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Thermal performance in zebrafish (Danio rerio)

Thermal acclimation capacity in wild and domesticated zebrafish

Gunnar Dresler

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Supervisor: fredrik jutfelt, IBI

Norwegian University of Science and Technology
Department of Biology

Master thesis

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B.Sc. Gunnar Dresler

Norwegian University of Science and Technology /
Norges Teknisk-Naturvitenskapelige Universitet (NTNU)

Supervisor: Associate Prof. Ph.D. Fredrik Jutfelt

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1 Abstract

In times of imminent threat of climate change, clear evidence of which physiological mechanisms are limiting thermal performance remains scarce. This thesis involved measuring different performances, over a wide range of temperatures, and included a comparison between wild and lab-strain zebrafish (*Danio rerio*). We hypothesised that thermal optima of different performances will vary like in the multiple performance multiple optima model (MPMO, Clark et al., 2013; Gräns et al., 2014), and that the lab-strain zebrafish, which have been selected to optimise performance at 25-28°C since the 70´s, have reduced the capacity to acclimate to non-optimal temperatures. To test this hypotheses, we performed a pilot study, and two large-scale acclimation experiments on zebrafish, one in summer (n=560) and one in fall (n=600) of 2017. We measured a range of performances including survival, growth rate, acute thermal tolerance (CT_{max}), and swimming speed. In the pilot and the first acclimation experiment only wild-caught F1 generation zebrafish from India (n=560) were used. Half of the fish used in the second experiment were wild-caught F1 zebrafish (n=300), and the other half were from a lab-strain (AB-WT line, n=300). Both acclimation experiments lasted at least for four weeks to temperatures ranging from 10°C to 36°C/38°C with a difference of 2°C between each, after final temperatures were reached. Mortality differed between all experiments but was especially high at the upper end of the thermal spectrum. Generally, lab fish displayed higher specific growth rates across temperatures than wild fish. CT_{max} rose with acclimation temperature in all populations but lab fish showed a lower CT_{max} at colder acclimation temperatures when compared to the wild population. CT_{max} was close to equal from temperatures above 26°C. Also, wild fish displayed a better swimming performance at highest temperatures, while the optima of the lab-strain was around 26 to 28°C. Lab-strain zebrafish appear to have maintained their capacity for some performances, e.g. survival and growth, during thermal acclimation, even after decades of adaptation to constant optimal temperatures. Overall the results still suggest a reduced acclimation capacity in lab-strain fish, and also shows varying optima to growth performance in wild zebrafish. Whilst more research is needed to fully investigate the first hypothesis, this thesis adds valuable information on varying optima between growth and swimming speed data, which will differ no matter how metabolic scope would turn out.

2 Introduction

From the tropics around the equator, where oxygen poor dead zones are growing, to the Arctic with its especially decreasing level of thick sea ice, rising water temperatures and increasing CO_2 levels are altering the world´s ocean and its ecosystems (NSIDC; Vose et al., 2012; Millero, 1995; Feely et al. 2004). In a rapidly changing surrounding, the weight of selection becomes heavier on the gene pool of many species. These adaptations can take place in different instances, e.g. through altered behaviour to increasing temperatures by migration to another habitat or by changing physiology with a metabolism that is more resistant to upcoming temperature changes e.g. due to improved heat shock mechanisms due to previous heat impact (Lindquist., 1986).

This abrupt climate shift challenges especially organisms living in or near the water and relying on their surroundings, such as aquatic ectotherms. Their environment is often already threatened by overfishing and increasing plastic in the ocean, but climate change imposes another threat. Most fish species are ectothermic and therefore, have a metabolism nearly completely relying on the temperature of the water around them. Better understanding of fish populations could help to improve future migration patterns, and could also save a market that displays a great food supply to humankind.

To do so it is necessary to study the thermal biology of organisms and evaluate the thermal window in which an organism can survive and develop successfully. Unfortunately, the area of thermal performance in aquatic ectotherms is not well enough understood yet and is therefore in the centre of greater interest in recent research.

