermal extremes can increase thermal tolerance in subsequent tests (Sidhu *et al*. 2014; Kingsolver *et al*. 2016; Morgan *et al*. 2018), among other factors, due to increased production of heat shock proteins and changed saturation of fatty acids beneficial for protecting the cells in extreme temperatures (reviewed by Hoffmann *et al*. 2003). The study by López-Olmeda & Sánchez-Vázquez (2011) comparing constant and variable maintenance temperature for zebrafish found clear effect of acclimation temperature on upper thermal tolerance, and also points at the influence of temperature conditions through the whole fish's life. They showed improved thermal tolerance linked to variable temperature, and same results for fish maintained in cycles and in stochastic regime within 28 ± 6 °C. Common garden experiments by Shaefer & Ryan (2006) also highlight the importance of thermal history when thermal tolerance is evaluated. As HIGH, LOW and RANDOM got the same treatments prior to and in the experiments, these potential effects on thermal tolerance should not have affect the observed tendencies in this study.

Method evaluation

The developed method for testing CTmin was based on recommendations for CTmax protocols. As there were limited literature confirming the effect of 0.3 °C min^{-1} cooling rate and the direct transference back to acclimation temperature of 28 °C, the physical conditions observed during the pilot were important for these decisions. Contrary to the CTmax tests, as the water cooled in CTmin, the fish began to swim slower or even stopped. To confirm LOE, the observer had to touch

the fish to see whether it reacted and continued swimming or had lost the righting reflex and could be picked out. For both CTmin and CTmax tests, observer bias could have occurred, but this was hopefully reduced by training and by the fact that the observer could not look at the temperature while evaluating the fish. The performance of HIGH, LOW and RANDOM in the CTmax test were close to what were measured for other F_4 fish in the lab, confirming that the method was implemented correctly and making the results from the developed, similarly conducted CTmin test more trustworthy. A minimum of seven days between the CTmin and CTmax trials was set to allow the fish to recover and return to 28 °C acclimation state.

Concluding remarks

This study showed that selection on acute thermal tolerance to high temperatures affects the ability to tolerate cold. Regarding thermal scope between CTmin and CTmax, directional selection for CTmax seems not to be beneficial, rather the opposite, as line HIGH expressed a slight shift upwards in their thermal window, and line LOW had significantly narrower scope compared to RANDOM. Individuals with a high thermal tolerance appear to be generally high performers and low thermal tolerance low performers. Among the selected lines, HIGH would be better suited to cope with climate change but may already have reached their upper limit. LOW performers in CTmax tests did not show improved CTmin compared to RANDOM and HIGH, which contradicts the hypothesis that this line inherit genes advantageous for cold tolerance instead. Zebrafish can live in a broad range of water temperatures and survive in seasonal fluctuations. However, heat waves and global warming can cause major thermal stress and eventually natural selection on the individuals with genes promoting thermal tolerance.

References

Animal Welfare Act (2009). Dyrevelferdsloven (2009-06-19, No. 97). Available on https://www.regjeringen.no/en/dokumenter/animal-welfare-act/id571188/.

Baer, C. F., & Travis, J. (2000). Direct and correlated responses to artificial selection on acute thermal stress tolerance in a livebearing fish. *Evolution*, *54*(1), 238-244.

Beitinger, T. L., Bennett, W. A., & McCauley, R. W. (2000). Temperature tolerances of North American freshwater fishes exposed to dynamic changes in temperature. *Environmental biology of fishes*, *58*(3), 237-275.

Bennett, W. A., & Beitinger, T. L. (1997). Temperature tolerance of the sheepshead minnow, Cyprinodon variegatus. *Copeia*, 77-87.

Becker, C. D., & Genoway, R. G. (1979). Evaluation of the critical thermal maximum for determining thermal tolerance of freshwater fish. *Environmental Biology of Fishes*, *4*(3), 245-256.

Bozinovic, F., & Pörtner, H. O. (2015). Physiological ecology meets climate change. *Ecology and evolution*, *5*(5), 1025-1030.

Brett, J. R. (1956). Some principles in the thermal requirements of fishes. *The Quarterly Review of Biology*, *31*(2), 75-87.

