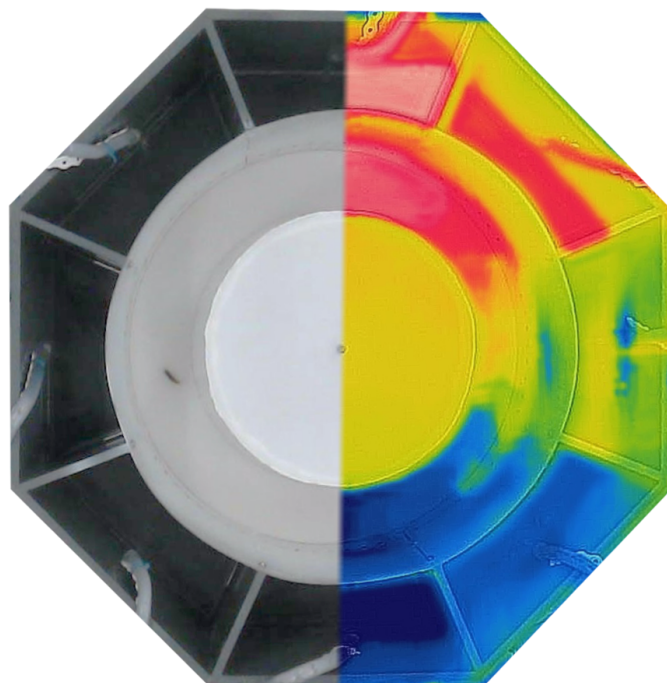Anna H. Andreassen

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Master's thesis in Biology
Supervisor: Fredrik Jutfelt
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NTNU
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First, I would like to thank my supervisors Fredrik Jutfelt and Rachael Morgan for guidance, support and encouragement. Rachael, for being awesome, patient and having a response time that exceeds all possible expectations, and Fredrik for his inspiring approach to science and setting a great example on how to balance research with other fun activities (i.e. celebrations and bouldering). I am grateful for being included in the zebrafish team and would like to thank the rest of group: Tine and Hanne for providing a solid study system; Marga for contributing valuable hours in the lab and making it possible to run trials all day during the experimental period; Mette for being a moral support and providing clarifications regarding the statistical work; and Josefin and Jeff for being inspiring collaborators and "extra supervisors" during the years in the research group. Eirik has played a special part during these years, as both a close friend and an extraordinary college. It has been interesting, frustrating and amazing to work, play and travel together with him. I look forward to many years of collaborations with this group! I would also like to thank my family and friends for support and motivation though my five years in Trondheim: Anine has been of great help in reviewing the manuscript and an invaluable friend; Agnes and Kristine, my former flatmates, have made the student life a wonderful experience; my hiking and climbing-buddies have helped maintain my physical and mental health and provided hours of fun between intense and exciting periods; and a special thanks to Henrik and Frost for being fantastic and for disrupting the work with this thesis.

Abstract

Behavioural thermoregulation allows ectotherms to control body temperature by choosing habitat temperatures. This is an essential survival strategy for aquatic ectotherms as they are challenged by both increasing water temperatures and stochastic heating events from climate change. Acute heat stress can be a strong selective force, and adaptation to warming may indirectly affect traits correlated to acute thermal tolerance. To predict how organisms will adapt to environmental change, research on correlated traits is crucial. In this study we tested whether selection for thermal tolerance led to differences in the thermal preference of zebrafish. A population of wild-caught zebrafish has been selected for upper thermal tolerance in three treatments, each kept in two replicate lines: one treatment is selected for increased tolerance (High), the second is selected for decreased tolerance (Low) and the third is a control treatment of randomly selected thermal tolerance (Random). Thermal preference of the fourth generation of these selected treatments were measured in a custom-built annular arena where individuals were tested in an even thermal gradient from 22 to 35°C. In addition, critical thermal maximum was measured for all individuals to correlate individual measures of thermal tolerance and preference. Through continuous recording of video, logging of temperature using 24 thermal sensors, and tracking the position of the fish with an automated tracking software, we achieved high-resolution data of individual temperature choices. We validate that the presented setup and method for data collection is suitable for research on thermal preference of a small eurythermal species. Based on our findings we present recommendations for future research using annular arenas. No difference in thermal preference (median occupied temperature) or range of thermal preference (interquartile range of occupied temperatures) was found between the treatments. There were, however, indications of a wider range of preferred temperatures for the Low treatment. The findings are currently inconclusive regarding whether correlations exist between these disparate traits within thermal biology (acute physiological tolerance and behavioural thermoregulation).

Samandrag

Gjennom åtferdsbasert temperaturregulering kan ektoterme dyr regulera kroppstemperaturen ved å velje habitatområder med ulike temperaturar. Dette er livsviktig for akvatiske ektotermer som blir utsett for aukande vasstemperaturar samt ei auke i omfanget og frekvensen av hetebølgjer med klimaendringane. Akutt varmeeksponering kan medføra seleksjon for høg temperaturtoleranse og slik seleksjon kan ha indirekte effektar på andre trekk. For å kunne føreseie korleis organismar vil tilpasse seg miljøendringar er det difor viktig å forske på potensielt korrelerte trekk. I dette studiet har vi testa om seleksjon for temperaturtoleranse har ført til endra preferanse for temperaturar hos sebrafisk. Ein populasjon av villfanga fisk har blitt selektert for øvre temperaturtoleranse i tre dupliserte linjer: ei er selektert for auka toleranse (High), den andre får lågare toleranse (Low), og den tredje er ei kontrollinje med tilfeldig vald temperaturtoleranse (Random). Preferansen for temperaturar vart målt for den fjerde generasjonen av desse selekterte linjene i eit spesialbygd ringkammer kor individa vart testa i ein jamn temperaturgradient mellom 22 og 35 °C. I tillegg vart den øvre kritiske temperaturgrensa målt for alle individa for å undersøkje korrelasjonen mellom temperaturtoleranse og -preferanse. Gjennom kontinuerleg videoopptak, temperaturlogging med 24 temperatursensorar og å spore posisjonen til fiskane med ein automatisert programvare, fekk vi data av høg oppløysing på kvart individ sitt temperaturval. Vi validerer at det presenterte oppsettet og metoden for datainnsamling eignar seg for forsking på temperaturpreferanse av små fisk som toler eit vidt spenn av temperaturar. Basert på funna har vi gjett anbefalingar for vidare forsking med ringkammer. Det vart ikkje funne ulik temperaturpreferanse (medianen av temperaturane fisken oppheldt seg i) eller spreiing på temperaturpreferanse (kvartilbredda av temperaturane fisken oppheldt seg i). Likevel fann vi indikasjonar på at preferansetemperaturane i Low-linja kunne ha ei større spreiing. Funna er ikkje einstydande angåande om det er ein korrelasjon mellom akutt toleranse og åtferdsbasert temperaturregulering.

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Introduction

It is predicted that climate change will include global warming accompanied by an increasing frequency and severity of stochastic heating events (Seneviratne *et al.*, 2014). Extreme fluctuations in temperature might be a considerable challenge to organisms in freshwater environments (Burggren, 2018). In order to predict how these animals might respond to abrupt changes we must increase our knowledge about the thermal limits of animals and their current ecological limitations (Stillman, 2019, MacMillan, 2019).

Adaption, acclimatisation and migration are considered the main coping mechanisms for animals in response to environmental challenges (Bowler, 2005, Somero, 2010, Sandblom *et al.*, 2016). Genetic adaption allows phenotypes in future generations to better match the new environment when conditions change in one direction over multiple generations. This is, however, a slow process and when short-term and abrupt fluctuations occur, the result could be a genetically determined phenotype-environment mismatch (Burggren, 2018). Acclimatisation allow alterations of phenotypes without changing the genotype and can therefore increase the individual resilience to rapid environmental changes. Such phenotypic plasticity can be reversible adjustments to changes, or irreversible changes (e.g. developmental plasticity) (Beaman, White and Seebacher, 2016).

An additional method of compensation for changes in the environment, perhaps previously underestimated, is behavioural thermoregulation (Sunday *et al.*, 2014). The body temperature of ectothermic organisms fluctuates with the ambient temperature, which thus directly affects their physiology, biochemical rates and behaviour (López-Olmeda and Sánchez-Vázquez, 2011). Consequently, aquatic ectotherms face a great challenge when water temperature rises. Freshwater species might be especially challenged due to their geographically restricted habitats (Perry *et al.*, 2005, Woodward, Perkins and Brown, 2010). Ectotherms can control their body temperature in a thermally heterogeneous environment by moving to a location of a preferred temperature (López-Olmeda and Sánchez-Vázquez, 2011). By this behavioural thermoregulation, individuals can indirectly change rates of physiological processes. This active choice of temperature, known as the animal's thermal preference (Fry, 1947), has been correlated to temperatures optimal for growth and metabolism, and has been described as a behavioural mechanism that can maximise fitness (Fry, 1947, Jobling, 1981). Furthermore, thermal preference is a mechanism of avoiding potentially harmful temperatures and is thus an

essential survival strategy when aquatic ectotherms are challenged by both stochastic heating events and long-term increasing water temperatures.