This thesis is focusing on deepening the understanding of thermal performance with the use of a large sample size of two zebrafish strains. Thereby, two questions will be of main interest: Which mechanism limits the thermal performance of ectothermic animals, and furthermore, has the thermal capacity of laboratory zebrafish been lost due to adaptation to stable optimal temperature over decades?

2.1 Thermal ecology of ectotherms

While endotherms rely predominantly on producing their own body heat by metabolic activities, environmental temperature mainly sets the life boundaries for ectothermic animals. The mechanism behind this is that biochemical reactions directly depend on temperature to determine the performance of ectothermic fish (Huey & Kinsolver, 2011). As a result, temperature has been termed an abiotic "master factor", due to its influence on behaviour, physiology, and distribution of aquatic organisms (Brett, 1971).

Different types of organisms can be defined on the width of their thermal tolerance breadth (TTB) between lower and upper critical temperature (T_{crit}) around their optimal environmental temperature (T_{opt}). Eurythermal organisms have a wider range of temperatures that they can live in and acclimatise to, and are traditionally found in higher latitudes and elevation (Payne & Smith, 2017). On the other hand, stenothermal organisms are often considered "specialists" and occupy rather narrow thermal niches and sustain a smaller thermal window in a temperature stable environment. These organisms typically appear in the tropics where they live close to their upper thermal tolerance, or in polar waters with extreme but stable low water temperatures that permit acclimatisation to warmer temperatures (Somero, 2010). After Janzen 1967 the supposedly increase in TTB with increasing latitude or elevation was described as an adaptive response of an organism´s physiology to wider variability in temperature. This built the basis for the temperature variability hypothesis, as adaptation to varying temperature as main factor.

In the end, the whole-animal performance is set by temperature´s effect on processes of lower levels like the cellular metabolism and reactive rates. It was also proposed that the supply of oxygen to the tissue sets the capacity for thermal tolerance (Pörtner, 2004).

Payne and Smith (2017) investigated if the TTB in stenotherms compared to eurytherms differs when T_{crit} and T_{opt} are expressed as equivalent biological rates (fig. 2.1 right). Their idea, the biological rate hypothesis, was that global trends in ectotherm thermal tolerance could be a result of dependence of biological rates, rather than by adaptation of species to global trends in environmental temperature variability. What they found was that aquatic ectotherms surprisingly exceeds higher rate-equivalent TTBs close to the equator when compared to the poles, which is supporting their idea.