Brett, J. R. (1971). Energetic responses of salmon to temperature. A study of some thermal relations in the physiology and freshwater ecology of sockeye salmon (Oncorhynchus nerkd). *American zoologist*, *11*(1), 99-113.

Cortemeglia, C., & Beitinger, T. L. (2005). Temperature tolerances of wild-type and red transgenic zebra danios. *Transactions of the American Fisheries Society*, *134*(6), 1431-1437.

Cowles, R. B., & Bogert, C. M. (1944). A preliminary study of the thermal requirements of desert reptiles. *Iguana*, *83*, 53.

Cox, D. K. (1974). Effects of three heating rates on the critical thermal maximum of bluegill. *Thermal ecology*, 158-163.

Crawshaw, L.I. & Podrabsky, J.E. (2011). Temperature Preference: Behavioural Responses to Temperature in Fishes.

Currie, R. J., Bennett, W. A., & Beitinger, T. L. (1998). Critical thermal minima and maxima of three freshwater game-fish species acclimated to constant temperatures. *Environmental Biology of fishes*, *51*(2), 187-200.

Engeszer, R. E., Patterson, L. B., Rao, A. A., & Parichy, D. M. (2007). Zebrafish in the wild: a review of natural history and new notes from the field. *Zebrafish*, *4*(1), 21-40.

Fangue, N. A., Hofmeister, M., & Schulte, P. M. (2006). Intraspecific variation in thermal tolerance and heat shock protein gene expression in common killifish, Fundulus heteroclitus. *Journal of Experimental Biology*, *209*(15), 2859-2872.

Fox, J., Weisberg, S., Adler, D., Bates, D., Baud-Bovy, G., Ellison, S., ... & Heiberger, R. (2012). Package 'car'. *Vienna: R Foundation for Statistical Computing*.

Hoffmann, A. A., Sørensen, J. G., & Loeschcke, V. (2003). Adaptation of Drosophila to temperature extremes: bringing together quantitative and molecular approaches. *Journal of thermal Biology*, *28*(3), 175-216.

Ho, D. H., & Burggren, W. W. (2012). Parental hypoxic exposure confers offspring hypoxia resistance in zebrafish (Danio rerio). *Journal of Experimental Biology*, *215*(23), 4208-4216.

Huey, R. B., & Kingsolver, J. G. (1993). Evolution of resistance to high temperature in ectotherms. *The American Naturalist*, *142*, S21-S46.

IPCC 2013. *Climate change 2013: The Physical Science Basis* (eds Stocker, T. *et al*.). Cambridge University Press.

Kassambara, A. (2017). ggpubr: "ggplot2" based publication ready plots. *R package version 0.1*, *6*.

Kern, E. M., Robinson, D., Gass, E., Godwin, J., & Langerhans, R. B. (2016). **Correlated evolution** of personality, morphology and performance. *Animal behaviour*, *117*, 79-86.

Kingsolver, J. G., MacLean, H. J., Goddin, S. B., & Augustine, K. E. (2016). Plasticity of upper thermal limits to acute and chronic temperature variation in Manduca sexta larvae. *Journal of Experimental Biology*, *219*(9), 1290-1294.

Kuznetsova, A., Brockhoff, P. B., & Christensen, R. H. B. (2017). lmerTest package: tests in linear mixed effects models. *Journal of Statistical Software*, *82*(13).

Lawrence, C. (2007). The husbandry of zebrafish (*Danio rerio*): a review. *Aquaculture*, *269*(1-4), 1-20.

López-Olmeda, J. F., & Sánchez-Vázquez, F. J. (2011). Thermal biology of zebrafish (Danio rerio). *Journal of Thermal Biology*, *36*(2), 91-104.

Morgan, R., Finnøen, M. H., & Jutfelt, F. (2018). CT max is repeatable and doesn't reduce growth in zebrafish. *Scientific reports*, *8*(1), 7099.

Moyano, M., Candebat, C., Ruhbaum, Y., Álvarez-Fernández, S., Claireaux, G., Zambonino-Infante, J. L., & Peck, M. A. (2017). Effects of warming rate, acclimation temperature and ontogeny on the critical thermal maximum of temperate marine fish larvae. *PloS one*, *12*(7), e0179928.