The thermal biology of fishes has been described by three aspects: 1) the behavioural thermoregulation described above, 2) the thermal range and optimum for growth performance, and 3) the upper and lower thermal tolerances (Fry, 1947). Opposing hypotheses have been proposed to explain the second aspect. There is evidence supporting the oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis implying that the temperature which maximises aerobic scope, the scope over which an animal can increase its metabolism, will maximise growth and other crucial performances such as reproduction, and behaviour (Pörtner and Knust, 2007, Pörtner and Farrell, 2008). Other evidence supports the multiple performances – multiple optima (MPMO) view, which states that various physiological mechanisms have different optimum temperatures (Clark, Sandblom and Jutfelt, 2013). The third aspect, thermal tolerance, describes the temperature range, bounded by upper and lower critical thermal limits (CT_{max} and CT_{min}, respectively), where an organism can function. Outside this range, performance ceases (e.g. loss of motor function or death) (Beitinger and Lutterschmidt, 2011, MacMillan, 2019). Meta-analyses show global changes in the geographic distribution of a range of species, and that the shifts correspond with the predictions based on their thermal tolerance (Parmesan and Yohe, 2003, Root *et al.*, 2003, Sunday, Bates and Dulvy, 2012). Underlying mechanisms of thermal tolerance and the relevance of different traits used to measure thermal limits is thus a focal point for multiple studies. Acute heat stress is a strong selective force that might indirectly affect traits correlated with thermal tolerance (Santos, Castañeda and Rezende, 2012). It is thus important to study changes in the whole thermal biology of populations occurring with selection for thermal tolerance.

Syndrome research is one approach to understand correlated traits. Traditionally, this field of research has centred on differences between closely related species and populations in different habitats, but it could also be applied to describe covariation in traits between individuals within a population (Réale *et al.*, 2010). Both life-history, behavioural and physiological traits have been connected in a syndrome framework (Rey, Digka and MacKenzie, 2015, Goulet, Thompson and Chapple, 2017, Goulet *et al.*, 2017). The Pace-of-Life Syndrome (POLS), predicts that physiological traits characteristic for "fast" living organisms (e.g. high metabolic rate, early maturation and early death) will be correlated with higher levels of certain behavioural traits such as aggressiveness, boldness and exploratory behaviour (Goulet,

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Thompson and Chapple, 2017). Despite how strongly temperature is known to affect both physiological processes and the behaviour of animals, little is known about the connection between personality traits and temperature (Biro and Stamps, 2010). Goulet *et al.* (2017) suggested that thermal traits should be included in the POLS with a cold-hot axis comparable to a shy-bold axis. According to this model, cold-type individuals might prefer lower temperatures, perform optimally at lower temperatures and have lower thermal tolerance, than hot-types. Studies have supported the inclusion of thermal preference in the syndrome framework by reporting correlations between higher thermal preference and: proactiveness (Rey, Digka and MacKenzie, 2015); higher optimal performance temperature, higher activity and boldness (Goulet *et al.*, 2017); and warmer habitats (Michelangeli *et al.*, 2018).

Traditionally, thermal preference has been categorised as either acute preference or as a final preferendum (Fry, 1947, Reynolds and Casterlin, 1979). The acute thermal preference is often measured within two hours in a thermal gradient. This can be positively, negatively or not affected by recent thermal experience (Johnson and Kelsch, 1998). Alternatively the final thermal preferendum of a species can be determined in long-term experiments lasting between 24 to 96 hours (Fry, 1947, Reynolds and Casterlin, 1979). The final preferendum becomes equal to the self-chosen acclimation temperature by allowing a sufficient amount of time for the fish to gravitate towards and reacclimate to the preference temperature.

Thermal preference has been studied in multiple experimental setups (Fry, 1958, McCauley, 1977). Fry (1958) divided the main approaches to study environmental gradients into two categories, vertical and horizontal. Vertical thermal gradients take advantage of the vertical stratification of water of different temperatures, but they are susceptible to confounding effects of pressure and light intensity along the gradient. Horizontal setups were divided into linear, annular, transverse, rosette and concentric gradients. The most commonly used method has been the linear versions, either in form of a trough (Mathur, Schutsky and Purdy Jr, 1982, Lafrance *et al.*, 2005, Rey, Digka and MacKenzie, 2015) or a shuttlebox (Neill, Magnuson and Chipman, 1972, Macnaughton *et al.*, 2018). Although still widely used, an important drawback of these designs is that the thermal choices can be affected by thigmotactic orientation due to the uneven distances to corners and walls. Additionally, the shuttlebox system might require a higher learning capacity, as the experimental animal must choose between two tanks of different temperatures and the movement of the organism determines the temperature in the tanks (Lagerspetz and Vainio, 2006). An annular arena has the advantage that it presents the same

range of temperatures to all experimental animals. Furthermore, the site-specific bias, as well as uneven light conditions and pressure, can be avoided in this type of setup. The annular arena design must ensure predictable currents and mixing of water, and allow a sufficiently high water column while preventing thermal stratification. By improving previous annular arena designs, Myrick, Folgner and Cech Jr (2004) developed a system with constant flow rates and temperatures, allowing a wide, yet fine scaled gradient suitable for eurythermal species. Following their setup, we created a custom-built annular arena to test individual thermal preference of zebrafish (*Danio rerio*). Using continuous video recording, logging of temperature with thermocouples, and tracking the position of individual fish with an automated tracking software, this system provided high-resolution data of individual temperature choices.

The eurythermal zebrafish, native to Eastern South Asia, live in temperatures from 14 to 39 °C (López-Olmeda and Sánchez-Vázquez, 2011). They inhabit shallow waters, such as rice paddies and slow-moving streams, strongly affected by the monsoon climate with considerable seasonal variation (Engeszer *et al.*, 2007, López-Olmeda and Sánchez-Vázquez, 2011). This small species has a short generation time and is easy to breed, making it suitable for experimental studies. It has become one of the most important model organisms in several research fields, such as genetics, developmental biology and physiology (López-Olmeda and Sánchez-Vázquez, 2011). Not only is it important to study the thermal biology of this species in itself: it is also a suitable model species for investigating thermal biology, and for gaining knowledge that can be applied to aquatic ectotherms in general.

The first aim of the present study was to develop an experimental setup and protocol to test thermal preference of small, eurythermal fish. We compared the first and the last period after the fish was introduced to the preference arena to assess whether the individuals discriminated between and chose different temperatures. The second aim was to test whether disparate traits within thermal biology, behavioural thermoregulation and acute thermal tolerance, covary and align in a syndrome. We hypothesised that selection for thermal tolerance would affect thermal preference if these traits are correlated. To test this, we compared the median occupied temperatures and the breadth of the thermal preference of individual zebrafish selected for thermal tolerance by CT_{max} tests. We predicted that the individuals selected for high thermal tolerance would show a higher thermal preference than individuals selected for lower thermal tolerance.

Methods

Selection treatments

Zebrafish (*Danio rerio*) originating from wild populations collected in West Bengal, India in 2016 and brought to the Norwegian University of Science and Technology (NTNU), Trondheim. Three treatments, each kept in two separate replicate lines, were selected from the ancestral population (n=1200) based on thermal tolerance by critical thermal maximum tests (CT_{max}). The six replicate lines were named after their selection treatment: High (H1, H2); Low (L1, L2); and Random (R1, R2) (Figure 1 (a)). The Random lines were kept as a control for domestication effects and were created and maintained from randomly selected fish after the CT_{max} tests. The High lines were created and maintained from the fish with the 33% highest CT_{max} values from each selection event, and Low from the 33% lowest. All treatments were duplicated to separate the selection pressure from CT_{max} from genetic drift.

Experimental animals and husbandry

From each selection treatment, 84 zebrafish of the F_3 selected generation were used to reproduce a F_4 generation for this experiment. All experimental animals were kept in the animal facility at NTNU. Each of the six replicate lines was divided into two holding tanks (50x30x30 cm, Figure 1(a)) filled with 35 L water, resulting in groups of 20 individuals per tank. The tanks were placed in a stratified manner in the room based on distance to the door and shelf height. Lights were on in a 12 h:12 h light-dark cycle with one hour of light-ramping in both directions, and the water temperature was maintained at 28 °C. Each tank contained carbon-filtered water, added salt (0.37 ppt) and water conditioner (AquaSafe, Tetra, Blacksburg, VA, USA), plastic plants as environmental enrichment and was aerated using two foam filters. The fish were fed *ad libitum* with ground fish food flakes (TetraPro Energy, Tetra, Blacksburg, VA, USA) twice a day throughout the experiment. Temperature, water quality and salinity were routinely controlled.

