

Jonas Arntsen

# Thermal preference in both temperature acclimated and thermal tolerance-selected zebrafish (*Danio rerio*)

Master's thesis in Biology

Supervisor: Fredrik Jutfelt

Co-supervisor: Anna H. Andreassen

July 2021



Jonas Arntsen

**Thermal preference in both  
temperature acclimated and thermal  
tolerance-selected zebrafish (*Danio  
rerio*)**

Master's thesis in Biology  
Supervisor: Fredrik Jutfelt  
Co-supervisor: Anna H. Andreassen  
July 2021

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



## **Acknowledgment**

I would like to thank my main supervisor Fredrik Jutfelt for guidance and support in both writing working on my master's thesis as well as bouldering. I would also like to thank my "co"-supervisor Anna H. Andreassen for acting as my main supervisor when Fredrik was out of country. It would not have been possible to complete my master's thesis without their support. In addition, I would like to thank Mette Finnøyen for giving me much needed feedback on my writing. Lastly, I would like to thank my friends and family for providing me with all their support during these past two years



## Abstract

The severity and frequency of stochastic heating events are currently increasing as the climate is changing, making the ability to cope with extreme temperatures more essential. Ectotherms' physiological functions are highly affected by their surrounding temperature and behavioural thermoregulation is therefore perhaps the most important coping mechanism for ectotherms in response to temperature. In this study we performed two experiments to test how thermal preference is affected by selection for thermal tolerance (critical thermal maxima,  $CT_{max}$ ) and how thermal acclimation affects thermal preference. The selection experiment consisted of zebrafish (*Danio rerio*) from a wild-caught population that had been reared for seven generations by selecting the individuals with the 33% highest  $CT_{max}$  from each generation (Up-selected treatment) and the individuals with the 33% lowest  $CT_{max}$  from each generation (Down-selected treatment). In the acclimation experiment, zebrafish acclimated to three different temperatures (Cold; 20°C, Optimum (control); 28 °C, Warm; 34°C) were used. Utilizing a custom-built annular arena, individuals were able to swim freely in a range of temperatures during a trial. Continuous videorecording as well as temperature logging through the use of 24 thermocouples were analyzed using an automated tracking software that gave data on individual temperature preference. To only analyze data from individuals expressing an active thermal preference, interquartile range of temperature was compared for all individuals and a threshold was set to remove individuals not showing a thermal preference. There was no significant effect of neither selection nor acclimation on thermal preference. This indicates that conservational efforts could be necessary as zebrafish's thermal preference, and thus their optimum, are not evolving with an increased thermal tolerance. We recommend performing another thermal acclimation experiment on zebrafish as previous experiments on thermal acclimation have given varied results in addition to our results showing a positive trend in response to thermal acclimation.





## Sammendrag

Det forventes at klimaendringer vil føre til en økning i plutselige varmeøkninger noe som gjør det viktig å ha evnen til å tilpasse seg det nye miljøet. Ektoterme arter sine fysiologiske funksjoner blir svært påvirket av deres eksterne temperaturer noe som gjør adferdsbasert termoregulering til kanskje den viktigste temperaturresponsen deres. I dette studiet har vi utført to eksperimenter hvor vi har testet hvordan temperaturepreferanse påvirkes av seleksjon på økt varmetoleranse (critical thermal maxima,  $CT_{max}$ ) og hvordan den påvirkes av akklimatisering. I seleksjonseksperimentet ble det brukt sebrafisk (*Danio rerio*) fra en villpopulasjon som i fangenskap har blitt selektert i åtte generasjoner ved å avle individene med 33% høyest  $CT_{max}$  med hverandre i hver generasjon (opp-selektert), samtidig som individene med 33% lavest  $CT_{max}$  ble avlet på hverandre i hver generasjon (ned-selektert). I akklimatiseringseksperimentet ble sebrafisk akklimatisert til tre ulike temperaturer (Kald; 20°C, Optimal (kontroll); 28 °C, Varm; 34°C). En spesiallaget, ringformet, tank ble brukt slik at individene fritt kunne velge hvilke temperaturer å oppholde seg i under forsøket. Fiskene ble kontinuerlig filmet og temperaturen ble kontinuerlig logget ved bruk av 24 thermocouples. Kun individer som gjorde et aktivt temperaturvalg ble brukt i analyser. Interquartile range ble brukt for å sammenligne aktiviteten til de testede individene for å fjerne de individene med høy interquartile range fra analysene. Det var ingen signifikant effekt av hverken seleksjon eller akklimatisering på temperaturpreferanse, noe som kan tyde på at bevaring vil være nødvendig siden sebrafisk sin temperaturpreferanse ikke utvikles med temperaturtoleranse. Vi anbefaler at det blir gjennomført et nytt akklimatiseringseksperiment på sebrafisk da resultatene varierte veldig, i tillegg til at vi kunne se en generell trend i responsen til temperaturakklimatisering.

# Table of contents

<b>Acknowledgment</b> .....	I
<b>Abstract</b> .....	III
<b>Sammendrag</b> .....	V
<b>Introduction</b> .....	1
<b>Methods</b> .....	6
Selection for Down-selected, Up-selected and Control treatments.....	6
Critical thermal maxima .....	6
Experimental preparations .....	7
Experimental setup.....	8
Selection experiment, protocol .....	10
Acclimation experiment .....	11
Analyses and data-collection .....	12
<b>Results</b> .....	14
CT <sub>max</sub> selection experiment .....	14
Acclimation experiment .....	16
<b>Discussion</b> .....	20
Acclimation experiment.....	20
Selection experiment.....	22
Effect of setup.....	23
Concluding remarks.....	24
<b>References</b> .....	25
<b>Appendix</b> .....	28

## **Introduction**

As climate change is predicted to aggravate global warming accompanied by an increasing frequency and harshness of stochastic heating events (Seneviratne, Donat, Mueller, & Alexander, 2014), species will need to adapt to the new climate. Some species are more resistant than others, and how each species copes with the incoming changes will vary (Duffy, Lefcheck, Stuart-Smith, Navarrete, & Edgar, 2016). It is therefore vital to understand if and how species are able to adapt to these changes, in order to consider if conservational efforts are needed.

Migration, acclimatization and adaptation have traditionally been considered as the three main coping mechanism in response to changes in temperature (Somero, 2010). For some species of fish, migration, which is the change from one habitat to another, is not a viable strategy. In addition to the cost and risks associated with habitat change (Chapman et al., 2013), some species of fish, especially freshwater species, are confined to their current habitat due to geographical restraints or dependence on other species (Labbe & Fausch, 2000). For these species, other coping mechanisms such as adaptation, acclimation (gradual physiological adjustment) or behavioural thermoregulation can be necessary. Adapting to a changing environment is a long-term and gradual strategy (Cuenca Cambronero, Beasley, Kissane, & Orsini, 2018). When the environment is rapidly changing, adapting through evolution can be ineffective and other coping mechanisms are needed for survival (Sih, Ferrari, & Harris, 2011).

In more recent years, behavioural thermoregulation has been recognized as an important coping mechanism to heat stress (Haesemeyer, 2020). Behavioural thermoregulation is the act of behaviourally regulating internal temperature for example by translocating to a new area with a different temperature (Ward, Hensor, Webster, & Hart, 2010). Ambient temperature is perhaps the most significant variable affecting aquatic ectotherms as body temperature affects nearly all aspects of physiology and behavior, such as growth, locomotion and sensory function (Angilletta, Niewiarowski, & Navas, 2002). Lacking the ability to thermoregulate through physiological processes, ectotherms use behavioural thermoregulation, making temperature arguably the most essential ecophysiological factor affecting their performance (Angilletta et al., 2002). Another important aspect to consider in behavioural thermoregulation is the difference between acute thermal preference and the final preferendum. While acute thermal preference can be defined as the immediate thermal preference in an individual, Fry (1947)

defined the final preferendum as the temperature at which preference and acclimation are equal, and an animal in a thermal gradient will finally gravitate towards, regardless of previous thermal experience (acclimation). Behavior is considered to be one of the most capable thermoregulatory responses due to its ability to prevail over longer time scales as well as its low energy demand compared to costly internal regulation (Rey, Digka, & MacKenzie, 2015). As many aquatic ectotherms likely will experience a temperature increase in their habitat, it is important to know if their behavioural thermoregulation changes with the temperature.

Acclimation allows individuals to cope with a new or changing environment and involves the phenotypic altering of behavioural, physiological or morphological characteristics (Woods & Harrison, 2002). There are two categories of acclimation that are recognized: reversible and developmental. Reversible acclimation are controlled responses in relation to changes in the environment such as days and seasons, whilst development acclimation are permanent changes as a response to the environment happening in early ontogeny (Beaman, White, & Seebacher, 2016). Both forms of acclimation can be beneficial to the individual, but there are likely energetic costs associated with them both. One such cost is the reallocation of energy to acclimation efforts which could have been used on other functions, e.g. growth (Hoffmann, Chown, & Clusella-Trullas, 2013).

Acclimation allows for compensatory physiological performance in an environment outside the optimum (Golovanov, 2006) and is an important ability for species living in fluctuating environments. However, extreme temperatures can render acclimation insufficient as every species has both an upper and a lower thermal limit (Díaz, Sierra, Denisse Re, & Rodríguez, 2002). Tropical species adapted to stable environments are expected to be sensitive to small changes in temperature. It is therefore predicted that these species will have a narrow thermal reaction norm, compared to other tropical species (Foray, Desouhant, & Gibert, 2014). A narrow thermal reaction norm means a species is poorly able to change its thermal phenotype to an environmental stressor, whilst a broad thermal reaction norm means a species that have a broader ability to change its thermal phenotype to compensate for the lowered fitness in response to a phenotype (Araya-Ajoy, Mathot, & Dingemanse, 2015). Considering the importance of thermal relations in ectotherms, studying their ability to acclimate to rapid

temperature change is essential in understanding the consequences of global warming for tropical communities of fish.

Critical thermal maximum ( $CT_{max}$ ) is an experimental measurement of an ectotherm's thermal limit and is perhaps the leading measure for upper thermal limits (Morgan, 2020). The process consists of warming an individual gradually at a certain rate, until the individual reaches loss of equilibrium (LOE) and locomotory activity becomes disorganized. For fish, LOE is further often defined as when the individual is unable to uphold its body and starts laying on its side, without the ability to return upright (Becker & Genoway, 1979). The properties of  $CT_{max}$  are not lethal in itself, however, after experiencing LOE individuals will perish if they are not removed from that temperature. In the wild LOE would make an individual incapable of escaping from the thermal conditions and predators, threatening the individual's survival. It has been shown that  $CT_{max}$  is affected by acclimation, but to what degree it is affected varies between species, where a species capable of withstanding an increase in temperature by increasing its  $CT_{max}$  is considered resistant to thermal stress (Christensen, Norin, Tabak, van Deurs, & Behrens, 2021). Previous studies on aquatic ectotherms have focused on the relationship between  $CT_{max}$  and physical factors such as growth and weight (Morgan, Finnøen, & Jutfelt, 2018), as well as physiological factors such as aerobic scope and metabolism (Chen et al., 2015). However, few studies have tested the relationship between  $CT_{max}$  and behavioural response. Even with the physical and physiological ability to survive an increase in ambient temperature, absence of a behavioural response would surely prove threatening for species relying on behavioural thermoregulation. If a species' physiological traits evolve to occupy a higher temperature, behavioural traits such as thermal preference should co-evolve so the species would occupy the new optimum temperature. Zebrafish is great species to study this relationship in, as they are already experiencing temperatures close to what appears to be the maximum thermal limit (Morgan, 2019).