Ospina, A. F., & Mora, C. (2004). Effect of body size on reef fish tolerance to extreme low and high temperatures. *Environmental Biology of Fishes*, *70*(4), 339-343.

Pang, X., Fu, S. J., & Zhang, Y. G. (2017). Individual variation in metabolism and thermal tolerance in juvenile qingbo (Spinibarbus sinensis). *Marine and freshwater behaviour and physiology*, *50*(5-6), 359-373.

R Core Team (2018). R: A language and environment for statistical computing: R Foundation for Statistical Computing; Vienna, Austria.

Recsetar, M. S., Zeigler, M. P., Ward, D. L., Bonar, S. A., & Caldwell, C. A. (2012). Relationship between fish size and upper thermal tolerance. *Transactions of the American Fisheries Society*, *141*(6), 1433-1438.

Regulation on the use of animals in research. (2015). Forskrift om bruk av dyr i forsøk (FOR-2015-06-18-761). Available on: https://lovdata.no/dokument/SF/forskrift/2015-06-18-761.

Research Council of Norway (2009). Fish in research – environmental requirements and welfare indicators for fish. A review of research needs.

Rey, S., Digka, N., & MacKenzie, S. (2015). Animal personality relates to thermal preference in wild-type zebrafish, Danio rerio. *Zebrafish*, *12*(3), 243-249.

Ruxton, G., & Colegrave, N. (2016). *Experimental design for the life sciences*. Oxford University Press.

Sandblom, E., Clark, T. D., Gräns, A., Ekström, A., Brijs, J., Sundström, L. F., Oldeström, A., Adill, A., Aho, T. & Jutfelt, F. (2016). Physiological constraints to climate warming in fish follow principles of plastic floors and concrete ceilings. *Nature communications*, *7*, 11447.

Sarasamma, S., Varikkodan, M. M., Liang, S. T., Lin, Y. C., Wang, W. P., & Hsiao, C. D. (2017). Zebrafish: A premier vertebrate model for biomedical research in Indian scenario. *Zebrafish*, *14*(6), 589-605.

Schaefer, J., & Ryan, A. (2006). Developmental plasticity in the thermal tolerance of zebrafish Danio rerio. *Journal of fish biology*, *69*(3), 722-734.

Schulte, P. M., Healy, T. M., & Fangue, N. A. (2011). Thermal performance curves, phenotypic plasticity, and the time scales of temperature exposure. *Integrative and comparative biology*, *51*(5), 691-702.

Seneviratne, S. I., Donat, M. G., Mueller, B., & Alexander, L. V. (2014). No pause in the increase of hot temperature extremes. *Nature Climate Change*, *4*(3), 161.

Sidhu, R., Anttila, K., & Farrell, A. P. (2014). Upper thermal tolerance of closely related Danio species. *Journal of fish biology*, *84*(4), 982-995.

Singleman, C., & Holtzman, N. G. (2014). Growth and maturation in the zebrafish, Danio: a staging tool for teaching and research. *Zebrafish*, *11*(4), 396-406.

Spence, R., Fatema, M. K., Ellis, S., Ahmed, Z. F., & Smith, C. (2007). Diet, growth and recruitment of wild zebrafish in Bangladesh. *Journal of Fish Biology*, *71*(1), 304-309.

Spence, R., Gerlach, G., Lawrence, C., & Smith, C. (2008). The behaviour and ecology of the zebrafish, Danio rerio. *Biological reviews*, *83*(1), 13-34.

Tongnunui, S. & Beamish, F.W.H. (2017). Critical Thermal Maximum, Temperature Acclimation and Climate Effects on Thai Freshwater Fishes. *EnvironmentAsia*, *10*(1), 109-117.