Ethical statement

The experiment was approved by the Norwegian Animal Research Authority (Permit Number: 8578) and all procedures performed were in accordance with the Norwegian Animal Welfare Act and the Regulation on the use of Animals in research.
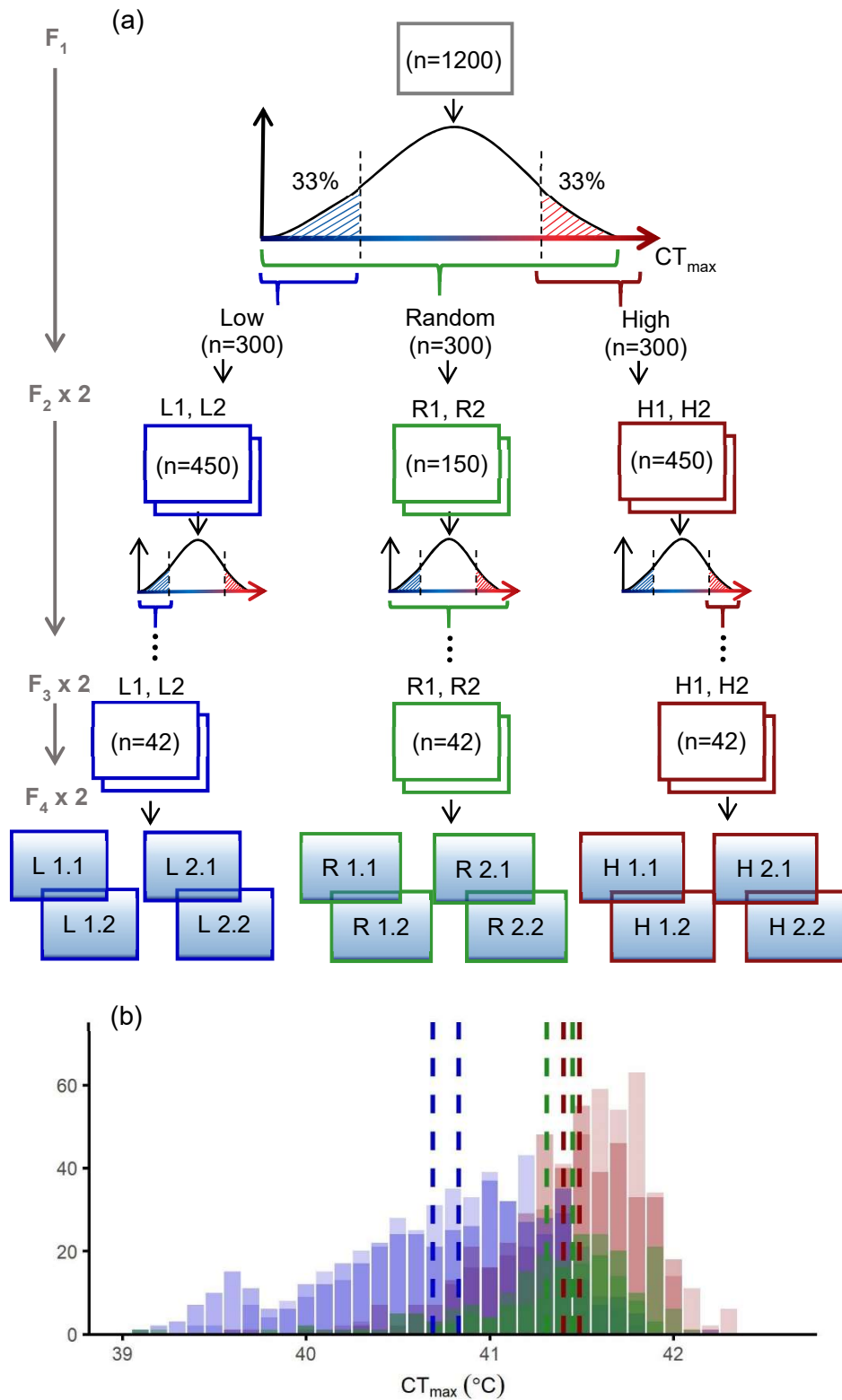
Figure 1 (a) Outline of the selection experiment creating two replicate lines of each treatment selected for increased (High), decreased (Low) and a control treatment with randomly selected (Random) thermal tolerance by CT_{max} tests. Each of the six replicate lines were divided into two holding tanks. (b) CT_{max} results from the F_4 generation in the main selection experiment. Dashed lines are mean values for each replicate line.

Experimental setup

Water supply system design

Five header tanks (57x39x42 cm) were built to heat water to supply the preference arena. Carbon filtered water was supplied to these via float valves (Fluidmaster Universal, Tubman, Gustavsberg, Sweden) keeping the water at approximately 40 L, avoiding overflow and allowing a continuous flow to the preference arena. Seacock outlets with hose nipples were placed close to the bottom of each header tank and connected to the arena via tubing. Two valves were placed along each tube: a valve proximate to the header tank for turning water flow on and off, and closer to the arena a valve to control flow rates. The header tanks were equipped with an air supply, titanium heaters (18x100 W and 12x500 W, TH-100, Aqua Medic, Bissendorf, Germany) controlled by thermostats (8xITC-306T, Inkbird, Shenzhen, China) and all tanks were insulated (11 mm Armaflex, Armacell, Münster, Germany). They were placed on a table covered with expanded polystyrene, allowing water to be supplied from beneath the tanks to the float valves and keeping the tanks 0.5 m above the preference arena. The hydrostatic pressure from the height difference was used to create a stable flow of water through the preference arena. Each header tank was controlled to hold one of five temperatures: 22, 26, 30, 34, and 38 °C, and the oxygen saturation was maintained high (dissolved oxygen > 95% air saturation) in all tanks. (Supplementary material, Figure S1).

Preference arena design

A 60 cm wide preference arena was designed and custom built in collaboration with the workshop at NTNU. The arena was divided into eight outer compartments, a middle swimming channel and an inner area with a drain in the middle (Figure 2). Nine evenly distributed holes (5 mm diameter) were made between each outer compartment and the swimming channel, five cm up on the dividing wall, allowing an even waterflow inwards and creating a gradient in the swimming channel ranging between 22 and 35 °C (Figure 3 (a)). The swimming channel had a 42 cm outer diameter and a 30 cm inner diameter, creating a 12 cm wide channel for the fish to swim in, with a four cm high water column. (Supplementary material, Figure S2).

Water from the header tanks was supplied to the eight outer compartments. One tube from the coldest (22 °C) and one from the hottest (38 °C) header tank led to outer compartments on opposite sides of the arena. Each of the three header tanks with intermediate temperatures supplied two outer compartments through pairs of tubes, the two compartments closest to the 22 °C compartment were filled with water of 26 °C, the two middle were filled with water of

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30 °C and the ones closest to the 38 °C compartment were filled with water of 34 °C. The flow rate from each tube was kept at 8 mL s^{-1}. The inner wall of the swimming channel comprised three rows of holes. The upper and lower were of 38 holes of 3 mm diameter to drain the water to the central part of the arena. The middle row contained 24 holes of 1.5 mm diameter. These were placed so that the three holes were evenly distributed towards each outer compartment and were created to evenly position thermocouples in the swimming channel. (Supplementary material, Figure S2).
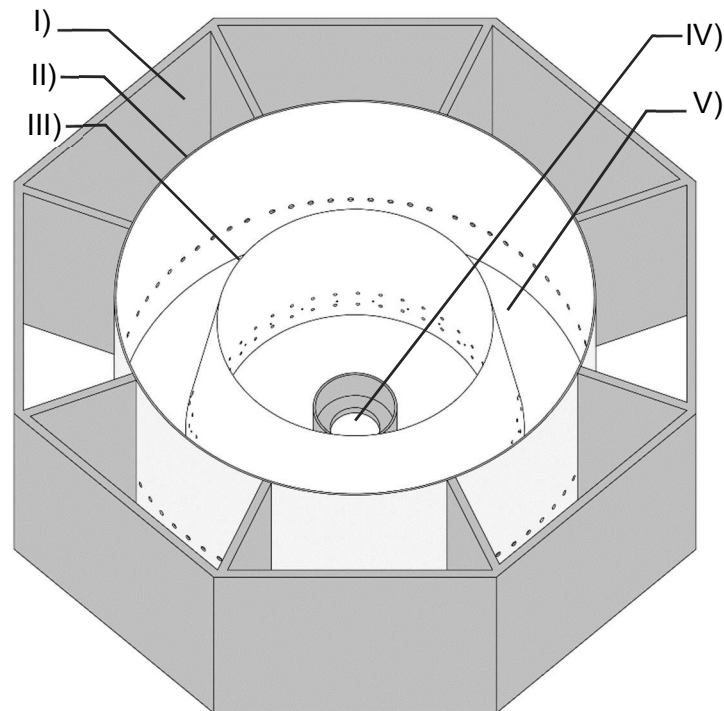

Figure 2 Illustration of the annular arena comprised of I) eight outer compartments, II) the outer wall of the swimming channel, III) the inner, cone shaped wall of the swimming channel, IV) the central drain and V) the swimming.

Number, placing and size of the holes, as well as flow rate, were chosen to optimise the gradient by minimising the vertical and horizontal stratification whilst still being suitable for the experimental fish. This was determined through multiple tests by monitoring the thermal gradient with fluorescent dye, an infra-red camera (Figure 3 (b)) and by taking manual temperature measurements of vertical and horizontal transects of the swimming channel with a high-precision thermometer (Testo-112, Testo, Lenzkirch, Germany). The setup was tested with fish of the same age and size as the experimental individuals to ensure that the flow rate was not too high for normal swimming and that the holes were suitable for the fish by not allowing them to swim through or get stuck.