Certain phenotypic traits, such as high metabolic rate and early maturation, are often consistently linked between individuals and is referred to as a syndrome. These traits are on a continuum with extremes represented as tendencies, such as shy-bold and late maturation -early maturation, and co-align for different traits (Réale, Reader, Sol, McDougall, & Dingemanse, 2007). Syndrome research has primarily focused on life history, behaviour and dispersal in the

past, with little physiology incorporated (Cote & Clobert, 2007; Dingemanse, Dochtermann, & Wright, 2010). Perhaps the most popular aspect of syndrome research is currently the Pace-of-Life syndrome (POLS) hypothesis, which predict that behavioural traits will covary with physiological (e.g., metabolic and hormonal) and life-history traits. Several studies have demonstrated a positive relationship between metabolic rate and a behavioural trait, such as boldness, aggression or activity (Biro & Stamps, 2010). Further, the hypothesis predicts that each individual can be placed along a slow-fast continuum where “fast” individuals would exhibit traits such as high metabolic rates, high growth and high fecundity (Goulet, Thompson, Michelangeli, Wong, & Chapple, 2017). Goulet et al. (2017) proposed that the POLS hypothesis could be extended to include a cold-hot axis under the premise that behaviour and thermal physiology would covary and created a model that supported the inclusion of the cold-hot axis (e.g. optimal performance temperature, selected body temperature and critical thermal tolerances). Thermal preference has been studied in a variety of different ectotherm species, such as shrimps, lizards, and fish (González et al., 2010; Li, Wang, Mei, & Ji, 2009; Nay, Johansen, Habary, Steffensen, & Rummer, 2015) with varying conclusions to its effect. There has been a lot of focus on the effect of acclimation (González et al., 2010; Li et al., 2009) with some studies focusing on the effect of genetic adaptation (Andreassen, 2019; Pilakouta et al., 2019). Thermal preference in ectotherm fish have most commonly been measured by using linear tanks, such as the shuttlebox system (Macnaughton, Kovachik, Charles, & Enders, 2018), which allows the tested individual to decide between two temperatures by moving freely from one tank to another. We used an alternative to the common linear approach, which allowed us to quantify acute thermal preference by use of an annular arena.

Zebrafish (*Danio rerio*) is a small fish belonging to the *Cyprinidae* family. Originally discovered in the Ganges delta, zebrafish has become one of the most essential study species as a model organism in several fields, including genetics, physiology and biomedical research (Egan et al., 2009; López-Olmeda & Sánchez-Vázquez, 2011; Spence et al., 2006). It's popularity as a study species originates from its short generation time, number of eggs produced, willingness to breed and more (Briggs, 2002). They generally inhabit slow-moving or standing water bodies, in particular rice fields, but they have in addition been found to occupy rivers and hill streams, ponds, pools, and lakes (Rey et al., 2015; Spence et al., 2006). Zebrafish naturally inhabits areas from India, Bangladesh and Nepal to Pakistan (Rey et al., 2015; Sundin et al., 2019), all being tropical regions. Water temperatures in these regions are relatively high ranging

from 24°C to 32°C (Morgan, Sundin, et al., 2020; Rey et al., 2015). In addition, tropical regions vary substantially in their precipitation creating an ever-changing freshwater habitat for the species located in those areas. Due to the nature of the habitat zebrafish inhabit, it is not uncommon for them to become trapped in smaller water areas due to changes in water elevation (Spence et al., 2006). These happenings in combination with the rising temperature and extreme heat waves through global warming mean that zebrafish must tolerate even higher temperatures than currently (Seneviratne et al., 2014). It is therefore vital to know how they will adapt genetically in order to know if conservation measures must be taken (Sundin et al., 2019).

In this experiment our first aim was to validate the effectiveness of an annular arena built by Andreassen (2019) by testing thermal preference in a small eurythermal (able to tolerate a wide range of temperatures) fish. Two different experiments were performed: one experiment on zebrafish selected for high and low  $CT_{max}$ , and one experiment on zebrafish acclimated to three different temperatures (Cold; 20°C, Optimum (control); 28°C, Warm; 34°C). The second aim was to test if either selection or acclimation in zebrafish would affect their thermal preference. To test the effect of selection and acclimation on thermal preference, the median occupied temperature and the interquartile range were compared for individual zebrafish selected for high and low thermal tolerance, as well as comparing zebrafish acclimated to three different temperatures. We predicted that selection for thermal tolerance, as well as acclimation would both lead to an increase in preferred temperature.

## Methods

### Selection for Down-selected, Up-selected and Control treatments

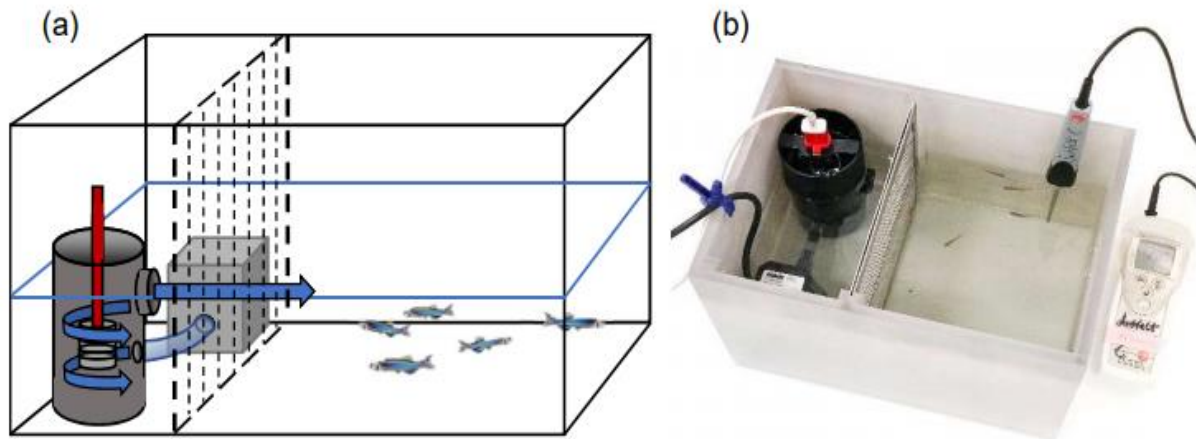
In 2016 wild zebrafish were caught in West Bengal (India) and transported to the Norwegian University of Science and Technology (NTNU) in Trondheim (Norway), by Morgan (2019). Fish were split into three treatments (Down, Control and Up) depending on thermal tolerance by critical thermal maximum tests ( $CT_{max}$ ). Each treatment was then again split into two separate replicates to control for genetic drift. From the F1 population ( $n=1200$ ), fish with the 33% lowest  $CT_{max}$  created the Down-selected treatments (Replicate L1, Replicate L2), fish with the 33% highest  $CT_{max}$  created the Up-selected treatments (Replicate H1, Replicate H2) and randomly selected fish created the Control treatments (Replicate R1, Replicate R2). The fish were always kept in laboratory water during housing and experiments, except for the relatively short period during preference trials. Laboratory water consisted of carbon filtered water with a mixture salt (0.37 ppt) and Aquasafe (Tetra®, Blacksburg, VA, USA).

### Critical thermal maxima

#### *Experimental setup*

To test  $CT_{max}$ , a custom created tank was used. The tank measured 25x22x18cm and contained 9L of carbon filtered water. A metal mesh separated the tank into two sections; one large section (the main compartment) for the fish to swim freely, and a smaller section for the pump (Eheim Universal 300, Germany) and heater. The metal mesh kept the fish from getting too close to the heater and the pump. The heater used was a custom-made cylindrical steel heating case consisting of an inflow nipple, an outflow and a 300W coil heater, connected to a water pump in order to ensure a homogenous water temperature throughout the entire arena ( $< 0.1^{\circ}C$ , Figure 1). A high precision digital thermometer with a  $\pm 0.1^{\circ}C$  accuracy (testo-112, Testo, Lenzkirch, Germany) gave continuous measurements of water temperature in the fish compartment (Morgan et al., 2018).





**Figure 1** (a) Illustration of the  $CT_{max}$  custom test tank. Hard lines are the outer walls. The dotted lines are a steel mesh preventing entry to the pump (grey cylinder) pumping water throughout the tank. (b) Photograph of the  $CT_{max}$  tank from above with a thermometer fastened (Morgan et al., 2018).

### *Experimental procedure*

The  $CT_{max}$  test was conducted during the period April 13<sup>th</sup>-17<sup>th</sup>, 2020. Fish from the same treatment were tested in groups of eight and placed in the main compartment of the  $CT_{max}$  tank at 28 °C. Water temperature increased by 0.3 °C min<sup>-1</sup> (Morgan et al., 2018). We used loss of equilibrium (defined as disorganized and uncontrolled swimming for two seconds) and inability to regain equilibrium as a threshold of  $CT_{max}$  and an approximation of individual thermal tolerance. When an individual lost equilibrium they were netted out before temperature, identity, weight and length were recorded, after which the individual was humanely sacrificed by being placed in a small tank with ice water mixed with MS-222.

### **Experimental preparation**

Between January 9<sup>th</sup>-14<sup>th</sup> 2020, fish from the 6<sup>th</sup> generation were used to produce offspring for the 7<sup>th</sup>. Each breeding box contained three males and three females from the same treatment to continue the selection process. In total, 221 eggs from the Down-selected treatment, 1093 eggs from the Control treatment and 1057 eggs from the Up-selected treatment were produced. Shortly after hatching the larvae were fed zebrafeed (Sparos) ad libitum several times a day and was changed to adult feed once they became juvenile. Replicate lines were separated into several tanks (50x30x30) to prevent stress.