Wickham, H. (2016). *ggplot2: elegant graphics for data analysis*. Springer

Appendix 1: Basis for the selected lines of zebrafish

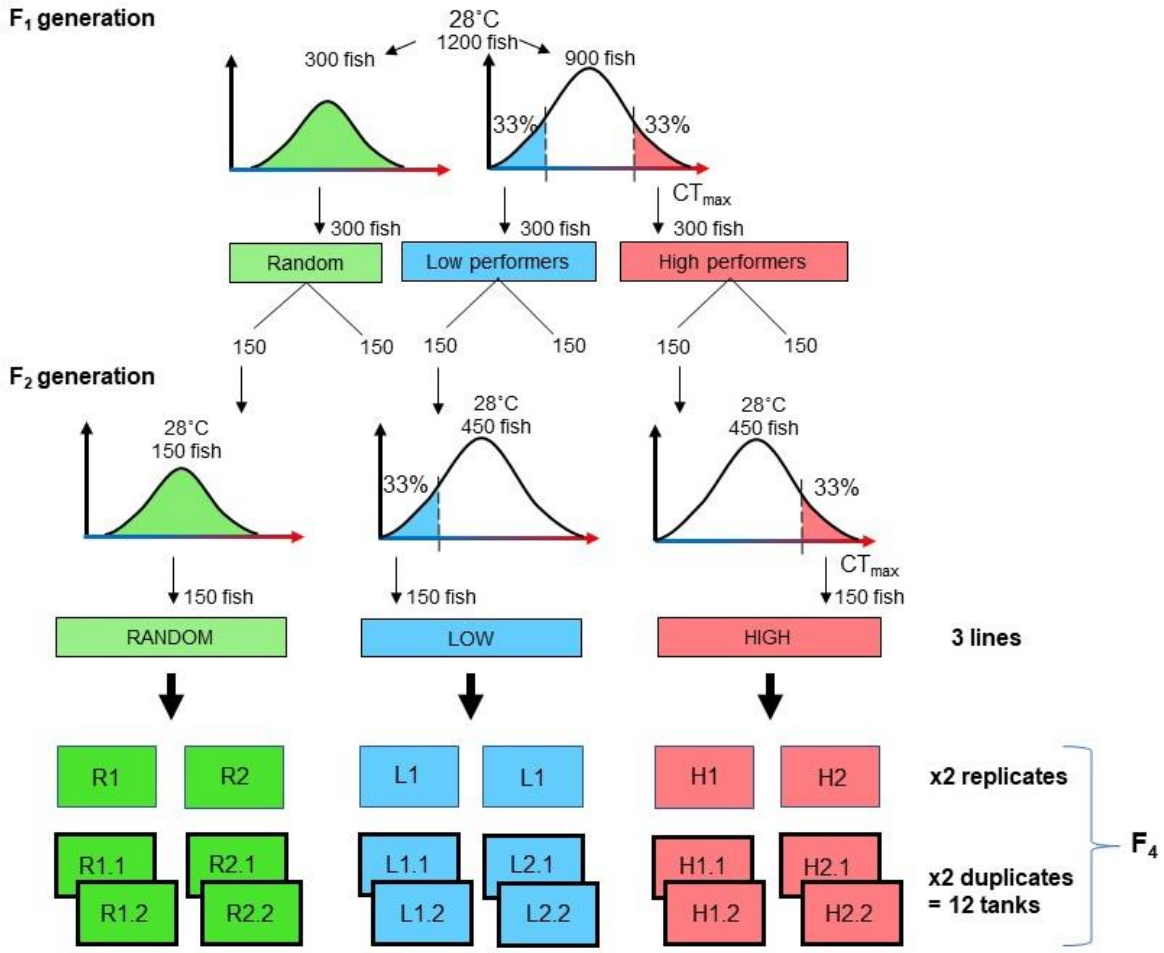


Figure A1. Design of CTmax selection experiment for the two first generations of zebrafish reproduced in the Jutfelt ecophysiology lab at NTNU Trondheim. Same selection design of lines (RANDOM, LOW and HIGH) was carried out between each reproduction. Each line was divided into two replicates in the selection. The fish used in the present experiment were fourth generation (F_4) of all replicates, each reproduced and split into two tanks, separately housed in the acclimation room. Figure adapted from Rachael Morgan.