The thermal gradient in the swimming channel (Figure 3 (a)) was measured by twenty-four type K thermocouples (10 m, Pico Technology, Cambridgeshire, UK). The thermocouples were thread up through the central drain of the arena to prevent any visual covering and were evenly spaced along the inner wall of the swimming channel through 24 holes. They were connected to USB port thermocouple data loggers (TC-08, Pico Technology, Cambridgeshire, UK) in an observation room. The inner wall of the swimming channel was cone shaped (base: 30 cm diameter, top: 24.5 cm diameter) to allow view of the whole channel by a USB camera (C1, Kurokesu, Vilnius, Lithuania) mounted 2.1 m above the arena. To create an even background for video analysis and tracking, the swimming channel was painted white.
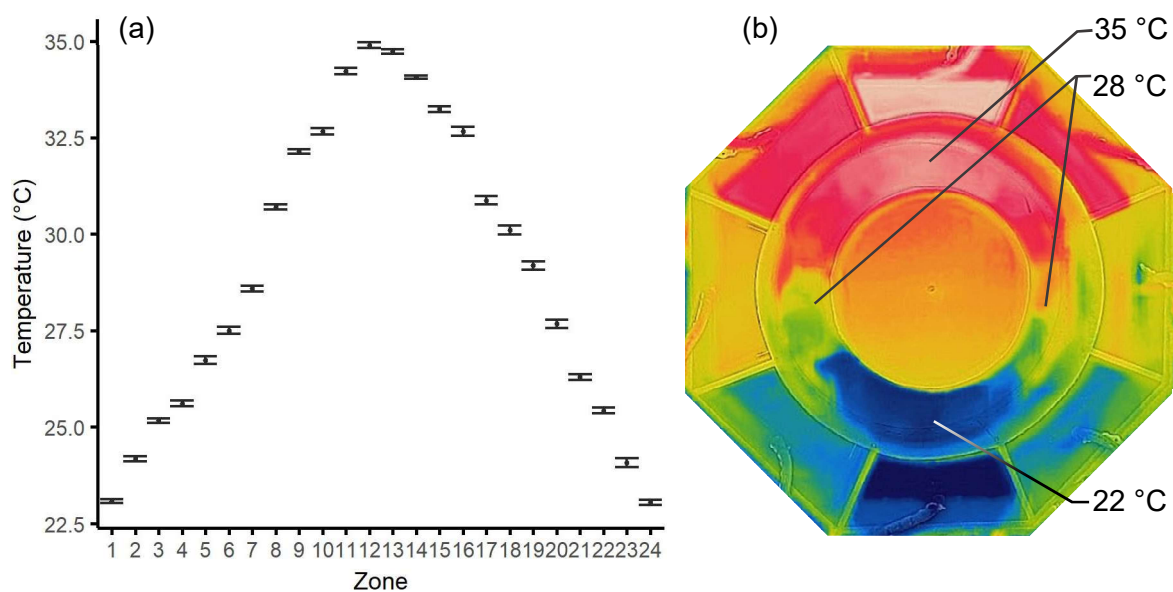

Figure 3 (a) Water temperatures of all trials (mean ± SE) measured in 24 zones equally distributed around the inner side of the swimming channel. (b) Infrared image of the annular arena showing the thermal gradient ranging from 35 to 22 °C. The gradient between the thermal extremes are mirrored.

Habituation tanks

To allow the fish to habituate to the setup and being in isolation, four tanks were built to simulate the preference arena and were kept in the same room as the trials were performed. They were made by placing a 15 cm diameter, white bucket inside a white bucket with a 31 cm diameter bottom, creating an 8 cm wide swimming channel. Water levels were kept at 4 cm in the acclimation channel to resemble the arena. Holes were drilled in the inner bucket and air was supplied to this with an air stone to ensure mixing of water in the outside channel. The habituation tanks were covered with lids and water temperature was controlled by the room

temperature. The lids were transparent so that the fish experienced the natural light cycle. All the habituation tanks were placed behind curtains, 45 cm above the ground to limit disturbance from the running trials. (Supplementary material, Figure S1).

Critical thermal maxima (CT$_{max}$) design

CT$_{max}$ was measured in the setup described by Morgan, Finnøen and Jutfelt (2018) to determine individual acute upper thermal tolerance. The test was done in 25x22x18 cm tanks filled with 9 L of water and using a heating rate of 0.3 °C min^{-1}. The experimental tanks were divided into a main and a heating compartment separated by a mesh to prevent the fish from swimming into the heating compartment. A 300 W coil heater within a steel cylindrical case was connected to a water pump (Eheim Universal 300, Deizisau, Germany) to ensure circulation of water to create homogenous temperature in the main compartment. A thermometer (Testo-112, Testo, Lenzkirch, Germany) was mounted on the side wall of the main compartment to continuously display the water temperature. (Supplementary material, Figure S3)

Experimental procedure

During November 8th and 9th 2018, 96 individuals were tagged (n=8 per tank) with two visible implant elastomer tags (Northwest Marine Technologies, Shaw Island, WA, USA) with a colour combination unique to the tank. The tags were located right and left of the dorsal fin, based on the site shown to be the best suited for tagging (Hohn and Petrie-Hanson, 2013). Prior to tagging the fish were anesthetised with 110 mg L^{-1} buffered tricaine methane sulfonate (MS-222). After tagging, while still anesthetised, all fish were weighted to nearest 0.01 g and standard length was measured to nearest 0.01 cm using a digital calliper. The fish were left to recover in an aerated tank before transferred back to their previous holding tanks. Six fish from each tank were used in the experiment (N=72), providing two spare fish in case of mortality or unsuccessful tests.

Thermal preference

Thermal preference trials were performed between November 18th and December 11th. Four fish were tested per day with continuously rotating the order of treatments (Random, High, Low) and replicate lines (R1, R2, H1 etc.) of the first, second, third and fourth fish tested, to control for time of day effects. Fish were transferred to the habituation tanks in isolation, 24 hours before their thermal preference test. During this period, they were not fed and there was minimal disturbance. Water temperature in the habituation tanks were measured at the start and

end of the acclimation period to ensure it was 28.1 ± 0.3 °C (mean $\pm$ SD). Each trial was started by gently netting a fish out from the respective habituation tank with a fine-meshed hand net allowing the fish to remain partly submerged in water while being moved. We attempted to let the fish swim into the net by guiding it with another hand net. After leaving the experimental room, the executor would start the video recording and temperature logging from the observation room. The trials were ended after two hours and the fish were brought back to their respective holding tanks. After half the trials were conducted, the thermal gradient was rotated 90 ° to check for room and setup effects such as light intensity or disturbance from the observation room. This was done by moving the tubes from the header tanks, two compartments over in the same direction. As a pilot experiment for prolonged exposure to the gradient, one replicate tank of Random fish was tested twice in the arena and tested for 16 h in their second trial.

CT_{max}

The CT_{max} test was performed on all tagged individuals on December 21st. The eight tagged fish from each replicate line were tested in groups and transferred to the CT_{max} main compartment with water of 28 °C. The water was heated at a rate of 0.3 °C min^{-1} and individual fish were netted out as they reached CT_{max}. Loss of equilibrium and inability to regain equilibrium was used as thresholds for CT_{max} and used to estimate the individual thermal tolerance. Once reaching CT_{max} the fish were immediately transferred to individual containers with MS-222 sitting in an ice bath. CT_{max} temperatures were recorded, individual fish IDs were determined, and weights and standard lengths were measured. After measurements were taken the fish were kept in ice water and humanely sacrificed.

Data collection and analyses

Data collection

Videos were recorded to a computer in the observation room and temperature was continuously logged in a data logging software (PicoLog 6, Pico Technology, Cambridgeshire, UK) with two second intervals from the 24 thermocouples spread around the inner wall of the arena. EthoVision (XT 12.0, Noldus IT, Wageningen, Netherland) was used to track individual fish in the recorded videos. The area of an individual was specified for every trial to ensure proper tracking of all fish. The swimming channel was divided into 24 zones in the tracking software, based on the placing of the thermocouples. For every trial, each zone was assigned a temperature corresponding to the mean logged temperature for the last hour of a given trial to

ensure that variations in the gradient between trials would not influence the results. Data on the cumulative duration in each zone for each individual, was collected from EthoVision.

Statistical analyses

The statistical analyses were performed with R 3.5.0 (R Core Team, 2018) using the nlme (Pinheiro *et al.*, 2017) and car (Fox *et al.*, 2012) packages. Cumulative duration in each area for the individual fish was used to create a distribution of occupied temperatures. Median occupied temperature, hereafter called preference temperature, and the interquartile range (IQR) between the first and third quartiles of the distribution was used as measures of thermoregulatory behaviour. Three linear mixed-effect models were fitted to the data on which two-way ANOVAs were performed. The first model tested the mean between-group differences in preference temperature. The second model was performed to assess the change in IQR from the first twenty minutes to the last twenty minutes of the two-hour trials, and to test whether the IQR was different between the treatments. Preference temperature was modelled with the independent categorical variable Treatment (Random, High and Low), and the covariates Order of daily tested individuals (1-4) and Weight measured at the end of the experiment. Both Order and Weight was mean centred to have the mean value of both in the intercept. The model for IQR was similar but included the factor Period (First twenty minutes, Last twenty minutes). Holding tank and replicate number was added to both models as random factors to account for the nested random structure. The third model tested between-group difference in CT_{max} and the effect of being tested in the preference arena (Tested, Untested) on the dependent variable. The latter grouping factor, Tested, comprised unequal observations per group (72 vs 20), and thus the analyses accounted for an unbalanced design. Observations were evaluated for being potential outliers if they were more extreme than 1.5 times IQR outside the lower first or third quartiles of the distribution, and three times standard deviation from the mean. Model selection was performed on models fitted by maximum likelihood using Akaike's Information Criterion (AIC). A change in AIC-value above two (ΔAIC>2) was used as an indication for models of better fits. Estimates were considered statistically significant when *p*-values were lower than 0.05, and interaction terms when *p*-values were lower than 0.25. In unbalanced models with interaction terms included, ANOVAs were specified with Type III Sums of Squares (SS). If interactions were removed due to better fits of additive models, Type II SS was used. All model assumptions were evaluated using residual plots. Results are presented as means ± standard error (SE) if nothing else is stated. Lastly, a dataset was created from the recorded temperatures to simulate results from fish that had occupied all zones for equally long periods.