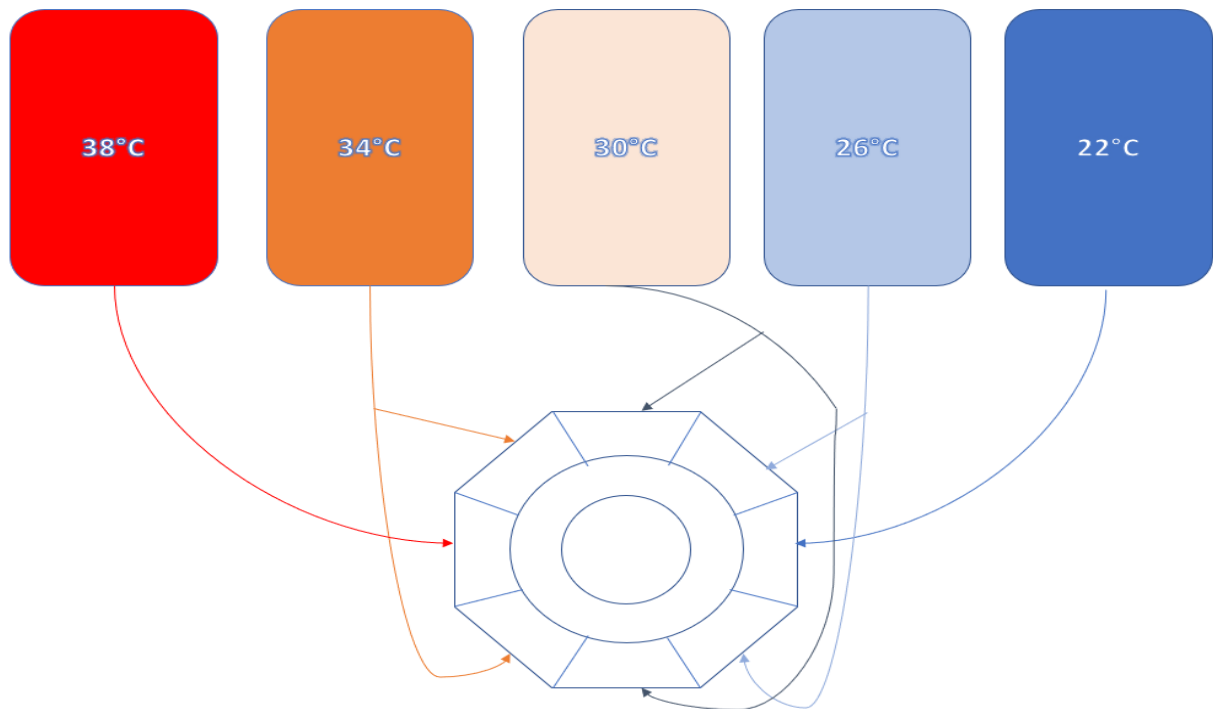
Once the fish juveniles had reached an adequate size, between 25<sup>th</sup> and 26<sup>th</sup> of February, 2020, 96 fish (32 from each treatment) were tagged with two visible elastomer tags (VIE, Northwest Marine Technologies, Shaw Island, WA, USA) with colour combinations that allowed identification of each individual. Using BD MicroFine+ 0.5mm insulin syringes (BD, Franklin lakes, NJ, USA), markers were placed on the right and left side of the dorsal fin, following the procedure based on the site shown to be the best suited for tagging (Hohn & Petrie-Hanson, 2013). Before tagging, fish were submerged into a solution consisting of 110 mg L<sup>-1</sup> buffered tricaine methane sulfonate (MS-222) to anesthetize the fish. Fish were considered anesthetized when equilibrium was lost and there was no response to prodding of the tail. Anesthetized individuals were measured to the nearest 0.01 g for weight using a precision scale (AC88, Mettler Toledo Ltd, Melbourne Australia) and nearest 0.01 cm for standard length using a Digital ABS Caliper (500-706-20, Mitutoyo Corp, Kawasaki, Japan). Once measurements were completed the fish were placed in an aerated tank (21x12x13 cm) to regain consciousness before being transported to their new holding tanks.

## **Experimental setup**

### *Room setup*

In the test room for thermal preference, five insulated header tanks (57x39x42) were placed at an elevated surface to serve as the water source for the test chamber. Each tank was supplied with a continuous flow of carbon filtered water where the water level was automatically regulated by float valves. Water within the header tanks ranged from 22°C to 38°C increasing with 4°C from one tank to the next. To maintain the different temperatures in the header tanks we used heating rods and thermostats (ITC-306T, Inkbird, Shenzhen, China). From the header tanks, plastic tubes ran down into a test arena placed in the middle of the room with water flowing at a rate of 8 ml/sec. Water from the header tanks went into the outer chambers of the test arena where the outer chamber on the right side received the coldest water (22°C) and the other outer chambers received water which increased incrementally (4°C increments) with the

leftmost outer chamber receiving water with the highest temperature (38°C, figure 2, Appendix; figure 3 and 4).



**Figure 2:** the header tanks and preference arena illustrated from above. The rounded squares are header tanks containing water of different temperatures. The arrow lines are tubes with water flowing from the header tanks into the outer chambers of the preference arena.

### *Preference arena*

The preference arena was designed by Andreassen (2019) based on previous designs by Myrick, Folgner, and Cech (2004). It is a 60 cm wide tank that consists of eight equally large outer chambers. A continuous waterflow is supplied from the header tanks. The outer chambers surround a donut shaped water compartment, referred to as the swimming channel, with a width of 12 cm. The inner wall of the swimming channel is cone shaped, thus allowing tracking of fish through the entire channel. The inner wall of the swimming channel has a diameter of 30 cm and the outer a diameter of 42 cm, at the water surface. From each outer chamber nine evenly distributed holes of 5 mm in diameter ensured an even flow of water into the swimming chamber. Water flows from the swimming chamber into the inner area through 38 holes of 3 mm diameter split into two rows, and further into a drainage hole that removed the water. Smaller holes, 24 in total, with a diameter of 1.5 mm were evenly placed along the inner wall, between the two rows of 3mm holes. These holes served to distribute 24 thermocouples evenly along the swimming chamber.

## **Selection experiment, protocol**

### *Pre-trial*

Approximately 15 hours before individuals were tested for thermal preference, they were placed in their own habituation tank over night to get accustomed to the setup. The habituation tanks were made up of a white bucket with a 31 cm diameter bottom with a second white bucket with the diameter 15 cm glued upside down in the middle of the large bucket (Appendix, figure 3). Little holes were drilled into the middle bucket to mirror the preference arena. There were four habituation tanks in total. Each tank had air provided by an air stone which was placed inside the middle bucket as to not disturb the fish. Transparent lids on top of the habituation tanks helped keep the temperature at  $28 \pm 0.02^\circ\text{C}$  ( $\mu \pm \text{S.E.}$ ) and allowed the fish to experience a natural day-night cycle from the automated light cycle in the room. Curtains were hung 45 cm above the ground covering the tanks from the rest of the room to prevent disturbance during the experiment. During the entire process fish identity was known through use of the colour combinations of the visible implant elastomer tags.

### *During trial*

At approximately 08:15 each day, the water flow was set up to range from approximately  $23^\circ\text{C}$  to approximately  $35^\circ\text{C}$ . When the correct temperature gradient was reached, the first fish was gently picked up from its habituation tank with a white hand net and placed into the swimming channel in the test arena. Immediately after a recording of the fish and logging of the temperature started. For two hours the fish was kept undisturbed with no one entering the room, whilst being monitored from a computer in the neighbouring room. After two hours the recording and temperature-logging was stopped, and the fish was transported to a new holding tank reserved for fish tested on the same day. Then the three other fish that had spent the night in habituation tanks were tested separately using the same procedure. To prevent an effect of placement within the swimming channel, fish were randomly placed in one of the areas with  $28^\circ\text{C}$  water (Appendix, figure 4).

## **Acclimation experiment**

### *Pre-trial*

The acclimation experiment started on October 2<sup>nd</sup> 2020, using fish from the F7 generation Control treatment. A total of 63 fish were separated into three different acclimation treatments with 21 individuals in each treatment. Furthermore, each treatment was separated into three different acclimation tanks with seven individuals in each tank. Fish were acclimated to either 20°C, 28°C or 34°C creating the Cold, Optimum (control) and Warm treatments, respectively. In total, the fish were kept in nine different tanks (50cm x 30cm x 30cm), three tanks per treatment. These were placed in a non-random order on a shelf with three height levels, and with room for only three tanks on each level. The top shelf had the placement order 20°C, 28°C, 34°C, with each shelf below having all placements shifted to the left. Temperature in the Cold and Warm treatment were adjusted by 2°C each day with both treatments having 28°C as the starting temperature, and the fish were acclimated over 5-6 weeks.

### *During trial*

The thermal preference tests for the acclimation experiment followed the same procedures as the selection experiment, with only a few differences. The same test arena as well as monitoring methods were used in the acclimation experiment as in the selection experiment. Temperature throughout all trials ranged from  $22.98 \pm 0.03^\circ\text{C}$  ( $\mu \pm \text{S.E.}$ ) °C to  $34.39 \pm 0.05^\circ\text{C}$  ( $\mu \pm \text{S.E.}$ ). Fish that were to be tested the following day were placed in habituation tanks containing water of the same temperature as their acclimation temperature. On test day, the individual being tested was placed in the same temperature as they were acclimated to in the swimming arena of the preference chamber, and each trial lasted 80 minutes, as the selection experiment had no difference in results after 80 and 120 minutes. Each day, five individuals were tested, usually between 08:00 and 15:00. Once each trial was complete, the tested individual was humanely sacrificed by being placed in water mixed with MS-222, after which the length and weight of the fish was measured.

## **Analysis and data collection**

### *Data collection*

Video recordings were collected using a computer in the observation room and behaviour was recorded with the software OBS (Bailey, Hugh. (2017) the OBS Project Contributors. *Open Broadcasting Service*). Whilst recording, temperature was logged continuously with a two second interval from the 24 thermocouples spread around the inner wall of the arena, recorded with the data logging software PicoLog 6 (Pico Technology, Cambridgeshire, UK). Video recordings were analyzed using the software EthoVision (XT 13.0, Noldus IT, Wageningen, Netherland) which tracked the movement of the tested fish. Using the tracking software, the swimming channel was separated into 24 different zones corresponding to the 24 thermocouples. Each trial was provided the mean logged temperature for each zone from only its own trial to ensure that inter trial variations would not affect the results. EthoVision quantified the cumulative duration spent in each zone for each individual.

Thermal preference was determined by the median temperature occupied by the individual during the last 20 minutes of each trial. Interquartile range (IQR), from the 25<sup>th</sup> percentile to the 75<sup>th</sup> percentile was used to assess the change in occupied temperatures during the trial. To determine if there was an active choice of temperature in tested individuals, the occupied temperatures during the first and last 20 minutes of trials performed in an annular arena were compared. Once inserted into the preference arena, fish usually responded with one of three different reactions: fast erratic movement, calm exploration or completely freezing. Individuals with a high IQR would be considered “non-choosers”, whilst individuals with a relatively low IQR were considered “choosers”. An IQR value of 5 was used as a cut-off point for the selection experiment based on comparisons of the IQR histograms of the different treatments (Appendix, figure 1). An IQR value of 4.3 was used as a cut-off point for the acclimation experiment based on comparisons of IQR histograms of the different temperatures (Appendix, figure 2). Recordings from the preference trials were split into twenty-minute intervals for comparing change in activity. Recordings of individuals that had an IQR of zero in both the first 20 minutes and the last 20 minutes were manually checked for activity throughout the 120 minutes. Fish that did not move for the entirety of the trial, were excluded in models. If there was movement between the first and last 20 minutes the test individual would be included in models. Some videos got damaged after being recorded and also had to be excluded, as well as one trial where the fish managed to escape the preference arena.