Appendix 2: Time schedule for the project

June:

- Plan the experiments. Start writing the master description.
- Read literature about the tests and study species. Learn a lot!
- Find out what is needed for the reproduction and general fish care during all parts.
- Pilot on CTmin and CTmax. *6 days*.
- Prepare equipment and aquaria.

July:

- Reproduction. *6 days*.
- Watch the eggs and larvae carefully during the first weeks.
- Set up the aquaria room and move larvae to these new tanks.

August – October:

- Make lists and detailed plans based on the learning period in the lab.
- Tagging and weighing. *4 days*.
- Pilot on behavioural experiments. *2 days*.
- Behaviour experiments. *8 days*.
- Do a pre-test to find out whether undergoing CTmin before the CTmax test will affect the results of the last test, and vice versa. *2 days*.
- Physiological experiments. *7 days*.

November 2018 – June 2019:

- Analyse the data.
- Write the master thesis.
- Present the results to the physiology group.

Appendix 3: Reproduction protocol

Table A1. Steps in the reproduction

Day	Procedure	Mark boxes/tank with
1	Separate males and females. Couple them up 3♀+3♂ in prepared selection boxes. Line the boxes up in the shelf. If not easy to decide whether it is a ♀or ♂, put in "random box" and eventually check after some days.	"H1 date". If not 3♀+3♂ available, write #♀+#♂ on the boxes.
2	Check for eggs in yesterday's boxes. Remove mesh. Keep the water level high in the boxes without cover.	
3	Wash eggs: pour the box water through a fine mesh so the eggs remain. Pour clean water over them from the same side, and then from the other side of the sieve so the eggs + ca. 3 cm water fill new boxes.	"W" (washed)
4	Separate (pipette) fertilized eggs and larvae over to new boxes with 3 cm clean water. Try to count individuals. Round down to the nearest 5, ex. 27→25, as it is expected some reduction in number.	"S" (separated) Number of fertilized eggs/larvae
5-6	When the larvae are swimming, increase the volume almost to the top and cover with cap. Start feed with larvae food and add fine filters. If very few larvae in boxes, it is possible to combine groups within the same treatment. If space enough, keep as many boxes as possible with respect to the diversity. Remove dead larvae.	
10+	Move fish larvae into aquaria in zebrafish room 2, with small amount of water (about 5 cm). Try to gather approximate equal numbers and diversity in each tank. Number of fish per aquaria: 100-300. Remove dead larvae.	Treatment + replicate "H11", "H12".. Number of larvae
~20	Fill the aquaria almost to top.	

Appendix 4: Pictures of tagging and individual measurements

Figure A2. Equipment for tagging and measurements of weight and length used on individual zebrafish. From left to right: buffered MS-222, aerated boxes (2.5 L) for pre- and post-tagged fish, sponge for holding fish during tagging, syringes with ink of six different colours, notes, scales for weight and length.