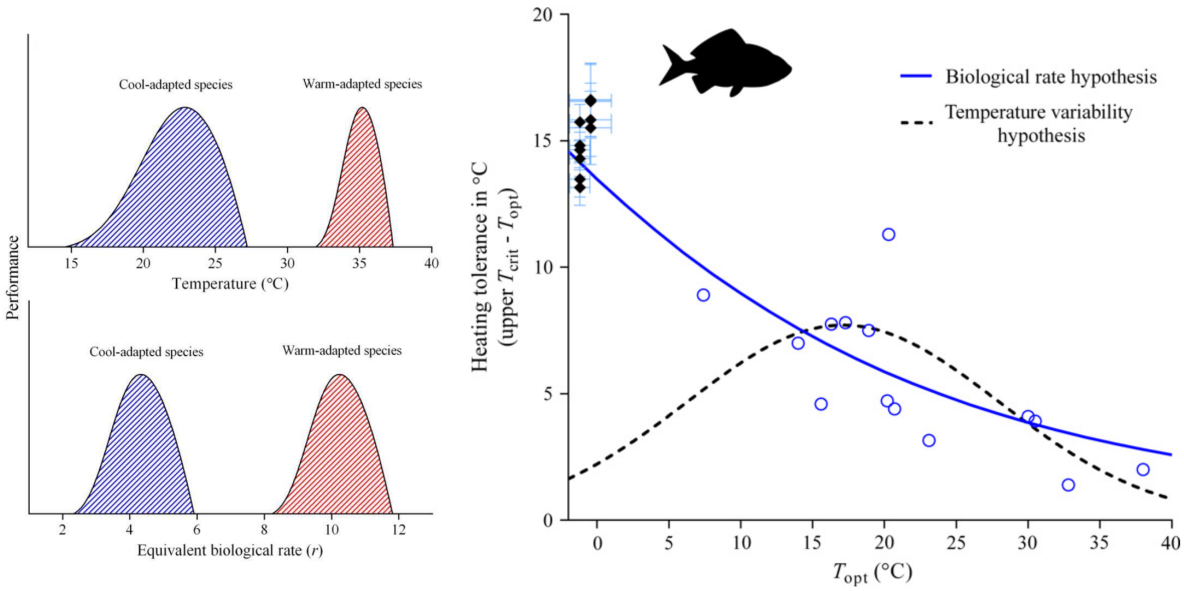

Figure 2.1 Left Performance as a function of temperature (up) and as equivalent biological rate (down) **Right** Heating tolerance depending on optimum temperature (T_{opt}). Displayed are the conceptualisation of the temperature variability hypothesis relating marine ectotherms (black dashed line), and the idea of the biological rate hypothesis (blue curved line) fitted for data on aerobic scope from fish of different altitudes (Payne & Smith, 2017)

With the suggestion of a biological rate hypothesis, it is easy to explain how wider thermal tolerance is possible in organisms living in colder environments, while ectotherms with higher T_{opt} are already close to approaching lethal limits in their habitat (fig. 2.1 left).

It has to be mentioned though that many measurements done and used by Payne and Smith for their calculations do not give certain information about long-term condition, because especially short-term data is often overestimating the T_{crit} and paints a picture of a wider niche than actually found in the environment (Sunday et al 2012; Peck et al. 2009; Peck et al 2014). Some species survive in short-term experiments temperatures up to 8°C to 18°C when temperature increases 1°C per day, while only 1°C to 6°C are possible for survival over a few months (Peck et al., 2009; Richard et al., 2012; Payne & Smith, 2017).

2.2 Climate change on aquatic ecosystems

Earth´s climate is changing and with it the physical, chemical and biological properties of the oceans are altered that define the ecosystems composition (Pörtner et al., 2014). Marine animals are confronted with higher temperatures, increased frequency of phenomenons like heat waves, and levels of CO_2 (Vose et al., 2012; Millero, 1995; Feely et al. 2004; Pörtner et al., 2014). Pörtner et al. (2014) concluded that based on fossil records and present research point to a clear link connecting key environmental drivers and responses to ocean ecosystems to climate change, meaning that already slower rates of climate change let to community shifts and extinction before (Pörtner et al., 2014).

Examples can be found in different areas from behaviour and distribution patterns of species to physiology. Changes have been seen in poleward distribution (Perry et al., 2005; Brander et al., 2003; Grebmeier et al., 2006), collapses in populations and local extinctions (Pörtner & Knust, 2007), increasing temperatures above critical temperatures probably lead to cardiac collapses in Fraser River sockeye that were seen in swimming failure and mortalities (Farrell et al., 2008), seasonal timing of biological events (Wiltshire & Manly, 2004) and food availability, species interactions and food web structure (Pörtner & Farrell., 2008).

Baseline for most organisms' vulnerability to changing conditions is clearly their physiology. It sets the limits for thermal tolerance, growth potential or geographic distribution of many species. Most threatened are polar regions and the tropics, while the first live in an environment with stable temperatures that made them adapt to narrow temperature ranges, the latter live already close to upper thermal limits. Physiology can be altered and there is evidence that genetic adaptation is taking place, but the question is if species can actually keep up with the intensive pace of the human created climate change (Pörtner et al., 2014). Unfortunately, knowledge about physiological mechanisms and temporal dynamics for thermal acclimation is little (Cossins & Bowler, 1987; Wang & Overgaard, 2007; Franklin & Seebacher, 2009; Healy & Schulte, 2012).