### *Stats*

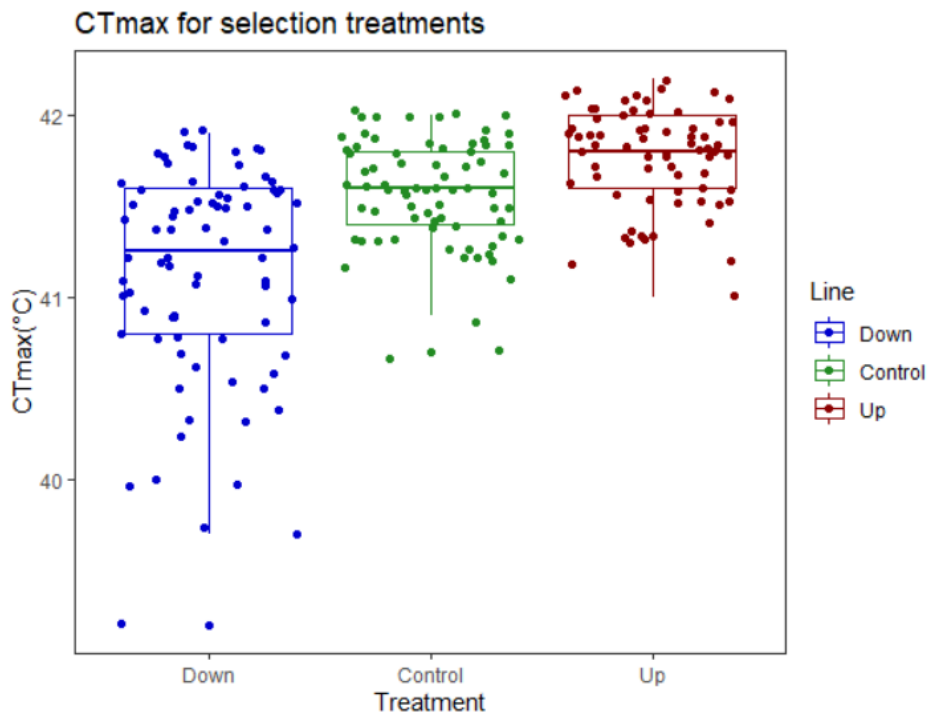
Statistical analyses were carried out in *R* (ver. 4.0.2). For mixed effects models the lme4 package and the lmerTest package were used to compare the means of medians of the position of individual fish during the last 20 minutes. We tested the effects of acclimation and selection on thermal preference in individual zebrafish using linear-mixed models. The possible effect of the nested structure on the variance in thermal preference was accounted for by adding holding tank as a random factor in both the selection model and the acclimation model. Models were fitted with factors thought to be biologically relevant and were included even if the effect was insignificant. Weight was included in all models while length was excluded because weight and length were highly correlated ( $R = 0.86$ ,  $p < 0.001$ , for selection and  $R = 0.52$ ,  $p < 0.001$ , for acclimation).

## Results

### CT<sub>max</sub> selection experiment

#### *Thermal tolerance*

In order to test the effect of selection on thermal tolerance in the eighth generation of selected zebrafish, CT<sub>max</sub> was measured (n = 240) for all selected treatments with the Down-selected (n = 80), Control (n = 80) and Up-selected (n = 80) treatment having a median±S.E. of 41.25±0.6°C, 41.60±0.3°C and 41.80±0.3°C, respectively (Figure 3). The Down-selected treatment ( $\beta \pm \text{S.E.} = -0.43 \pm 0.07$ ,  $p < 0.001$ ) affected CT<sub>max</sub> negatively whilst the Up-selected treatment ( $\beta \pm \text{S.E.} = 0.19 \pm 0.07$ ,  $p = 0.007$ ) affected CT<sub>max</sub> positively. Weight had a positive effect ( $\beta \pm \text{S.E.} = 1.42 \pm 0.49$ ,  $p = 0.04$ ) on thermal tolerance (Table 1).



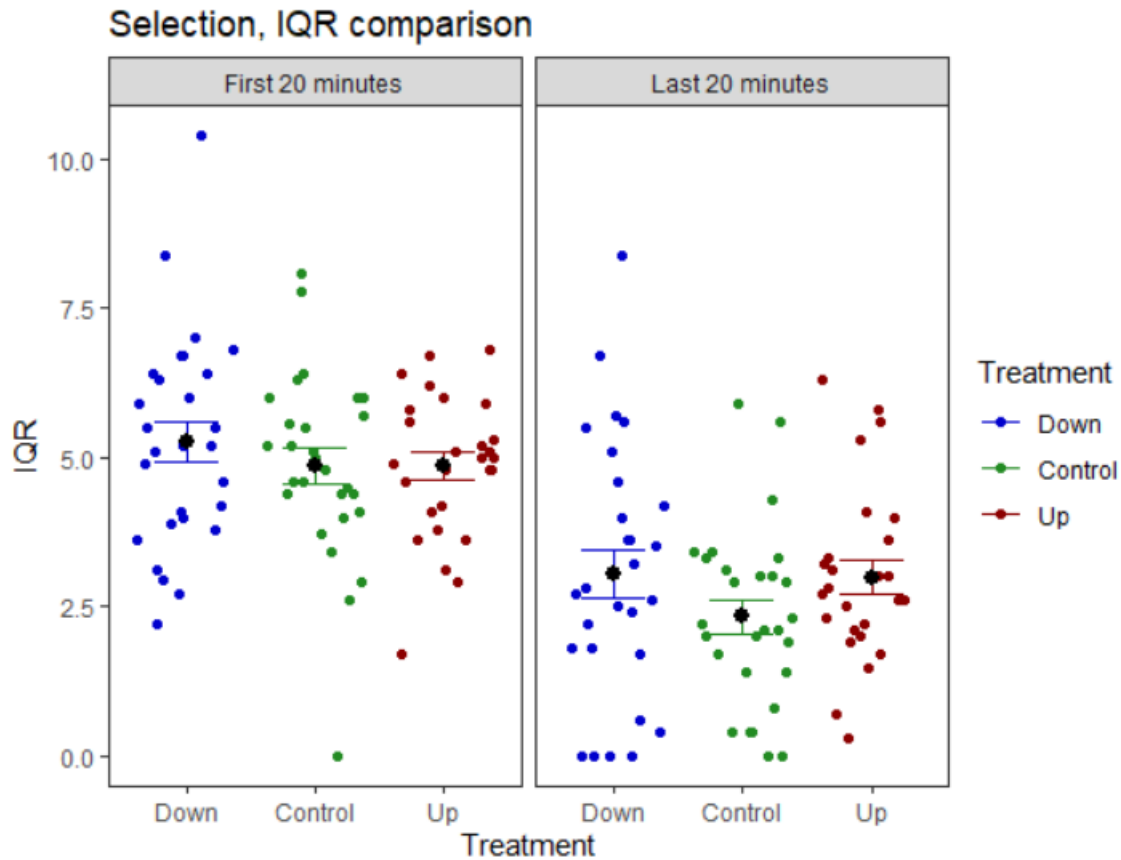
**Figure 1** Critical thermal maximum (CT<sub>max</sub>, °C) for the eighth generation of selected zebrafish, represented by a boxplot, showing the median (horizontal-colored lines), first quartile, third quartile and minimum and maximum values in the dataset. The datapoints are the CT<sub>max</sub> of one individual and are jittered horizontally to visualize each datapoint.

#### *Thermal preference*

The mean IQR ( $\pm \text{S.E.}$ ) of all individuals in all three selection lines (Down-selected; 3.04±0.28°C, Control; 2.25±0.28°C, Up-selected; 2.99±0.28°C) decreased significantly in the



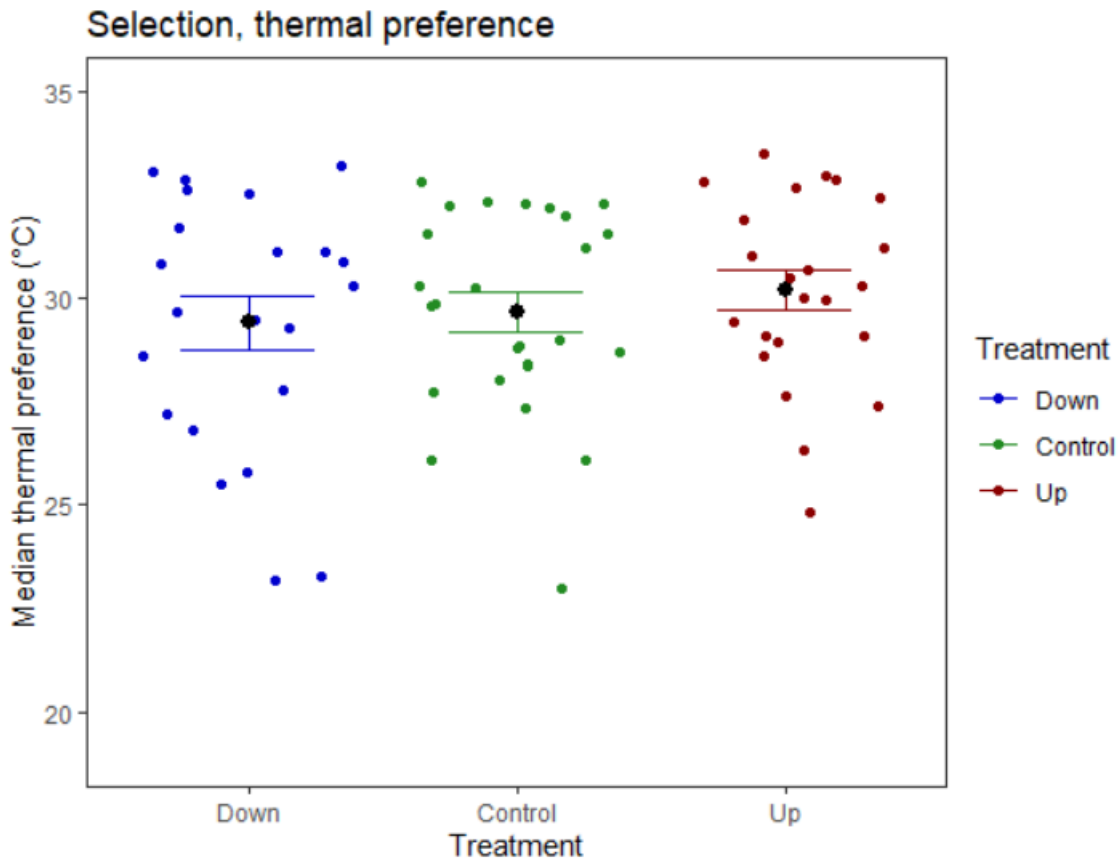
last 20 minutes compared to the first 20 minutes of the trials (Wilcoxon rank sum test,  $p$ -value  $< 0.001$ , Figure 4).



**Figure 2** Mean interquartile range of individuals occupied temperatures in the selection experiment during the 20 first (left) and 20 last (right) minutes shown for each treatment. Each coloured datapoint is the mean interquartile range (IQR) of temperature for one individual over 20 minutes and are jittered horizontally to visualize each datapoint. The mean interquartile range is showed with standard error (black dots and error bars).

Thermal preference was negatively affected by the Down-selected treatment ( $\beta \pm \text{S.E.} = -0.80 \pm 1.50$ ,  $p = 0.595$ ) and was positively affected by the Up-selected treatment ( $\beta \pm \text{S.E.} = 0.13 \pm 1.51$ ,  $p = 0.929$ , table 1), however, neither result were statistically significant. The Down-selected treatment ( $n = 22$ ) had an average median thermal preference of  $30.00 \pm 0.64^\circ\text{C}$ , Control ( $n = 26$ ):  $29.85 \pm 0.47^\circ\text{C}$ , and Up-selected ( $n = 23$ ):  $30.30 \pm 0.43^\circ\text{C}$ . Neither weight ( $\beta \pm \text{S.E.}$ :

= $0.01 \pm 0.06$ ,  $p = 0.860$ ) nor the real temperature of the habituation tanks ( $\beta \pm \text{S.E.} = -2.74 \pm 1.46$ ,  $p = 0.061$ ) had statistically significant effects on thermal preference (Table 1).