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Results

Method validation

To test whether an annular arena was suitable for detection of the thermal preference of individual zebrafish, the occupied temperatures during the first and last twenty minutes of two-hour trials were compared. When the fish were introduced to the swimming channel individuals responded in notably different ways. Some explored the arena more calmly, while others responded with erratic movements and fast swimming around the arena. Differences in interquartile range (IQR), were used to distinguish individuals that occupied a specific thermal range and individuals that did not discriminate between temperatures. The IQR in the three treatments was lower in the last twenty minutes compared to the first twenty minutes of the two-hour trials (-3.525 ± 0.250 °C, $p<0.0001$, Table1, Figure 3 (a)). Most fish occupied a narrow range of temperatures, well within an IQR of four degrees, at one or both of the two corresponding sides of the arena during the last twenty minutes of the trials. There was nevertheless still variation among individuals and some individuals retained a relatively high IQR, i.e. occupying a wide range of temperatures.
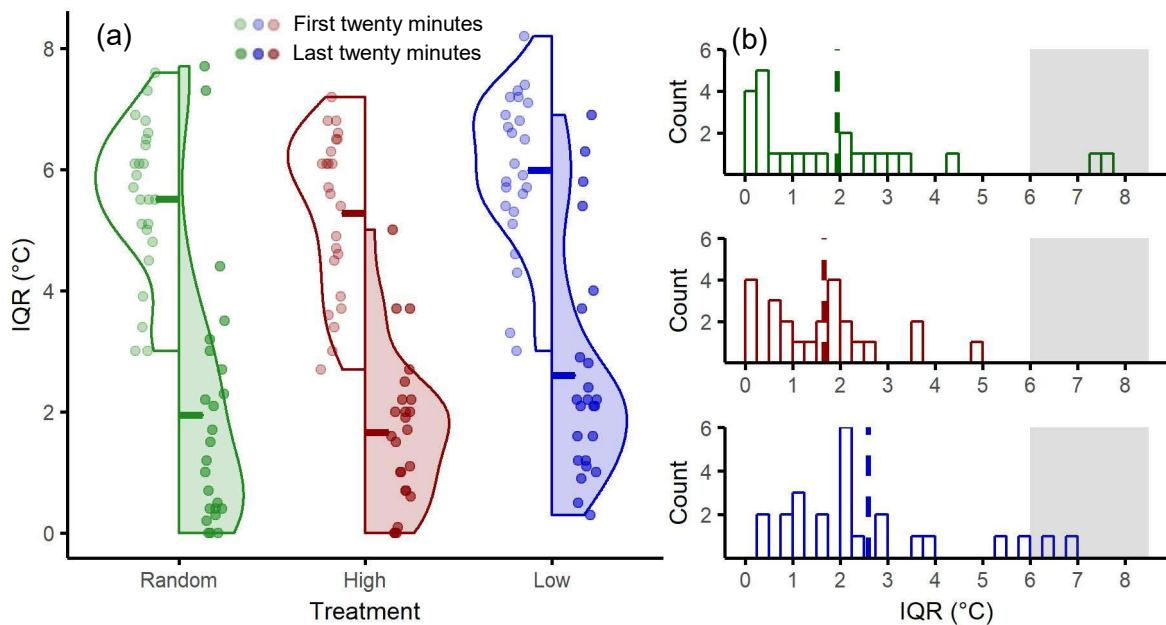

Figure 3 (a) Interquartile range (IQR) of occupied temperatures during the twenty first (filled) and twenty last (open) minutes. (b) Histograms of IQR during the last twenty minutes for the Random (control, upper), High (middle) and Low (lower) treatments. Dashed lines are mean IQR and the grey area is IQR if individuals had occupied all temperatures equally long.

The grey area of the histograms (Figure 3 (b)) represents the IQR estimated from the simulated dataset. It represents what the IQR would be if the fish had not discriminated between temperatures and occupied all zones of the swimming channel equally. The range of this area is caused by the variation in thermal gradients in the channel between trials. Based on the distributions in the IQR (Figure 3 (b)), fish with an IQR above 4 °C were considered as non-choosers of temperatures and excluded from the analyses on thermal preference. These individuals (n=8) represent 11% of all tested individuals (N=72) and varied between the treatments (Random=3, High=1, Low=4).

An example of a representative chooser and non-chooser from the Random treatment (Figure 4 (a)) illustrates that the IQR of the non-chooser does not change from the start to the end of the experiment. The heatmaps of the arena similarly shows that the non-chooser utilised the whole swimming channel during the last twenty minutes, while the chooser (Figure 4 (b)) only occupied a specific part of the thermal gradient during the last twenty minutes of the trial.
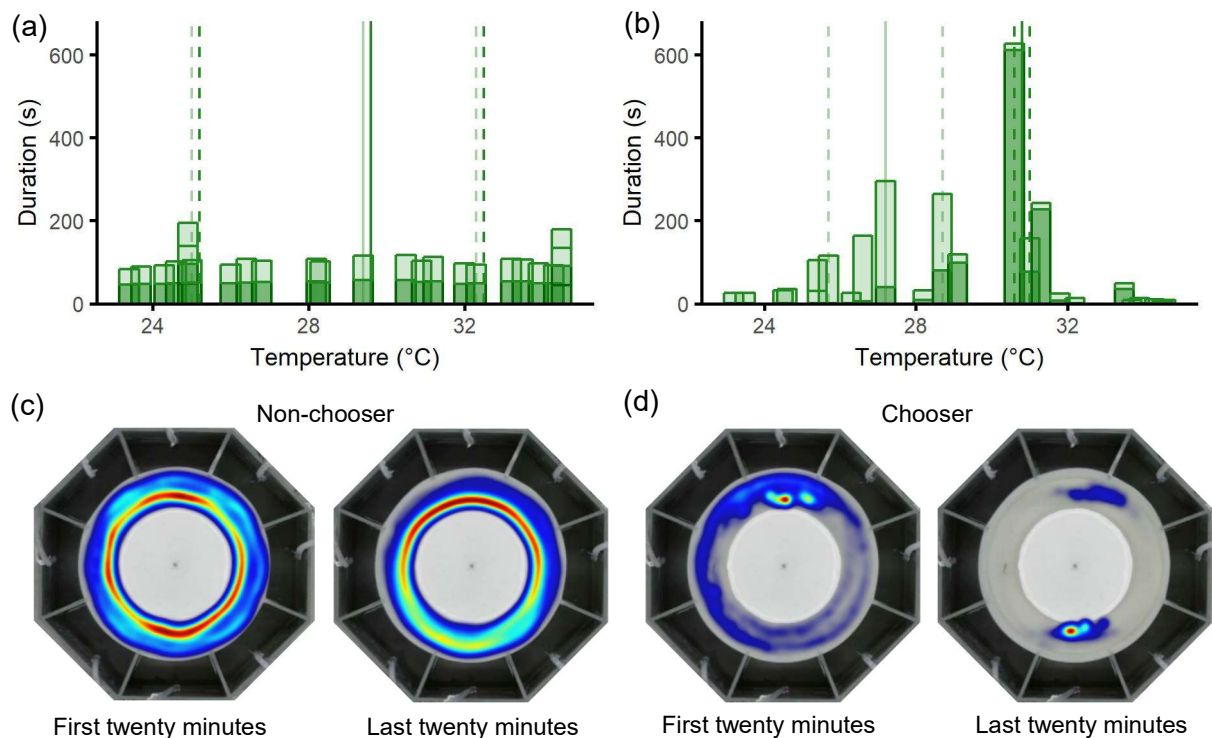

Figure 4 Duration in each temperature during the first twenty minutes (light green) and last twenty minutes (dark green) for a representative (a) "non-chooser" and (b) "chooser" from the Random (control) treatment. Solid lines represent the median selected temperature and dashed lines represent the first and third quartiles. Heatmaps of the occupied areas in the swimming channel during the first twenty (left) and last twenty minutes (right) from a (c) "non-chooser" and a (d) "chooser".

Thermal tolerance and preference

Preference temperature and the range of the thermal preference were compared in zebrafish selected for High, Low and Randomly selected (control), thermal tolerance. The individual preference temperatures were not significantly different between the treatments ($p=0.265$, Table 1, Figure 5 (a)). Likewise, the weight of individuals at the end of the experiment and order of tests was not found to significantly affect the preference ($p=511$, $p=0.271$, respectively, Table 1). The IQR of the last twenty minutes (Random: 1.95 ± 0.43 °C, High: 1.66 ± 0.26 °C, Low: 2.60 ± 0.38 °C, Figure 5 (b)) was not different between treatments ($p=0.130$, Table 1). There was no significant effect of end weight on IQR ($p=0.174$, Table 1) but an indication of an effect, however not significant, from the order of tests ($p=0.097$, Table 1).
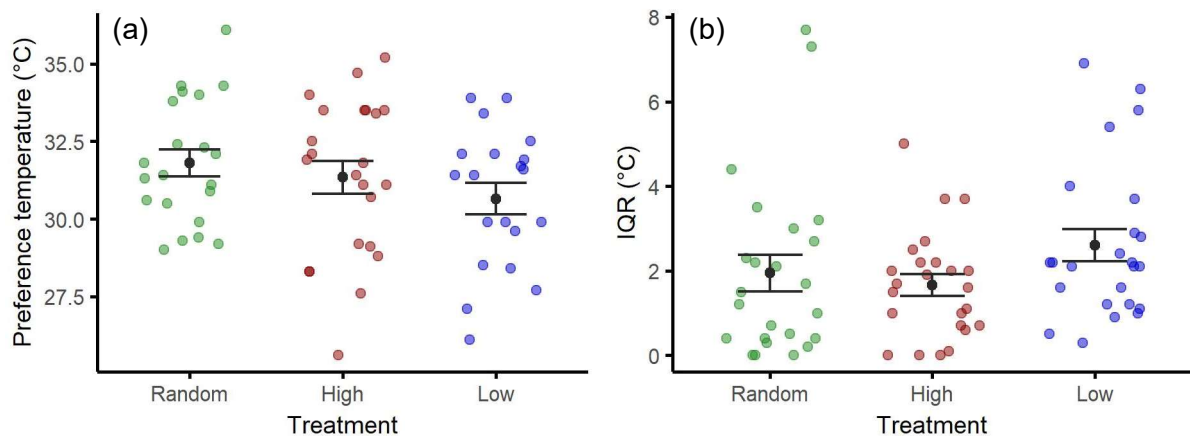

Figure 5 (a) Preference temperature and (b) interquartile range (IQR) of occupied temperatures for the F_4 generation of three selection treatments for thermal tolerance, Random (control), High and Low. Results are presented with means $\pm$ SE.