Furthermore, cases of reduced body sizes have been reported, and also changes in interspecific interactions have been taking place, e.g. by the northward shifting of plankton communities that drag a whole food chain behind them (Pörtner et al., 2014). Climate change may be favouring species that can adapt faster through smaller body and shorter generation times. These qualities are often found in species that behave invasive, making the current changing climate to a boom for invaders (Stachowics et al., 2002).

2.3 Thermoregulation and thermal acclimation

Like all other animals, ectotherms are specialized and bound to live in a so called thermal niche that is ultimately limited at a temperature where collapse of body functions and denaturation take place. Habitat choices can be based on different specifications like availability of food or the most sufficient oxygen supply like described by the oxygen and capacity-limited thermal tolerance (OCLTT) hypothesis (see 2.3 OCLTT hypothesis or MPMO model), to optimize performance in growth, reproduction and locomotion. These choices have to take place in the thermal niche itself while a specific habitat provides an acute thermal window to live in. Furthermore, this window has presumably evolved 'as narrow as possible to minimize maintenance costs (Pörtner & Farrell, 2008).

Body size, life stage, sex, or variation of gene expression in a population influence the limitations of different performances. Limitations of the thermal window are connected to body size and life stage, and it was suggested that for the aerobic thermal window especially early larvae and adult spawners have the narrowest thermal windows while juveniles should possess the widest (see fig. 2.2). The window has been suggested to narrow with increasing body size (Pörtner & Farrell, 2008). Based on the OCLTT hypothesis it was predicted that aerobic limitation will be experienced earlier by larger than by smaller individuals (Pörtner, 2004; Pörtner & Farrell, 2008).

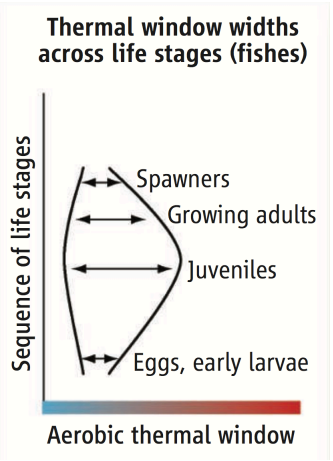

Figure 2.2 Thermal window widths across life stages (Pörtner & Farrell, 2008)

Acclimation, e.g. by altering gene expression, takes place in shifting the acute window in the niche of a species. While acclimation is a process achieved in the lab, the term acclimatization refers to a process that takes place in nature. Thermal acclimation is triggered by stressors in the environment, and leads to an adjustment of the thermal window to improve the organism´s function at non-optimal temperatures. It was suggested that the process of acclimation to another temperature takes at least one to three weeks, whether warmer or cooler does not matter (Barrioneuvo & Fernandes, 1998). An example for a physiological acclimation response is the acclimation of Atlantic cod to 5°C warmer water in an even longer timeframe of ten months, differences in gene expression could be displayed two different populations (Lucassen et al., 2006). The acclimation took place through down regulation in the liver´s capacity for aerobic metabolism (Lannig et al., 2005) and remodelling of mitochondrial functions (Mark et al., 2006; Pörtner, 2010). These mechanisms however come with a price (Krebs & Loeschcke,

1994; Angilletta, 2009), e.g. keeping production of heat shock proteins high for better thermal tolerance can be energetically costly. Minimizing energy turnover in relation to climate variability is beneficial and could be a reason that limits the shift of the thermal window in temperate species (Pörtner, 2001; Pörtner, 2002; Johnston et al., 1998; Hardewig et al., 1999; Pörtner et al., 2005; Pörtner, 2006). A change beyond the thermal niche itself takes only place over generations on an evolutionary time scale due to evolutionary adaptations (Pörtner., 2010). A change in temperature itself doesn't have to be the only reason to challenge an organism, especially the length of exposure to a specific temperature whether warmer or colder is crucial as well (Pörtner & Knust, 2007).