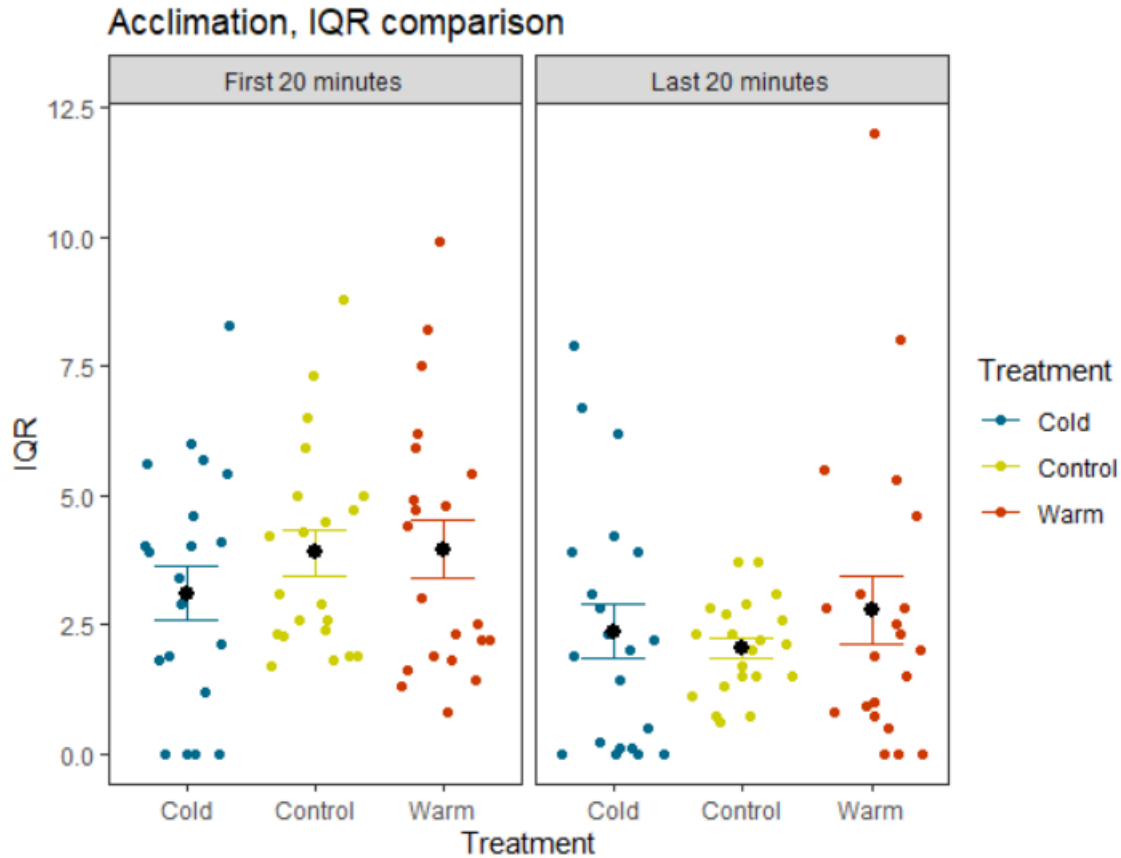


**Figure 3** Preferred temperature for the last 20 minutes of each trial in the Down-selected, Control and Up-selected treatment with an IQR < 5 in the selection experiment. Each datapoint is the median temperature the test individual occupied at the end of its trial and are jittered horizontally to visualize each datapoint. The median preferred temperature is presented with standard error (black dots and error bars).

### Acclimation experiment

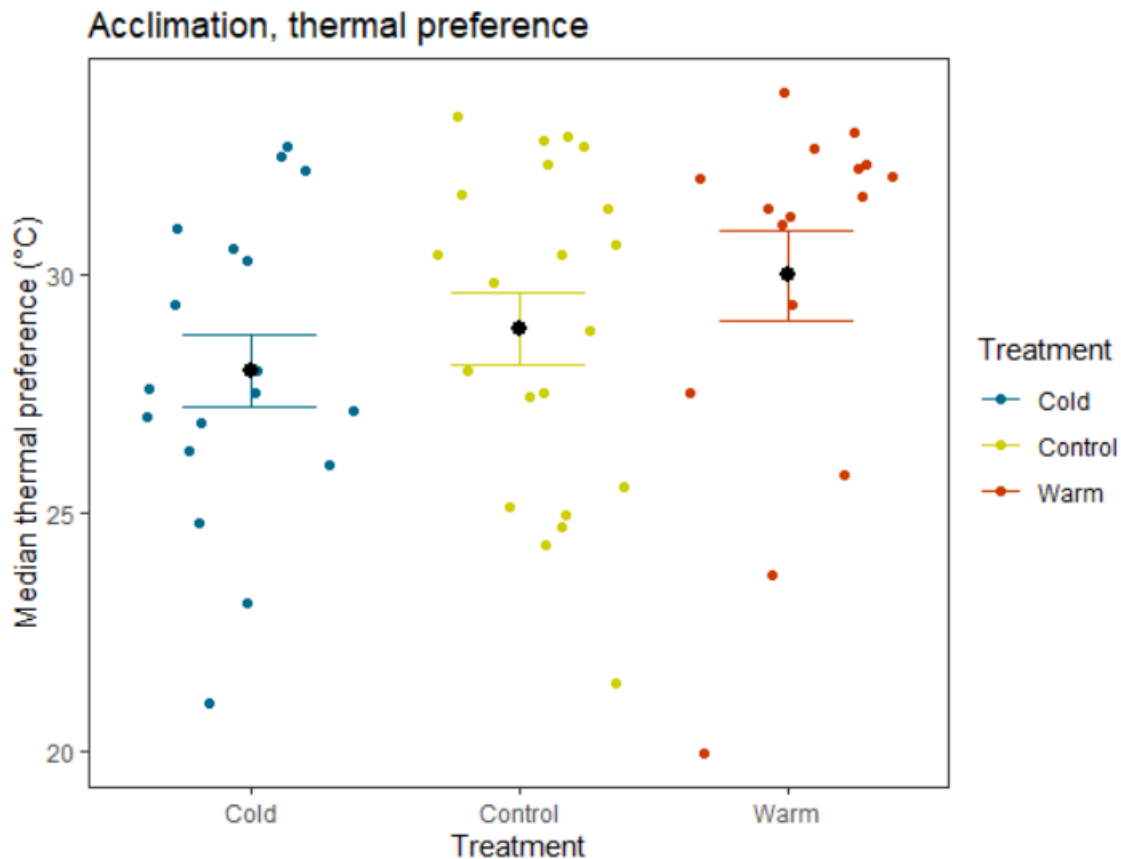
To test if acclimation affects thermal preference in zebrafish, 63 individuals were acclimated to three different temperatures (20°C, 28°C and 34°C) and thermal preference was compared between the three different acclimation temperatures. Overall, IQR decreased significantly in the last 20 minutes of the trials compared to the first twenty minutes of the trials (Wilcoxon rank sum test,  $p < 0.001$ ; figure 6). The mean IQR ( $\pm \text{S.E.}$ ) was lower in the last 20 minutes than the first 20 minutes of the trials for all treatments (Cold;  $2.35 \pm 0.52$  °C, Optimum (control);  $2.04 \pm 0.20$  °C, Warm;  $2.77 \pm 0.64$  °C). In all, 55 zebrafish from the Cold ( $n = 18$ ), Optimum

(control) ( $n = 21$ ) and Warm ( $n = 16$ ) treatments had IQR values low enough to be included in the analyses.



**Figure 4** Mean interquartile range (black points) of occupied temperatures during the 20 first (left) and 20 last (right) minutes of all trials shown for Cold, Optimum (control) and Warm treatment in the acclimation experiment. Each datapoint is the IQR for one trial and are jittered horizontally to visualize each datapoint. The mean IQR of occupied temperatures are showed with standard error.

The median preferred temperature for the Cold treatment ( $27.55 \pm 0.75$  C°) and the median for the Warm treatment ( $31.50 \pm 0.96$  C°) appeared, by visual inspection, to be lower and higher than the Optimum (control) median ( $29.8 \pm 0.75$  C°), respectively. However, neither the Cold treatment ( $\beta \pm \text{S.E.}: -0.66 \pm 1.75$ ,  $p = 0.706$ ) nor the Warm treatment ( $\beta \pm \text{S.E.}: 0.87 \pm 1.80$ ,  $p = 0.628$ ) affected thermal preference. Neither weight ( $\beta \pm \text{S.E.}: 0.00 \pm 0.05$ ,  $p = 0.979$ ) nor the habituation tank temperature ( $\beta \pm \text{S.E.}: -0.62 \pm 1.41$ ,  $p = 0.660$ ) had any effect on thermal preference (Table 1).



**Figure 5** Preferred temperature (black points) for last 20 minutes of each trial for the Cold, Optimum (control) and Warm treatment with an IQR of below 4.3 in the acclimation experiment. Each datapoint is the median temperature for one trial the test individual occupied in the trial and are jittered horizontally to visualize each datapoint. The median preferred temperature is presented with standard error.

For the thermal preference experiments (selection and acclimation) none of the factors included in the models had any effect on thermal preference (table 1). However, for the thermal tolerance ( $CT_{max}$ ) experiment the Up-selected treatment and weight affected thermal tolerance positively whilst the Down-selected treatment affected thermal tolerance negatively (table 1). Of the random effects for the selection experiment, tank accounted for the most variance ( $\sigma^2 = 1.01$ ). However, tank did not account for any of the variance in the acclimation experiment ( $\sigma^2 = 0.00$ ).

**Table 1:** Model output (estimate, standard error (S.E.) and  $p$ -value) for three linear mixed effects models from the selection, acclimation and  $CT_{max}$  experiment. Estimates for selection and acclimation experiments are median preferred temperature values and the  $CT_{max}$  experiment show estimates from mean  $CT_{max}$ . Bold  $p$ -values are significant ( $<0.05$ ).

Thermal preference							Thermal tolerance		
Predictors	Selection			Acclimation			CT <sub>max</sub>		
	Estimate	S.E.	$p$ -value	Estimate	S.E.	$p$ -value	Estimate	S.E.	$p$ -value
(Intercept)	29.81	1.38	<b>&lt;0.001</b>	30.58	2.51	<b>&lt;0.001</b>	41.36	0.09	<b>&lt;0.001</b>
Down	-0.80	1.50	0.595	-0.79	1.73	0.648	-0.43	0.07	<b>&lt;0.001</b>
Up	0.13	1.51	0.929	0.96	1.78	0.588	0.19	0.07	<b>0.007</b>
Weight	0.01	0.06	0.860	-0.00	0.05	0.979	1.42	0.49	<b>0.004</b>
Habituation-Temp	-2.74	1.46	0.061	-0.62	1.41	0.660			

Random Effects	
$\sigma^2$	5.31
$\tau_{00}$	12.58
	0.68 Group:(TankID:(Replicationline:Line))
	0.00 Aquarium:Line
	1.01 TankID:(Replicationline:Line)
	0.73 Line
	0.00 Replicationline:Line
	0.52 Line
N	21 Group
	9 Aquarium
	12 TankID
	3 Line
	6 Replicationline
	3 Line

## Discussion

### Acclimation experiment

Acclimation has been shown to not affect thermal preference in some species (González et al., 2010), whilst others found that acclimation affected thermal preference only at certain acclimation temperatures (Li et al., 2009; Schram et al., 2013; Wu, Hu, Dang, Lu, & Du, 2013). In this study we found that acclimation to eight degrees below and six degrees above optimal temperatures for 5-6 weeks did not affect thermal preference in zebrafish. Neither the Cold treatment nor the Warm treatment were significantly different from the Optimum (control) treatment, even though there was a general trend where cold acclimated fish had a lower thermal preference and the opposite trend was seen in warm acclimated fish, when looking at the means of each treatment. The temperature used for warm acclimated fish could be increased a couple of degrees before becoming lethal (Morgan, 2020) so both decreasing and increasing the acclimation temperature could yield different results. However, (Christensen et al., 2021) also showed that there was no difference in thermal preference in zebrafish acclimated to 10°C and 20°C, when testing their thermal preference in a shuttle tank system over a period of ~23 hours. Even though they did not measure acute thermal preference, it still gives some support to our results, indicating that thermal preference in zebrafish is not affected by acclimation.