The mean thermal preference of the Random replicate tank tested over 16.5 hours (n=6) was 31.55 ± 0.96 °C and the mean IQR was 1.07 ± 0.49 °C. Estimates from the simulated dataset showed that if no animals had discriminated between zones during the last twenty minutes the preference temperature would be 28.90 ± 0.07 °C and that the IQR would be 6.92 ± 0.05 °C.

Table 1 ANOVA tables from the thermal preference, IQR and CT_{max} linear mixed effects models. Order and Weight are mean centred.

	Preference model			IQR model			CT_{max} model		
	Chisq	d.f.	p-value	Chisq	d.f.	p-value	Chisq	d.f.	p-value
Treatment	2.659	2	0.265	4.097	2	0.130	4.484	2	0.106
Order	1.210	1	0.271	2.757	1	0.097			
Weight (g)	0.433	1	0.511	1.851	1	0.174	2.606	1	0.107
Period				198.753	1	<0.0001			
Test							5.177	1	0.023
Line:Test							6.807	2	0.033

Table 2 Estimates and SE from the thermal preference, IQR and CT_{max} linear mixed effects models. The Untested, Random Treatment is in the intercept. Order and Weight are mean centred.

	Preference model	IQR model	CT_{max} model
	Estimate ± SE	Estimate ± SE	Estimate ± SE
Intercept	31.720 ± 0.505	5.562 ± 0.350	41.907 ± 0.107
Treatment - High	-0.267 ± 0.710	-0.417 ± 0.466	-0.008 ± 0.151
Treatment - Low	-1.116 ± 0.716	0.514 ± 0.460	-0.431 ± 0.151
Order	-0.300 ± 0.272	0.192 ± 0.116	
Weight (g)	-3.094 ± 4.702	2.963 ± 2.178	0.657 ± 0.407
Period – Last twenty		-3.525 ± 0.250	
Test – Untested			0.162 ± 0.094
High:Untested			-0.154 ± 0.141
Low:Untested			0.233 ± 0.140

The critical thermal maximum (CT_{max}) test was performed on all experimental animals, including the extra fish that were not tested in the preference arena. The model thus tested the effect of being exposed to the thermal gradient in the preference arena and whether selection for upper acute thermal tolerance had led to contrasting thermal tolerance between the three treatments. Two observations were excluded from the final analyses as outliers due to their low CT_{max} values and effect on the distribution of the residuals. Both were from the Low treatment and one of them was tested in the preference arena.
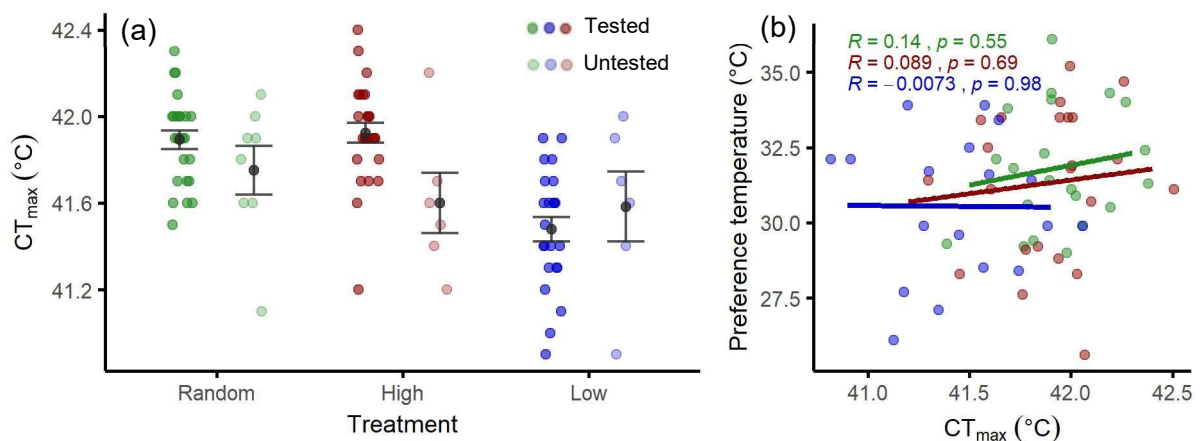

Figure 6 (a) Critical thermal maximum (CT_{max}, °C) presented with mean ± SE for the three selection treatments Random (control), High and Low. Dark points represent individuals that had been tested for thermal preference before the CT_{max} test and light points represent untested individuals. (b) Correlation between individual measures of CT_{max} (°C) and preference temperature for individuals tested for preference from the three selection treatments Random (green, control), High (red) and Low (blue).

An interaction was present between the factors Treatment and Test (p=0.033, Table 1). The mean CT_{max} for the Low, Tested individuals was $\Delta4.3$ °C lower than Random, Tested fish (Figure 6 (a), Table 2). No correlation was found between preference temperature and the CT_{max} values (Figure 6 (b)).

The mortality was low (n=1) during the experiment. The one fish that died was from the Low treatment and was not tested for thermal preference. On average the fish did, however, have poor growth over the experimental period reflected by the low increase in weight ($\Delta0.02 \pm 0.006$ g) and standard length ($\Delta0.88 \pm 0.08$ mm) over 42 days.

Discussion

Method validation

In this study, we demonstrated that annular arenas are suitable for detection of thermal preference in individual zebrafish. During the two-hour trials the experimental fish settled within 2 °C as shown by the interquartile range (IQR) values. The chosen temperature ranges were not in the thermal extremes, confirming that we are detecting individual thermoregulatory behaviour. Furthermore, the mean preference was approximately 2.5 °C higher than if all fish occupied all zones without discriminating between temperatures. These results confirm that the annular setup allowed high-precision measurements of thermal preference in zebrafish.

Annular arenas enable measurements of thermal preference without confounding effects such as pressure differences and uneven distances to corners and walls. However, it has been questioned whether previous studies using the annular setup to determine thermal preference have used suitable methods for data collection (Reiser *et al.*, 2013, Macnaughton *et al.*, 2018). Specifically, the frequency of measurements and the period of the trials when the position of fish has been recorded have often been limited by laborious approaches. Here we have presented an alternative method for data collection comparable to that described by Reiser *et al.* (2013). The method is a considerable improvement over methods using manual recording of individual position and/or temperatures. The tracking program we used in this study, EthoVision, worked well with tracks being lost in less than 6% of the trials, and the program provided simple methods for manually tracking the seconds where swimming trajectories were absent. By tracking the fish every second, our method produced a substantially finer resolution,

between 120 and 600 times higher, than previous studies with annular arenas (Myrick, Folgner and Cech Jr, 2004, Cocherell *et al.*, 2014).

Automated temperature recordings further improved our method compared to traditional methods. In some studies measurements have been taken manually at the position of the fish giving rise to an important source of disturbance of fish behaviuour during trials (Chen *et al.*, 2008). In other studies, measurements have been conducted before and after each trial giving a coarse estimate of the temperature that the fish occupied (McMahon, Bear and Zale, 2008). The automatic recording of the thermal gradient applied in this study required no disturbance during the trials and gave an accurate estimate of the temperature that the fish occupied. The real-time monitoring and contiuous logging allowed us to immediately detect any changes in the gradient and correct for this between trials. Furthermore, any temperature variations among trials were accounted for by matching the individually recorded temperatures to the recorded positions of each fish.

The described experimental setup enabled us to create a thermal gradient covering the natural range of the eurythermal zebrafish (12-39 °C) in which the preference temperature should occur (Clark, Sandblom and Jutfelt, 2013). Because this range is close to the lower and upper acute thermal limits of the species (12 °C and 40 °C, respectively), a narrower range of temperatures (22-35 °C) was applied in our trials. Even within this range, U-turns were observed in response to both extreme temperature areas of the thermal gradient. Although the avoidance of temperatures is an important part of thermoregulation, it is not desirable that the median occupied temperature merely represents an area in the gradient away from the avoidance temperatures, perhaps biased towards the temperature the fish avoids the least (Reynolds and Casterlin, 1979). An even narrower range of temperatures could thus be more suitable for detection of small differences between individuals or treatments. We would recommend future studies to apply a range between 25 and 35 °C based on the preference temperatures and IQRs established from this experiment.