2.4 OCLTT hypothesis and MPMO model

There are mainly two potential ideas trying to explain the relationship between temperature and thermal performance in the current literature. The oxygen and capacity-limited thermal tolerance (OCLTT) hypothesis is most prominent, which focuses on oxygen supply to the tissue as the main factor determining performance in growth, reproduction or locomotion (see fig. 2.3 Right). The basic claim of this hypothesis predicts that biochemical and physiological capacities of aquatic ectotherms have evolved to maximize aerobic scope in a given temperature span (termed as T_{optAS}). All other fitness-related performances should be optimized in this range as well (Pörtner, 2010).

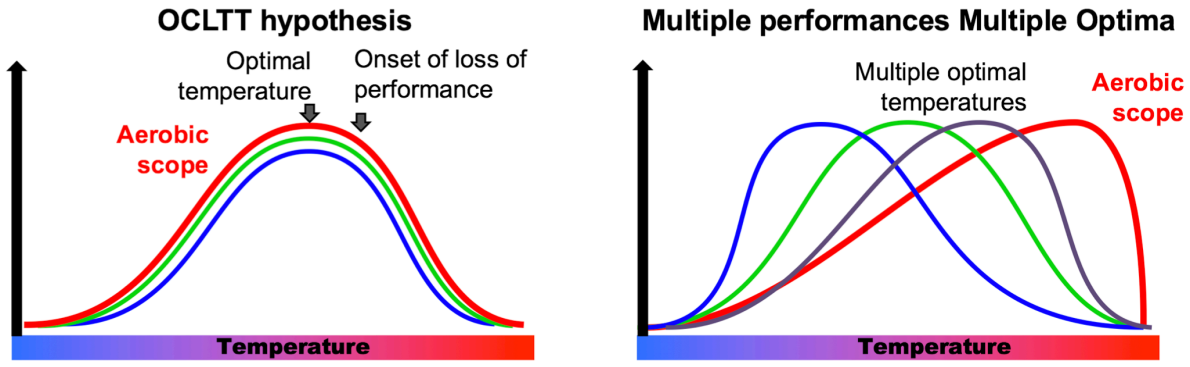

Figure 2.3 Different ideas for the concept of thermal tolerance **Right** OCLTT Hypothesis with optima sorted below the curve of aerobic scope (red) **Left** MPMO Model with different optima for different performances (Reference: Clark et al., 2013)

The OCLTT hypothesis still needs a lot of testing, because there are studies that provide supporting evidence (Pörtner & Knust, 2007), but also evidence that question its reliability (Gräns et al., 2014). The multiple performances multiple optima (MPMO) model was proposed by Clark et al. (2013) as an alternative explanation for thermal performance of ectotherm

organisms. It predicts that a specific performance, e.g. growth, aerobic scope, or swimming performance, would peak at different temperatures.

The OCLTT hypothesis itself would be an easy and elegant solution to measure performances with only one variable, which would make experiments that focus on the impact of climate change easier to perform. While the approach is good, the T_{optAS} ecological relevance for maintaining optimal fitness can be questioned (Clark et al., 2013).

The OCLTT hypothesis is supposed to be an integrative part for 'developing cause-and-effect understanding of the influence of climate change and variability on marine ecosystems, including food web structure, recruitment success and fish landings'. This statement was based on research done in eelpout (*Zoarcidae*) when limitations in aerobic performance produced decreased growth and abundance when maximum temperatures exceeded upper pejus temperatures in the summer season, thereby larger animals were affected first (Pörtner & Knust, 2007).

It was stated that these findings needed to be supported by long-term data. Gräns et al. (2014) tried to test the OCLTT hypothesis in this sense and performed long-term experiments with Atlantic halibut. The data in the long-term experiments showed that oxygen uptake was not the limiting factor for the given performance in growth. These results did not go in line with predictions based on the OCLTT hypothesis (Gräns et al., 2014) and led to further discussion about how and how not to evaluate the OCLTT hypothesis and measurement of aerobic scope (correspondence by Pörtner, 2014). It was argued that oxygen transport limitations may be the main mechanism behind thermal tolerance but could be compromised by choices of life, e.g. by choosing a higher possibility for food accessibility over oxygen supply (correspondence by Farrell, 2013). The OCLTT hypothesis is not universally accepted in the field, and many empirical studies do supply contradicting data (Brijs et al., 2015; Ekström et al., 2016). Also, many core assumption of the hypothesis have been redefined so that it is hard to test and that its predictive power was diminished (correspondence by Jutfelt et al., 2018).