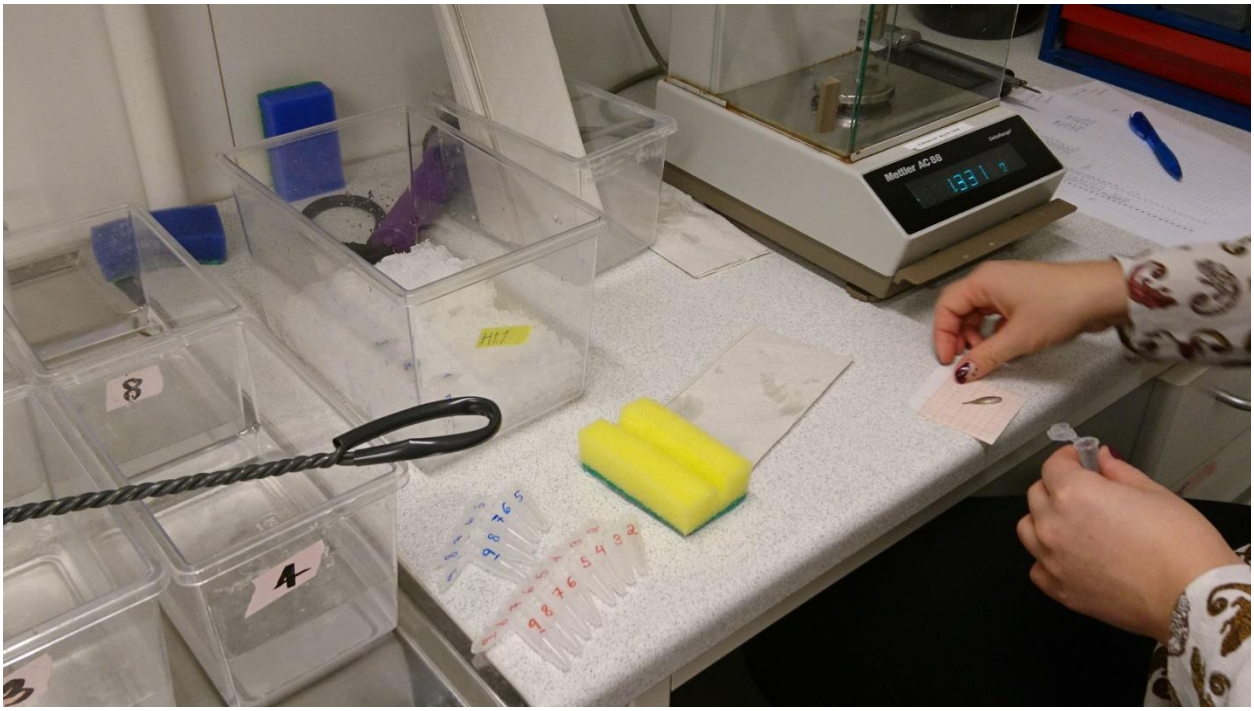


Figure A3. Equipment used for measuring weight and length of zebrafish. From left to right: numbered boxes (1 L) and Eppendorf tubes for individual zebrafish after CTmax-tests, box (2.5 L) with ice to keep the fish cold, UV flashlight, scales for weight and length, notes.

Appendix 5: Validation of factors for the model

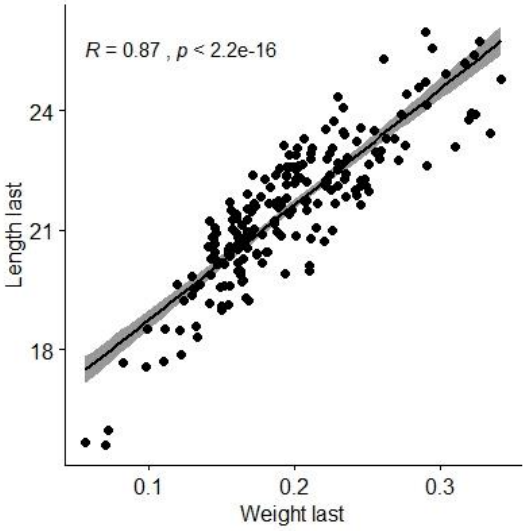


Figure A4. Correlation between length and weight for the last measurement of all fish (n=204) 90-95 days post fertilization. Regression line fitted by Pearson's with r = 0.87.

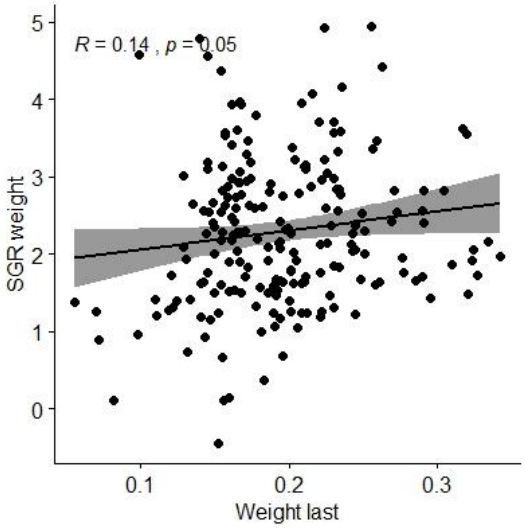


Figure A5. Specific growth rate as a function of weight for the last measurement of all fish (n=204) 90-95 days post fertilization. Regression line fitted by Pearson's with r = 0.14.