Fish have a tendency to explore temperatures deviating from the proposed optimal value and thermal preference could be viewed as a range around a central temperature (e.g. mean, mode or median) of the occupied temperatures (Magnuson, Crowder and Medvick, 1979, Reynolds and Casterlin, 1979, Jobling, 1981). This range, or precision of thermal choice, can be described by a measure of dispersion, for instance standard deviation, range of 95% of the observations

or the first and third quartiles as in the present study. By analysing the range of occupied temperatures, differences in the selectiveness to temperatures can become evident. As stated earlier, the ability to thermoregulate is crucial for ectotherms when environmental temperatures are close to their physiological limits and the measure of how specifically the animal choose temperatures is an important part of thermal preference. We therefore considered IQR an important component of thermal preference behaviour and investigated the response of IQR to selection for thermal tolerance in the same manner as for the median preferred temperature.

Creating an even thermal gradient was a fine balance between optimising factors that make the swimming channel suitable for the fish to swim in, and factors enabling an unstratified water column. An increased height and breadth of the water column was restricted by the higher flow rate required to prevent stratification. A higher flow rate would furthermore exhaust the fish in the arena and result in a larger demand for water heating as the exchange rate of the water in the arena would increase. The latter is especially important when performing experiments with long lasting thermal gradients (Reiser *et al.*, 2013). To establish and maintain the thermal gradient in our system, 7.8 kW was required to heat the water. The pilot experiment on long-term trials verified that this system enabled long-term tests.

Thermal tolerance and preference

To test our hypothesis on coevolution of disparate traits in thermal biology, we tested whether the three selection treatments, High, Low and Randomly selected (control) acute upper thermal tolerance, had led to differences in thermal preference. There were no significant differences in the preference temperature between the treatments, contrary to our hypothesis. Furthermore, we found no statistical evidence that the preferred thermal range was different between the treatments by testing differences in IQR. However, more Low selected fish were categorised as non-choosers, than from the other treatments. Additionally, the distribution of IQRs gave an indication that the Low lines could have a higher mean IQR. A higher IQR in the Low treatment might indicate that these fish were more flexible and had a broader preference. Alternatively, it might indicate that they were less selective to temperatures and had lower precision in their thermoregulation, or had a lower learning ability. From the main selection experiment the Low selected lines were found to have lower fecundity than the other selection treatments and inferior cold tolerance (unpublished data). These findings indicate a reduced performance at least in some traits and could be in line with the broader IQR showing a decreased ability to

behaviourally thermoregulate. This would be unfavourable in an environment with extreme temperature variations where thermoregulatory behaviour can be crucial for survival.

The larger response to selection for decreased thermal tolerance in the Low treatment than increased tolerance in the High treatment was in line with the results from the main selection experiment (Figure 1 (b)). The restricted response to selection for higher values of critical thermal maximum (CT_{max}), might be caused by the thermal tolerance already being close to the physiological limits (Sandblom *et al.*, 2016, MacMillan, 2019). Based on this we might expect a larger difference in thermal preference between the Low and Random treatments, compared to between the High and Random treatments, if coevolution with thermal tolerance has occurred. There was only a small difference in CT_{max} between the Low and Random treatments, however, suggesting that only a limited change in thermal preference could be expected. The considerable interindividual variation in preference temperature would require a large sample size for detection of small differences between the treatments.

There was a significant interaction between the different treatments and the effect from being tested in the annular arena. Moreover, the average values of CT_{max} in the tested fish from all our treatments were higher than in the main selection experiment (Random: $\Delta 0.51$ °C, High: $\Delta 0.46$ °C, Low: $\Delta 0.7$ °C). This could be an effect of being tested in the annular arena, but the limited sample size of untested fish made this difficult to test with the available data. The fish tested for thermal tolerance were exposed to a wide range of temperatures for a period of two hours and briefly encountered temperatures both above and below the optimal range. This exposure could have made the animals more robust to the acute thermal challenge in the CT_{max} test. Results from previous experiments on the same lines have revealed that acclimation to 34 °C significantly increases the acute thermal tolerance. Surprisingly, these effects were evident after two hours of exposure to the thermal gradient followed by 14 to 30 days of housing at their normal holding temperatures. It is possible to hypothesize that the individuals that preferred the highest temperatures in our setup might have had a higher effect on CT_{max}, but no correlation was found between the median occupied temperature and CT_{max}.

The mean preference temperature of all fish (31.28 ± 0.29°C) was found to be higher than the optimal temperature for growth rate previously measured on the F_1 generation. The growth rate was found to increase with acclimation temperature until 30 °C and decrease with acclimation to higher temperatures (unpublished data). Jobling (1981) proposed that measurements of the

final thermal preferendum could be used to estimate the optimal growth temperature for a species. The thermal preference was measured after 24 hours of habituation to the experimental setup in the habituation tanks followed by two hours of exposure to the thermal gradient in the preference arena. The thermoregulation measured in this study is thus acute thermal preference and could be affected by the previous thermal experience of the fish (Johnson and Kelsch, 1998). Although we attempted to minimise any acclimation differences between trials by closely controlling the temperature for the 24 hours preceding each trial, some variations may have occurred. The results from the long-term pilot experiment measured after 16.5 hours did not suggest that the fish gravitated towards the lower optimal growth temperature, but rather that they maintained the high thermal preference (31.55 ± 0.96 °C). Further experiments should be conducted on long-term exposure in the thermal gradient for assessment of the final preferendum.

Stress could potentially explain why we found higher thermal preference for the experimental animals than the optimal temperature for growth. It has been suggested that zebrafish produce behavioural fever by utilising higher temperatures in response to stress (Rey *et al.*, 2015). Some fish in our study responded with erratic movements when they were introduced to the arena, a response linked to stress behaviour in zebrafish (White *et al.*, 2017, Graham, von Keyserlingk and Franks, 2018). The low growth rates during the experiment could also be an indication of stress (Pickering, 1992). Faustino, Tacão-Monteiro and Oliveira (2017) found that zebrafish recovered more quickly from an aversive event in groups than alone. Similarly, White *et al.* (2017) found that the initial stress response was lower, with fewer erratic movements, for fish in groups than observed for individual fish. An attempt to track individual fish in groups of six in our experimental setup was made but the individual tracks were scrambled in EthoVision when fish crossed each other's swimming trajectories. Furthermore, measuring multiple individuals simultaneously in the same annular arena may not be optimal because the shoaling behaviour in zebrafish could suppress the inter-individual preference differences (Spence *et al.*, 2008) and it would be challenging to account for the social interactions and non-independence of the observations in the data analyses. In this experiment, data on individuals were needed to correlate preference measurements to the thermal tolerance of individuals and we therefore chose to perform trials on single fish. The research revealing that social context modulates the stress response in zebrafish together with findings supporting a fever response to stress could, nevertheless, be a possible explanation for why isolated individuals might have a surprisingly high thermal preference.

Future directions

Further experiments are required to assess whether selection for thermal tolerance affects thermal preference in zebrafish and whether these traits could be alligned on a cold-hot axis in a syndrome framework. We did not determine the individual repeatability and further experiments are needed to assess the thermal preference of zebrafish under various conditions. Future research on groups, long-term trials and comparisons between stressed and unstressed trials would elucidate the results from this study. It should also be investigated whether statistical models allowing for heteroscedasticity of the variance in the difference treatments could better explain the data (Cleasby and Nakagawa, 2011).

It was recently shown that multigenerational exposure to elevated temperatures and increased thermal tolerance in sticklebacks (*Gasterosteus aculatus*) did not lead to increased thermal preference (Pilakouta et al., 2019). Acute thermal tolerance is a strong selective force, but the three selection events on the treatments used in the present study might be insufficient to reveal coevolution in a disparate thermal trait. An additional preference experiment has been conducted on the same generation as tested in the present study. The sample size for thermal preference in the three treatments has consequently been doubled, and analyses of the additional dataset will provide clearer results.

Concluding remarks

Our study provides a validated method for testing the thermal preference in small, eurythermal species. Furthermore, the presented methods for measuring thermal preference considerably improves previous approaches lacking automation of data collection and analyses. Although we were unable to detect changes in thermal preference after three generations of selection for upper acute thermal tolerance, we cannot conclude from our results that the thermal preference and thermal tolerance are not correlating traits that align on a cold-hot axis in a syndrome framework. The indications from this experiment are encouraging for future research on coevolution of thermal traits.