Clark et al. (2013) summed up the overall measurement methods of aerobic scope and their comparability between species in a review published in 2013. A comparison between the OCLTT hypothesis and a introduced (MPMO) model (fig.2.3), which suggest different optimal temperatures for different processes and life history attributes, was exemplified (Fig. 7 in Clark et al., 2013). Furthermore, the authors collected evidence from multiple example studies that put the OCLTT hypothesis unintentionally to the test. Richter and Kolmes (2005) showed that the optimal temperature for spawning in Pacific salmonids (*Oncorhynchus*) is typically below 14°C, which is in contrast to the T_{optAS} in pink salmon (*Oncorhynchus gorbuscha*) of 21°C with

aerobic scope performance fading with decreasing temperature (Clark et al., 2011). It suggests that even though the T_{optAS} is at 21°C, reproduction at this temperature would likely fail. Another example stated that two coral reef fish species maintained a high aerobic scope (>70% of maximal) at 32°C after decline in aerobic scope while increasing temperature from 29 to 32°C, in spite of being closely to their estimated lethal temperature of around 33°C (Munday et al., 2009). This is in contradiction with an essential principle connected to the OCLTT hypothesis, because oxygen limitations only appear at exactly lethal temperature. Moreover, Claireaux et al. (2000) showed that the thermal profile of aerobic scope in the Atlantic cod is not as predicted by the OCLTT hypothesis on a stable maximum at an intermediate temperature range and declining to both ends (Pörtner & Knust, 2007; Pörtner & Farrell, 2008). Instead, aerobic scope increases steadily with temperature along the normal range (Claireaux et al., 2000, Clark et al., 2013).

To sum up, it can be said that the OCLTT hypothesis may has its importance in certain defined areas, but that it is not 'universally applicable'. In this context, the presented examples from recent studies, especially by Clark et al. (2013) and the findings of Gräns et al. (2014), suggest the MPMO model as more appropriate to explain thermal performance.

2.5 Study species: The zebrafish as a model organism

In this thesis, zebrafish (*Danio rerio*) that originate directly from the wild are used as the main study species. The parental generation was collected in 2016 at various location in Northern West Bengal, India, and should therefore display a high genetic variability. Wild zebrafish can be found in India, Nepal, and Bangladesh at temperatures ranging from as cold as 6°C in winter to 38°C and above in summer (Arunachalam et al. 2013; Spence et al. 2008; Engeszer et al. 2007). They are usually found in standing or slow-moving waters like on the edges of rivers or blind channels (Sterba, 1962, Talwar & Jhingran, 1991; Jayaram, 1999). The bred F1 generation of these zebrafish consequently is hypothesised to show a closer relation to the actual environment of wild zebrafish than any other line domesticated in the lab, and make predictions for the wild more

Figure 2.4 A zebrafish (Photo by Per-Harald Olsen, NTNU Trondheim)

accurate. The second strain used was supplied by the Yaksi lab, and is of the AB-Wildtype (AB-WT) line, originating from a purchase of fish from a pet shop in Albany, Oregon, USA in the later years of the 1970s. Since then, the line has been domesticated and kept at 25°C to 28°C to optimise performance in this range (Spence et al. 2008).

Zebrafish has been used as an experimental animal in various research areas. It is often used in the study of embryonic development (e.g. Sarmah & Marrs, 2016), because it develops rapidly, has a transparent larvae state, and displays a high fecundity in the lab. Also, it is used as genetic model even for human diseases (e.g. Tonon et al., 2016). Its genome is fully sequenced and has approximately 70% orthologues with human genes (Howe et al., 2014).

Previous experiments focusing on thermal performance practiced on various species, e.g. the eelpout (*Zoarces viviparous*; Pörtner & Knust, 2007), the Atlantic cod (*Gadus morhua*; Larsen et al. 1997), and Atlantic halibut (*Hippoglossus hippoglossus*; Gräns et al., 2014).