Despite not being significant, there is still an indication that acclimation affected thermal preference for both the Cold acclimated ( $\mu \pm \text{S.E.}: 27.55 \pm 0.75 \text{ C}^\circ$ ) and the Warm acclimated ( $\mu \pm \text{S.E.}: 31.50 \pm 0.96 \text{ C}^\circ$ ) fish when comparing mean median temperatures. When acclimated to a new temperature, physiological functions change to better suit the change in environment. One such physiological function has been shown in cold acclimated zebrafish, where the fish partially compensate for lowered activity by increased tail beat frequencies and locomotor performance in response to an increase in thyroid hormone (Little & Seebacher, 2013). When acclimated, the acute optimal temperature might have changed and a behavioural change to prefer the new temperature would maintain the individual's new physiological optimum. However, as acclimation is a compensatory measure, maintaining the original thermal preference would ensure that the individual is more likely to regain its "true" optimum temperature. Individuals in relatively stable environments, such as tropic regions, should in theory favour a strategy where the optimum temperature does not change. In fluctuating environments, individuals should favour a strategy where the optimum change to better suit the

new environment. This is supported by evolutionary theory which predicts that species specialized to a stable environmental conditions should have a narrow capacity to change its phenotype in response to change in the environment (Huey & Hertz, 1984).

Another unexpected result is the overall spread of the individual thermal preference. The acclimation experiment followed almost exactly the same methods as the selection experiment, yet the spread of data across all treatments is higher in the acclimation experiment (Cold; S.D. = 2.40 °C, Optimum (control); S.D. = 0.92 °C, Warm; S.D. = 2.97 °C) compared to the selection experiment (Down-selected; S.D. = 2.18 °C, Control; S.D. = 1.48 °C, Up-selected; S.D. = 1.46 °C). Interestingly, the mean ( $\pm$ S.E.) IQR values for all treatments in the acclimation experiment were lower (Cold; 2.35 $\pm$ 0.52 °C, Optimum (control); 2.04 $\pm$ 0.20 °C, Warm; 2.77 $\pm$ 0.64 °C) than the mean IQR values in the selection experiment (Down-selected;  $\mu$  $\pm$ S.E. = 3.04 $\pm$ 0.28 °C, Control; 2.25 $\pm$ 0.28 °C, Up-selected; 2.99 $\pm$ 0.28 °C), which means that individuals in the acclimation experiment occupied a narrower range of temperatures on average than the individuals in the selection experiment.

The cause of this is currently unknown, but it is possible that differences in the methods between the two experiments causes the difference in these results. Each trial in the acclimation experiment lasted 40 minutes shorter than the selection experiment. The decision to shorten the acclimation trials was based on data from the selection experiment which showed no significant difference in IQR between the 60-80 minutes and the last 100-120 minutes. Because the Optimum (control) treatment also showed this spread, it is unlikely that the intrinsic effect of acclimation is the source. Another possible cause of error from the methods could be the habituation tanks, as their placement differed slightly between the two experiments. To minimize disturbance, habituation tanks were placed as close to the preference arena as possible. In the selection experiment, habituation tanks were kept in the same room as the preference arena, whilst in the acclimation experiment habituation tanks were kept in a separate room (a consequence of having to maintain three different acclimation temperatures). The extra movement necessary to translocate zebrafish from the habituation tank to the preference arena could have caused extra levels of stress from handling and may have caused the large spread in thermal preference expressed by acclimated individuals.

### **Selection experiment**

The results show that selection for  $CT_{max}$  did not affect thermal preference significantly, giving little support to the hypothesis that this trait could be aligned on a cold-hot axis in a syndrome. Because the occupied temperature surrounding a fish affects physiological functions such as growth rate, immune response and metabolism, fish would choose to occupy a temperature close to their peak performance (López-Olmeda & Sánchez-Vázquez, 2011). Our tested fish ( $n = 96$ ) had a median occupied temperature of ( $\mu \pm S.E.$ )  $29.75 \pm 0.26$  °C which is above what is believed to be the optimum temperature for growth at  $28.5$ °C (López-Olmeda & Sánchez-Vázquez, 2011). Tested fish spent 16 hours in a habituation tank containing water around  $28$ °C. If the temperature in the habituation tanks were different from  $28$ °C, this could have affected the preferred temperature. This is further supported by habituation temperature's inclusion in the selection model ( $\beta \pm S.E. = -2.74 \pm 1.46$ ,  $p = 0.061$ ) showing it to be close to significant. It is, however, strange that thermal preference decreases as temperature in the habituation tank increases, and as the mean temperature of the habituation tanks were quite stable ( $\mu \pm S.E. = 28.3 \pm 0.2$ °C), it seems unlikely that this is the cause of variation.

IQR differed between the first and last 20 minutes for all treatments, but a visual inspection (figure 6) shows that the mean IQR values are quite similar. Interestingly, the Down-selected treatment had the most individuals excluded because their IQR was too high ( $n = 6$ ) and also had the most individuals with a zero value IQR ( $n = 4$ ). This means that the Down-selected treatment had both the most active fish as well as the most fish with no activity in the last 20 minutes of the trials.

We hypothesized that selecting for thermal tolerance would increase thermal preference if the two traits were to align in a cold-hot axis in a syndrome, but our results did not support this hypothesis. A possible reason for the absence of covariation is that  $CT_{max}$  is a lethal, short-term measurement. Selection pressure for higher  $CT_{max}$  would not necessarily make it advantageous to increase the optimal temperature for physiological functions. In a tropical environment that experiences monsoon seasons where temperatures vary a lot throughout the day and night, evolving a higher  $CT_{max}$  could be beneficial in order to survive in a confined space during daytime. These bursts of higher temperatures are not permanent, and it is therefore possible that there would be little to no advantage in increasing the optimal temperature for physiological functions and thus the thermal preference when selecting for higher thermal tolerance.



The 7<sup>th</sup> generation of zebrafish show a significant difference in  $CT_{max}$  across all treatments, however both the Control (SE = 0.3) and the Up-selected treatment (SE = 0.3) have a narrower spread of datapoints than the Down-selected treatment (SE = 0.6). Comparing these results to the previous generations, it becomes apparent that the Up-selected treatment hardly has had an increase in  $CT_{max}$  throughout eight generations. If the reason for the lack of increased  $CT_{max}$  originated from either the breeding methods or the zebrafish used, the Down-selected treatment would show little change in  $CT_{max}$  as well. As this is not the case, a possible conclusion is that zebrafish are nearing its absolute possible peak in increasing  $CT_{max}$  and are not able to evolve it further. Morgan, Finnøen, Jensen, Pélabon, and Jutfelt (2020) measured the  $CT_{max}$  of the 6<sup>th</sup> generation of zebrafish and found that the Up-selected treatment had an average  $CT_{max}$  of ( $\mu \pm S.E.$ )  $41.85 \pm 0.32$  °C, which is similar to our results ( $\mu \pm S.E.$ :  $41.80 \pm 0.3$  °C). Still, this indicates that zebrafish are nearing its thermal peak and further selection for increased  $CT_{max}$  might be impossible. Because there is a correlation between selection and  $CT_{max}$ , the inability to evolve a higher  $CT_{max}$  could explain why there is no effect of selection in the Up-selected treatment. It is however unlikely, as there is no effect of selection in the Down-selected treatment also.

### **Effect of setup**

Throughout both the acclimation experiment and the selection experiment, the tested individuals show several stress related behavioural traits such as freezing bouts and spontaneous rapid turns (Egan et al., 2009), which might have affected the results. The stress observed in our trials could originate from a variety of sources, with human handling perhaps being the most prominent source. Zebrafish in our lab are used to both activity and noise from people in the same room as their tanks, but handling of the fish is kept to a minimum. A likely source of stress is therefore the overall process of suddenly experiencing a large increase in handling compared to the relatively low amount they usually receive. In addition, both the habituation tanks and the preference arena are new environments, which could indeed stress the test individuals. Even if the habituation tanks are built to get the zebrafish accustomed to the setup of the preference arena, flowing water and a large variety of temperatures in the preference arena is still a new environment and a possible source of stress. Once stressed, the effect might have been amplified by the lack of depth in the setup. Even if the fish should have gotten

accustomed to the test arena by spending 15-hours in the habituation tanks, a common response in zebrafish when stressed is to swim downward (Egan et al., 2009), but because the preference arena is equally shallow across the entire arena, this is not possible. In addition, zebrafish are a shoaling species with a common stress reaction to increase shoal cohesion (Egan et al., 2009). Both performing the test in solitude and the lack of shoaling options when stressed could have affected the results.

In the wild, the ability to choose between a range of temperatures to occupy seems far more likely than having to choose between just two different temperatures, which makes the continuous thermal gradient in our preference arena more applicable to nature than the shuttlebox method. Additionally, certain acclimation effects begin almost immediately after entering a new temperature which suggests that the shuttlebox method might be affecting the “true” thermal preference of the individuals being tested.

### **Concluding remarks**

There have been a variety of studies focusing on behavioural thermoregulation, but we offer a unique perspective on the effect of selection with our 7<sup>th</sup> generation of selected zebrafish. The preference arena used also offer a great alternative to measuring acute thermal preference tracking the individual’s thermal preference at a continuous pace whilst also tracking temperature throughout the entire arena. Selection for a higher upper thermal limit in our zebrafish appear to have stagnated and in combination with the results showing that selection did not affect thermal preference, conservational efforts could be considered as our results indicate that zebrafish already are living close to the maximum thermal limit they can evolve. We found that acclimation does not affect thermal preference in zebrafish. However, as no results from the acclimation experiment were statistically significant, we recommend performing the same experiment again to determine whether the trend seen in the results were disturbed by methodical errors or if there was a biological effect.