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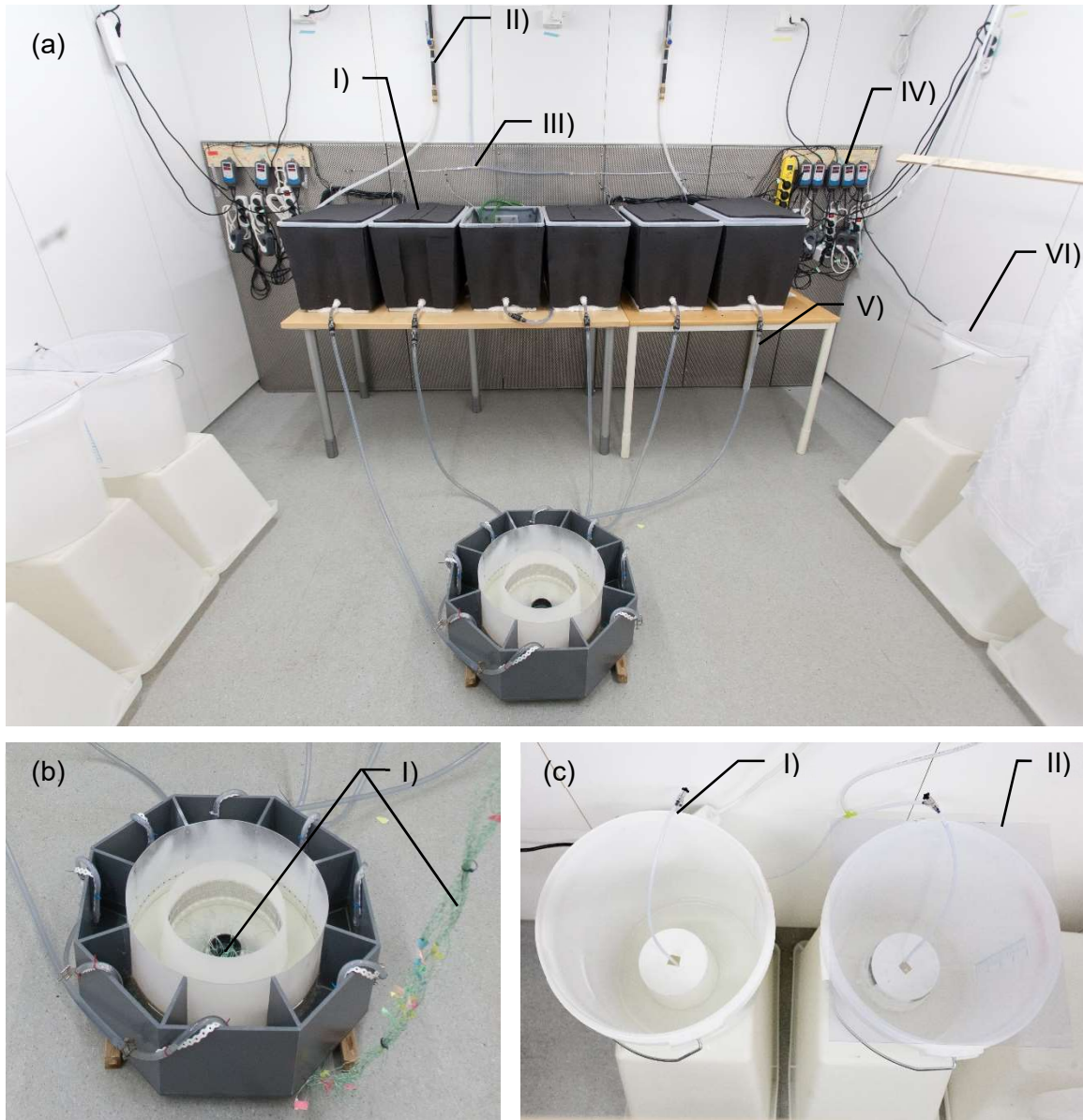
Supplementary Material

Figure S1 (a) Overview of the experimental setup showing: I) the insulated header tanks supplied with II) carbon filtered water and III) filtered air; IV) Thermostats controlling titanium heaters inside the header tanks ensuring stable temperatures; V) Hoses with closing and adjustment valves leading water to the outer compartments of the annular arena; VI) Placement of the habituation tanks behind curtains. (b) Annular arena with I) thermocouples pulled up through the central drain and attached to the inner wall of the swimming channel. (c) Habituation tanks with I) air supply and II) transparent lids to keep the water temperature high. Photos by Eirik R. Åsheim.
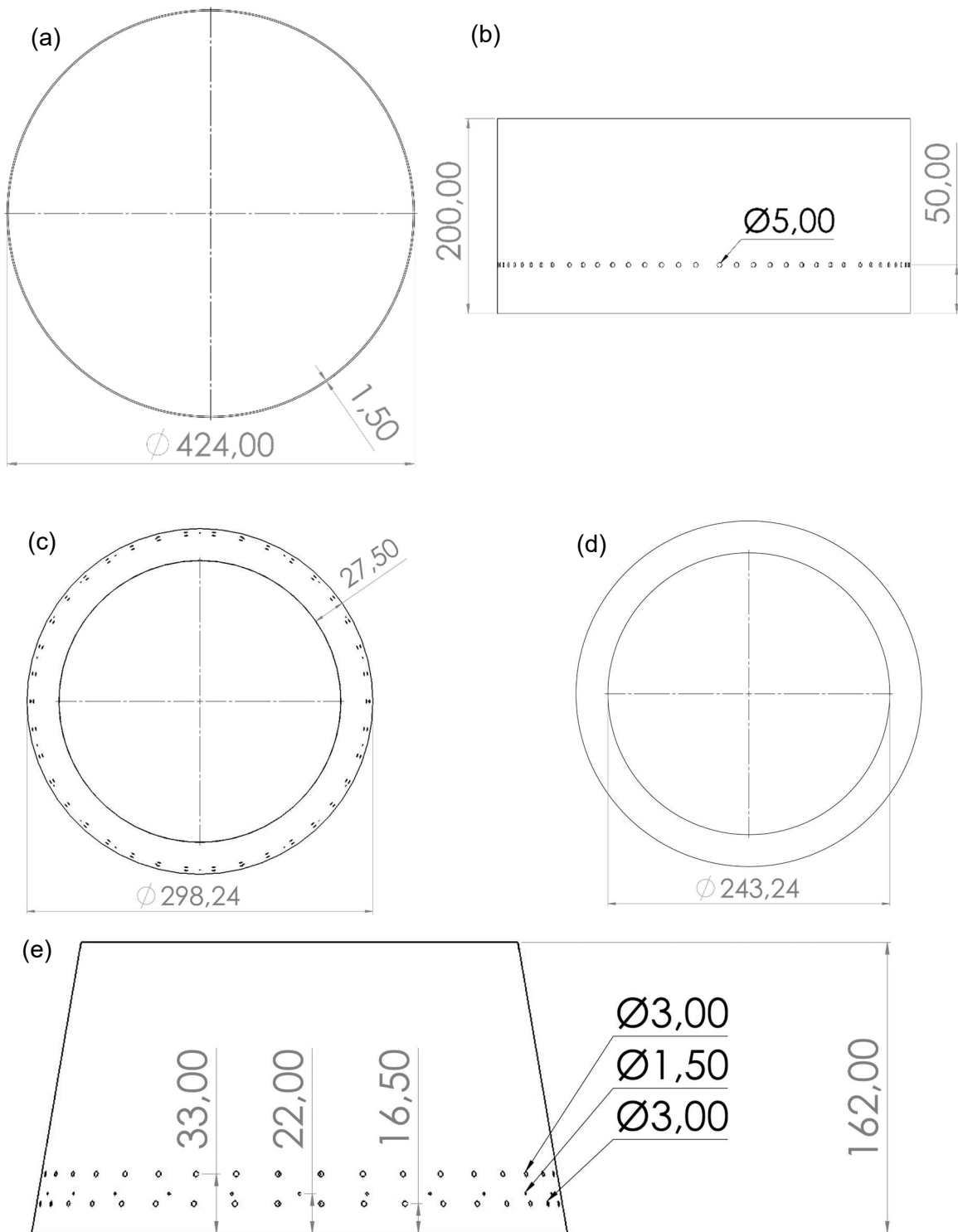
Figure S2 (a) Outer wall of the swimming channel and (b) placing of holes between the outer compartments and the swimming channel. (c) Outer and (d) inner diameter of the cone shaped inner wall of the swimming channel. (e) Placing of holes between the swimming channel and the central drain. The upper and lower row of holes are to ensure an even, unstratified thermal gradient. The middle row is for attaching the 24 thermocouples to monitor and record the thermal gradient. The illustrations are created in SolidWorks by Henrik Plassen and Eirik Bjørkedal Rømo.
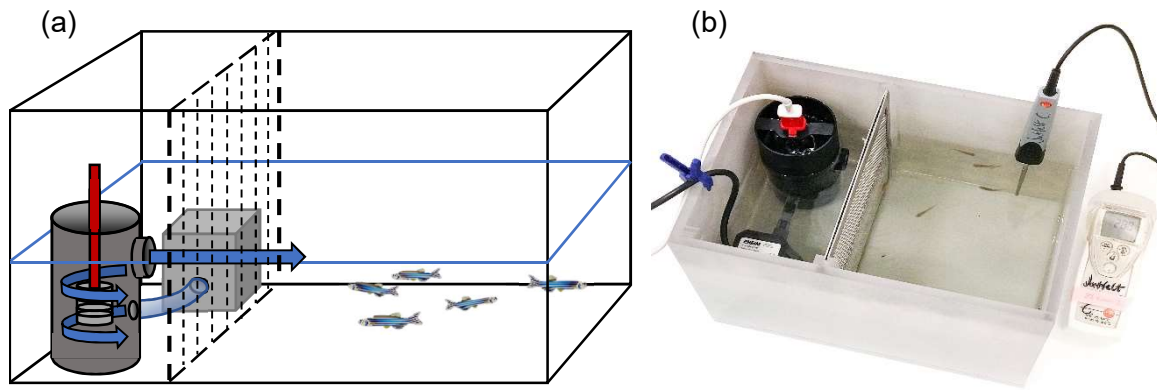
Figure S3 (a) Outline of the critical thermal maxima (CT$_{max}$) setup design. The heating compartment (left) is equipped with a 300 W coil heater within a steel cylindrical case connected to a water pump to ensure a homogeneous water temperature in the main compartment (right). (b) Photograph of the tank (25x22x18 cm) filled with 9 L of water, showing the dividing mesh between the two compartments and the thermometer mounted on the side wall of the main compartment to continuously display the water temperature. (Morgan, Finnøen and Jutfelt, 2018).