An advantage of zebrafish is that breeding is an easy and fast process due to its fast development and high number of eggs. Domesticated zebrafish can start to breed two months after hatching when the male testis are usually completely developed (Maack & Segner, 2003), while the directly from the wild originating fish in our lab need around three months (Morgan et al., unpublished). One female can lay up to a hundred eggs per mating cycle (Nasiadka & Clark, 2012). Long term experiments with other fish can take years and are mostly performed with a lower number of data points at higher costs.

Moreover, the zebrafish can be accommodated in smaller aquaria at higher numbers and perform experiments in compacter apertures due to its small adult body size and has been shown to respond with low stress to animal handling. The fish started to feed on the same evening after their arrival in our lab from India. A journey that included a flight and two days in a truck, which is a good indicator for stress resistance and acclimation potential. Other fish like Atlantic salmon (*Salmo salar*) need longer times to acclimate and lower their stress level (Nomura et al., 2009). Also, the critical thermal maxima test and growth measurements were performed on zebrafish before (Morgan et al. 2018).

The zebrafish is already an invaluable model organism that bridges between the taxa of insects like the common fruit fly (*Drosophila melanogaster*) and classical lab mammals like rats and mice. The usage of zebrafish in this research field could make comparisons easier, shrink research costs, improve animal welfare and produce better standardized data.

2.6 Experimental aim and relevance

This master thesis aims to find answers to two central aspects:

- What limits the thermal performance of ectothermic animals?
- Has the thermal capacity of laboratory zebrafish been lost due to adaptation to stable optimal temperature over decades?

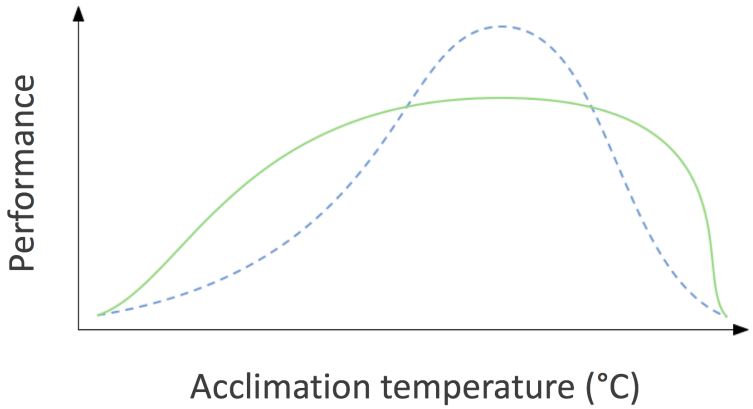

Figure 2.5 Expected performance curves for a wild (green) and a lab-strain population (blue) of zebrafish (*Danio rerio*). The lab-strain population shows a clear optimal peak at temperatures closer to the centre of the temperature spectrum (intended as 25°C to 28°C), and their performance decreases drastically to both ends of the spectrum, when they are shifted away from temperature they were domesticated at. In contrast, the wild population shows a lower overall performance, but a wider optimum stretching over a wide temperature range, which declines only closer to end of both extremes

Therefore, we will observe thermal performance through a spectrum of different techniques (survival, growth, acute thermal tolerance, and swimming speed) over temperatures ranging from 10°C to 38°C. We hypothesis that performances display optima at different temperatures (Claireaux et al., 2000; Clark et al., 2013; Gräns et al., 2014), and do not line up with the same optimal temperature, e.g. aerobic scope as predicted by the OCLTT hypothesis (Pörtner & Knust, 2007; Pörtner & Farrell 2008). For the second part, we predict that the lab-strain zebrafish have reduced the ability to acclimate to non-optimal temperatures besides 25°C to 28°C and that performances will be weaker than in the wild population (fig. 2.5). Lab-strain fish lived in labs without thermal extremes for decades (Spence, 2008), while wild zebrafish encounter fluctuations and eventual higher temperatures as stressors in their surroundings. The loss of thermal plasticity in form of lower thermal tolerance has already been shown in other domesticated fist species (Carline & Machung 2001).