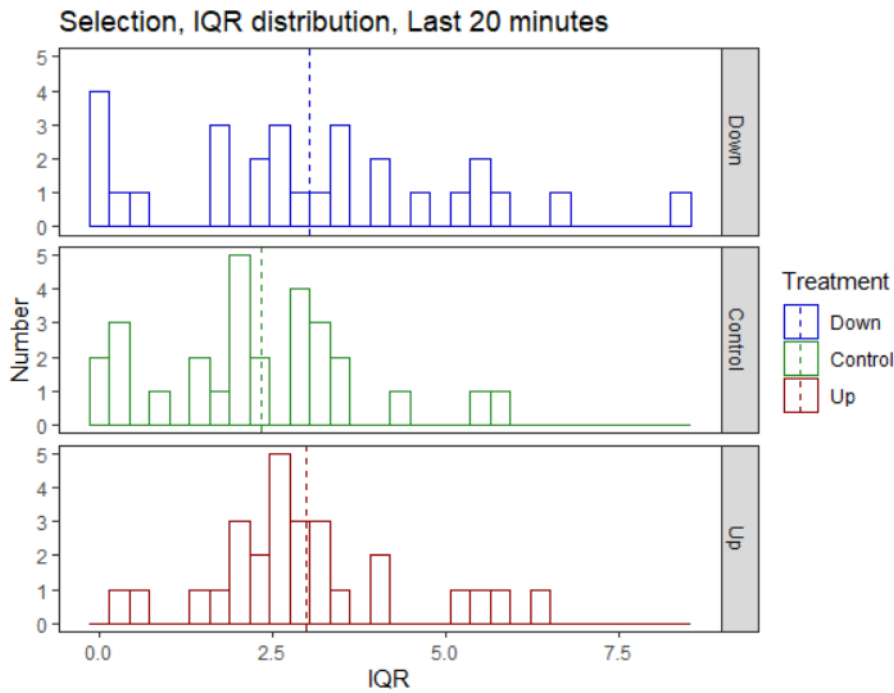
## References

- Andreassen, A. H. (2019). *Development of an automated annular arena and thermal preference of zebrafish selected for thermal tolerance*. NTNU,
- Angilletta, M. J., Niewiarowski, P. H., & Navas, C. A. (2002). The evolution of thermal physiology in ectotherms. *Journal of Thermal Biology*, *27*(4), 249-268. doi:[https://doi.org/10.1016/S0306-4565\(01\)00094-8](https://doi.org/10.1016/S0306-4565(01)00094-8)
- Araya-Ajoy, Y. G., Mathot, K. J., & Dingemanse, N. J. (2015). An approach to estimate short-term, long-term and reaction norm repeatability. *Methods in Ecology and Evolution*, *6*(12), 1462-1473. doi:<https://doi.org/10.1111/2041-210X.12430>
- Beaman, J. E., White, C. R., & Seebacher, F. (2016). Evolution of Plasticity: Mechanistic Link between Development and Reversible Acclimation. *Trends in Ecology & Evolution*, *31*(3), 237-249. doi:<https://doi.org/10.1016/j.tree.2016.01.004>
- 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. doi:<https://doi.org/10.1007/BF00005481>
- Biro, P. A., & Stamps, J. A. (2010). Do consistent individual differences in metabolic rate promote consistent individual differences in behavior? *Trends in Ecology & Evolution*, *25*(11), 653-659. doi:<https://doi.org/10.1016/j.tree.2010.08.003>
- Briggs, J. P. (2002). The zebrafish: a new model organism for integrative physiology. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *282*(1), R3-R9. doi:<https://doi.org/10.1152/ajpregu.00589.2001>
- Chapman, B. B., Eriksen, A., Baktoft, H., Brodersen, J., Nilsson, P. A., & Hulthen, K. (2013). A Foraging Cost of Migration for a Partially Migratory Cyprinid Fish. *PLoS One*, *8*(5). doi:<https://doi.org/10.1371/journal.pone.0061223>
- Chen, Z., Snow, M., Lawrence, C. S., Church, A. R., Narum, S. R., Devlin, R. H., & Farrell, A. P. (2015). Selection for upper thermal tolerance in rainbow trout (<em>&lt;em>Oncorhynchus mykiss</em></em> Walbaum). *The Journal of Experimental Biology*, *218*(5), 803. doi:<https://doi.org/10.1242/jeb.113993>
- Christensen, E. A. F., Norin, T., Tabak, I., van Deurs, M., & Behrens, J. W. (2021). Effects of temperature on physiological performance and behavioral thermoregulation in an invasive fish, the round goby. *Journal of Experimental Biology*, *224*(1). doi:<https://doi.org/10.1242/jeb.237669>
- Cote, J., & Clobert, J. (2007). Social personalities influence natal dispersal in a lizard. *Proceedings of the Royal Society B: Biological Sciences*, *274*(1608), 383-390. doi:[10.1098/rspb.2006.3734](https://doi.org/10.1098/rspb.2006.3734)
- Cuenca Cambronero, M., Beasley, J., Kissane, S., & Orsini, L. (2018). Evolution of thermal tolerance in multifarious environments. *Molecular Ecology*, *27*(22), 4529-4541. doi:<https://doi.org/10.1111/mec.14890>
- Díaz, F., Sierra, E., Denisse Re, A., & Rodríguez, L. (2002). Behavioural thermoregulation and critical thermal limits of *Macrobrachium acanthurus* (Wiegman). *Journal of Thermal Biology*, *27*(5), 423-428. doi:[https://doi.org/10.1016/S0306-4565\(02\)00011-6](https://doi.org/10.1016/S0306-4565(02)00011-6)
- Dingemanse, N. J., Dochtermann, N., & Wright, J. (2010). A method for exploring the structure of behavioural syndromes to allow formal comparison within and between data sets. *Animal Behaviour*, *79*(2), 439-450. doi:<https://doi.org/10.1016/j.anbehav.2009.11.024>
- Duffy, J. E., Lefcheck, J. S., Stuart-Smith, R. D., Navarrete, S. A., & Edgar, G. J. (2016). Biodiversity enhances reef fish biomass and resistance to climate change. *Proceedings of the National Academy of Sciences*, *113*(22), 6230. doi:[10.1073/pnas.1524465113](https://doi.org/10.1073/pnas.1524465113)
- Egan, R. J., Bergner, C. L., Hart, P. C., Cachat, J. M., Canavello, P. R., Elegante, M. F., . . . Kalueff, A. V. (2009). Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behavioural Brain Research*, *205*(1), 38-44. doi:<https://doi.org/10.1016/j.bbr.2009.06.022>

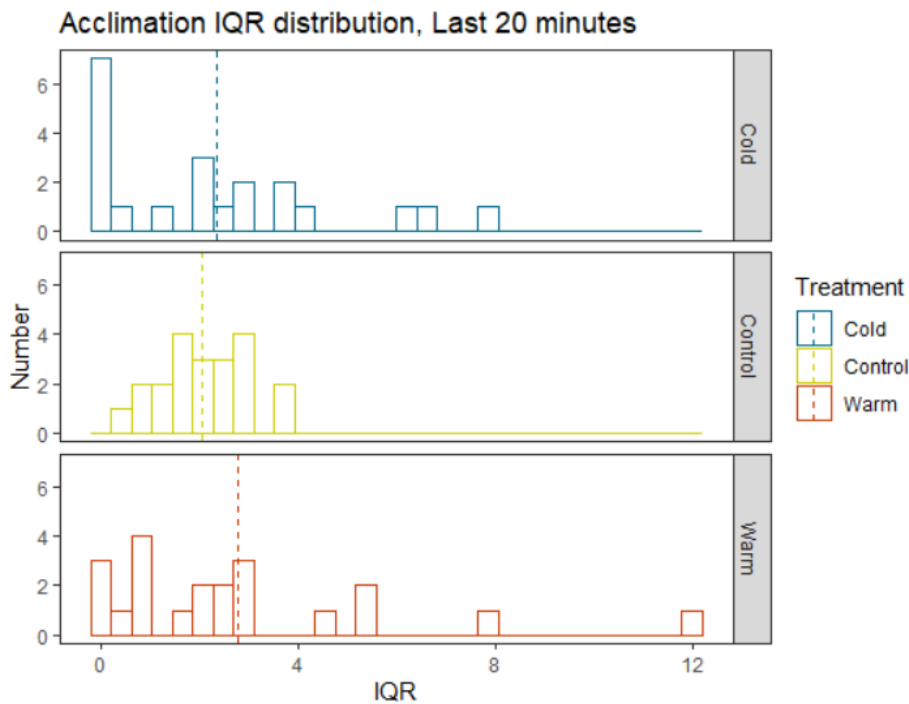
- Foray, V., Desouhant, E., & Gibert, P. (2014). The impact of thermal fluctuations on reaction norms in specialist and generalist parasitic wasps. *Functional Ecology*, *28*(2), 411-423. doi:<https://doi.org/10.1111/1365-2435.12171>
- Fry, F. E. J. (1947). Effects of the environment on animal activity. *Publ. Out. Fish. Res. Lab*, *55*(68), pp. 1-62.
- Golovanov, V. K. (2006). The ecological and evolutionary aspects of thermoregulation behavior on fish. *Journal of Ichthyology*, *46*(2), S180-S187. doi:<https://doi.org/10.1134/S0032945206110075>
- González, R. A., Díaz, F., Licea, A., Denisse Re, A., Noemí Sánchez, L., & García-Esquivel, Z. (2010). Thermal preference, tolerance and oxygen consumption of adult white shrimp *Litopenaeus vannamei* (Boone) exposed to different acclimation temperatures. *Journal of Thermal Biology*, *35*(5), 218-224. doi:<https://doi.org/10.1016/j.jtherbio.2010.05.004>
- Goulet, C. T., Thompson, M. B., Michelangeli, M., Wong, B. B. M., & Chapple, D. G. (2017). Thermal physiology: A new dimension of the pace-of-life syndrome. *Journal of Animal Ecology*, *86*(5), 1269-1280. doi:<https://doi.org/10.1111/1365-2656.12718>
- Haesemeyer, M. (2020). Thermoregulation in fish. *Molecular and Cellular Endocrinology*, *518*, 110986. doi:<https://doi.org/10.1016/j.mce.2020.110986>
- Hoffmann, A. A., Chown, S. L., & Clusella-Trullas, S. (2013). Upper thermal limits in terrestrial ectotherms: how constrained are they? *Functional Ecology*, *27*(4), 934-949. doi:<https://doi.org/10.1111/j.1365-2435.2012.02036.x>
- Hohn, C., & Petrie-Hanson, L. (2013). Evaluation of visible implant elastomer tags in zebrafish (*Danio rerio*). *Biology Open*, *2*(12), 1397-1401. doi:<https://doi.org/10.1242/bio.20136460>
- Huey, R. B., & Hertz, P. E. (1984). Is a jack-of-all-temperatures a master of none? *Evolution*, *38*(2), 441-444.
- Labbe, T. R., & Fausch, K. D. (2000). Dynamics of intermittent stream habitat regulate persistence of a threatened fish at multiple scales. *Ecological Applications*, *10*(6), 1774-1791. doi:[https://doi.org/10.1890/1051-0761\(2000\)010\[1774:DOISHR\]2.0.CO;2](https://doi.org/10.1890/1051-0761(2000)010[1774:DOISHR]2.0.CO;2)
- Li, H., Wang, Z., Mei, W., & Ji, X. (2009). Temperature acclimation affects thermal preference and tolerance in three *Eremias* lizards (Lacertidae). *Current Zoology*, *55*(4), 258-265. doi:[10.1093/czoolo/55.4.258](https://doi.org/10.1093/czoolo/55.4.258)
- Little, A. G., & Seebacher, F. (2013). Thyroid hormone regulates muscle function during cold acclimation in zebrafish (*Danio rerio*). *Journal of Experimental Biology*, *216*(18), 3514-3521. doi:[10.1242/jeb.089136](https://doi.org/10.1242/jeb.089136)
- López-Olmeda, J. F., & Sánchez-Vázquez, F. J. (2011). Thermal biology of zebrafish (*Danio rerio*). *Journal of Thermal Biology*, *36*(2), 91-104. doi:<https://doi.org/10.1016/j.jtherbio.2010.12.005>
- Macnaughton, C. J., Kovachik, C., Charles, C., & Enders, E. C. (2018). Using the shuttlebox experimental design to determine temperature preference for juvenile Westslope Cutthroat Trout (*Oncorhynchus clarkii lewisi*). *Conservation Physiology*, *6*(1). doi:[10.1093/conphys/coy018](https://doi.org/10.1093/conphys/coy018)
- Morgan, R. (2019). On the Observation of Wild Zebrafish (*Danio rerio*) in India. *Zebrafish*, *16*(6), 546-553. doi:[10.1089/zeb.2019.1778](https://doi.org/10.1089/zeb.2019.1778)
- Morgan, R. (2020). Physiological plasticity and evolution of thermal performance in zebrafish.
- Morgan, R., Finnøen, M. H., Jensen, H., Pélabon, C., & Jutfelt, F. (2020). Low potential for evolutionary rescue from climate change in a tropical fish. *Proceedings of the National Academy of Sciences*, *117*(52), 33365. doi:[10.1073/pnas.2011419117](https://doi.org/10.1073/pnas.2011419117)
- Morgan, R., Finnøen, M. H., & Jutfelt, F. (2018). CTmax is repeatable and doesn't reduce growth in zebrafish. *Scientific Reports*, *8*(1), 7099. doi:[10.1038/s41598-018-25593-4](https://doi.org/10.1038/s41598-018-25593-4)
- Morgan, R., Sundin, J., Finnøen, M. H., Dresler, G., Vendrell, M. M., Dey, A., . . . Jutfelt, F. (2020). Are model organisms representative for climate change research? Testing thermal tolerance in wild and laboratory zebrafish populations. *Conservation Physiology*, *7*(1). doi:[10.1093/conphys/coz036](https://doi.org/10.1093/conphys/coz036)

- Myrick, C. A., Folgner, D. K., & Cech, J. J. (2004). An Annular Chamber for Aquatic Animal Preference Studies. *Transactions of the American Fisheries Society*, 133(2), 427-433. doi:10.1577/02-154
- Nay, T. J., Johansen, J. L., Habary, A., Steffensen, J. F., & Rummer, J. L. (2015). Behavioural thermoregulation in a temperature-sensitive coral reef fish, the five-lined cardinalfish (*Cheilodipterus quinquelineatus*). *Coral Reefs*, 34(4), 1261-1265. doi:10.1007/s00338-015-1353-4
- Pilakouta, N., Killen, S. S., Kristjánsson, B. K., Skúlason, S., Lindström, J., Metcalfe, N. B., & Parsons, K. J. (2019). Temperature preference does not evolve in sticklebacks despite multigenerational exposure to elevated temperatures. *bioRxiv*, 615005. doi:10.1101/615005
- Réale, D., Reader, S. M., Sol, D., McDougall, P. T., & Dingemanse, N. J. (2007). Integrating animal temperament within ecology and evolution. *Biological Reviews*, 82(2), 291-318. doi:https://doi.org/10.1111/j.1469-185X.2007.00010.x
- Rey, S., Digka, N., & MacKenzie, S. (2015). Animal Personality Relates to Thermal Preference in Wild-Type Zebrafish, *Danio rerio*. *Zebrafish*, 12(3), 243-249. doi:10.1089/zeb.2014.1076
- Schram, E., Bierman, S., Teal, L. R., Haenen, O., Van De Vis, H., & Rijnsdorp, A. D. (2013). Thermal preference of juvenile dover sole (*Solea solea*) in relation to thermal acclimation and optimal growth temperature. *PLoS One*, 8(4), e61357.
- 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), 161-163. doi:10.1038/nclimate2145
- Sih, A., Ferrari, M. C. O., & Harris, D. J. (2011). Evolution and behavioural responses to human-induced rapid environmental change. *Evolutionary Applications*, 4(2), 367-387. doi:https://doi.org/10.1111/j.1752-4571.2010.00166.x
- Somero, G. N. (2010). The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine 'winners' and 'losers'. *The Journal of Experimental Biology*, 213(6), 912-920. doi:10.1242/jeb.037473
- Spence, R., Fatema, M. K., Reichard, M., Huq, K. A., Wahab, M. A., Ahmed, Z. F., & Smith, C. (2006). The distribution and habitat preferences of the zebrafish in Bangladesh. *Journal of Fish Biology*, 69(5), 1435-1448. doi:10.1111/j.1095-8649.2006.01206.x
- Sundin, J., Morgan, R., Finnøen, M. H., Dey, A., Sarkar, K., & Jutfelt, F. (2019). On the Observation of Wild Zebrafish (*Danio rerio*) in India. *Zebrafish*, 16(6), 546-553. doi:10.1089/zeb.2019.1778
- Ward, A. J. W., Hensor, E. M. A., Webster, M. M., & Hart, P. J. B. (2010). Behavioural thermoregulation in two freshwater fish species. *Journal of Fish Biology*, 76(10), 2287-2298. doi:https://doi.org/10.1111/j.1095-8649.2010.02576.x
- Woods, H. A., & Harrison, J. F. (2002). Interpreting rejections of the beneficial acclimation hypothesis: When is physiological plasticity adaptive? *Evolution*, 56(9), 1863-1866. doi:https://doi.org/10.1111/j.0014-3820.2002.tb00201.x
- Wu, M.-X., Hu, L.-J., Dang, W., Lu, H.-L., & Du, W.-G. (2013). Effect of thermal acclimation on thermal preference, resistance and locomotor performance of hatchling soft-shelled turtle. *Current Zoology*, 59(6), 718-724. doi:10.1093/czoolo/59.6.718

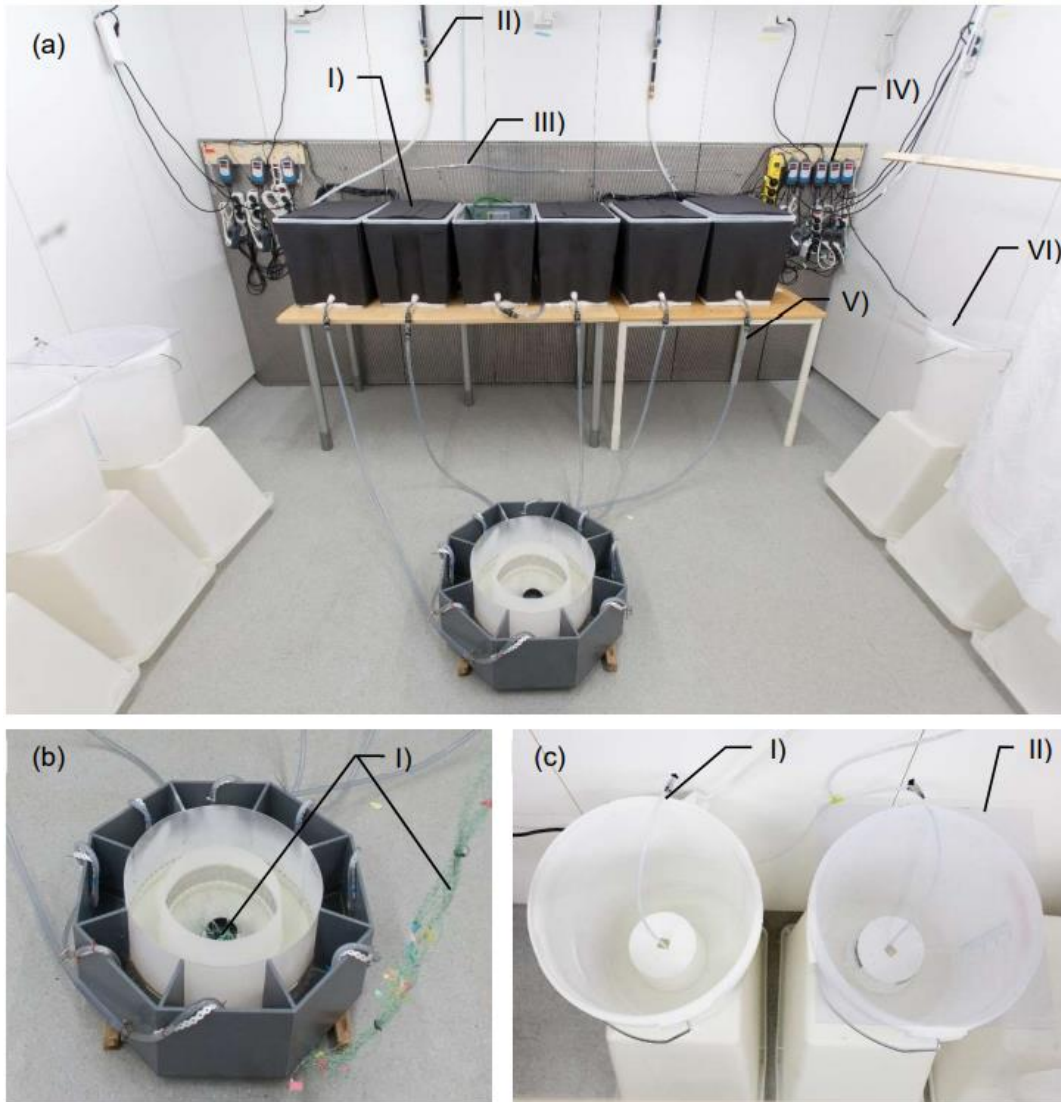
## Appendix



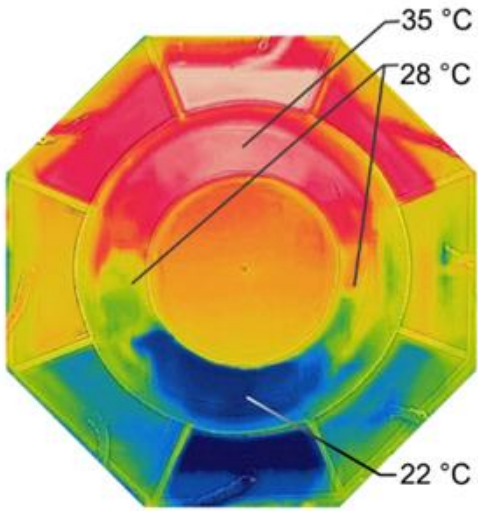
**Figure 1** Histogram of IQR of preferred temperature in the selection experiment during the last 20 minutes for the Down-selected, Control and Up-selected treatment. Dashed lines are the mean IQR for the given treatment. Each colour represents a different treatment and count is number of individuals with a given IQR.



**Figure 2** Histogram of IQR of preferred temperature in the acclimation experiment during the last 20 minutes for the Cold, Optimum (control) and Warm treatment. Dashed lines are the mean IQR for the given treatment. Each colour represents a different treatment and count is number of individuals with a given IQR.



**Figure 3** (a) Room setup for thermal preference trials showing: I) the five header tanks (plus one backup tank) containing II) carbon filtered water as well as III) filtered air; IV) Thermostats-controlled temperature inside the header tanks by heating titanium rods when temperature was below desired; V) Adjustable hoses supplying the outer chambers of the preference arena with water; VI) habituation tank placement (only habituation tanks on the left side were used and they were not elevated). (b) Photograph of the preference arena with the I) thermocouples entering from the drainage hole and being fastened in the inner wall. (c) I) Habituation tanks supplied with I) air and a II) transparent lid to maintain the correct temperature. Photos by Eirik R. Åsheim (Andreassen, 2019)



**Figure 4** The preference arena shown with water taken with a thermal camera showing approximate temperatures during a trial (Andreassen, 2019).