3 Material and methods

The whole study performed for this master thesis consists of three distinctive experiments that where performed in 2017. A pilot study performed in February was the first experiment, and will be following referred to as "Pilot". Moreover, two full sized acclimation experiments were run. The first one was performed in May and June, only with the F1 generation of the wild zebrafish from India, and will be further on mentioned as "Experiment 1", whereas the acclimation done in November and December, with wild and lab-strain zebrafish, will be called "Experiment 2".

3.1 Animal handling

The zebrafish in the lab at the institute of biology, belonging to the Norwegian University for Science and Technology in Trondheim, are kept in accordance with Mattilsynet (Statens tilsyn for planter, fisk, dyr og næringsmidler). An application via FOTS (Forsøksdyrforvaltningens tilsyns- og søknadssystem) was sent in and approved. The animal facility is not open to the public and access is only granted to researchers and competent, as well as approved animal handling employees. The fish labs contain LED lights that are dimmable that can provide sufficient simulated day rhythms.

Figure 3.1 Tank setup with fresh water supply, environmental enrichment, drain and foam filter

The fish tanks are custom-built of transparent glass and in the shape of a rectangular prism with a volume of 63 litres. One tank can accommodate up to hundred zebrafish, with a number of 48 main tanks for accommodation the fish lab contains around 5000 fish at this time and more are expected due to a planned breeding processes. The fish is wild caught zebrafish from

various locations in India, it was transported by air to Sweden and from there by truck to Trondheim in large oxygenated fish transportation bags. Moreover, the lab-strain fish (AB-Wildtype) used in Experiment 2 was supplied by the Yaksi group at the Kavli Centre in Trondheim.

Fresh water volume (left in fig. 3.1) of the facility tanks is exchanged daily, treated with Aquasafe (Tetra) and salt, and bubbled with oxygen. Normally accommodation takes place at 28 ±0.5°C. Every tank contains environmental enrichment, since zebrafish like to stay more often in an enriched part of a tank (Kistler et al., 2011), and aquaria are placed on dark coloured plates, because the fish prefer darker bottom colours (Pavlidis et al., 2013). Foam filters are used to provide oxygen through bubbling and to sustain better water quality by cultivating colonies of useful bacteria. Tagged nets are used to remove fish from the tanks, thereby a specific net is only used for a small number of tanks to prevent any spread of infections or diseases. Feed comes daily as dry feed (Tetra Pro), and living microorganisms (e.g. brine shrimp) to support natural hunting behaviour. Breeding takes places in smaller containers and at least three pairs due to more aggressive behaviour at low densities (Osborne et al., 2016). During the process the eggs fall through a net at the bottom of the tanks and are inaccessible for the parental generation.

3.2 Experimental setup

Pilot study: Two rooms were equipped with two aquaria each, one for adult fish and one for young juveniles (below one cm in body length). Environmental enrichment, a pump in the adult tank and a foam filter in the tank for young juveniles were present. Over the time of 19 days the room temperatures of both rooms were changed to test thermal limits. The temperature was changed around 2°C downwards each day in the cooled room starting around 24°C and 1°C upwards in the warmed room that started at 32°C. During this period feeding behaviour, shape and activity of the individuals were observed and noted to define when the fish suffer from stress and finally reach their thermal limits. The light was turned on from 08:00 to 20:00 every day, checks were performed two to three times a day and feeding took place once in the morning and once in the afternoon.

Experiment 1: F1 zebrafish (n=510), originating from a wild zebrafish population, were used in this experiment. These juvenile fish were randomly separated into 17 aquaria, which were changed to different temperatures ranging from 8°C to 40°C with a difference of 2°C in

between, during the first part of the acclimation process. As a result, 30 fish were accommodated at one temperature. Male and female individuals were in every experimental unit. Every aquarium contained environmental enrichment, a pump with foam filter inlay that kept the water clean and facilitates water mixing, a thermocouple to record the water temperature, and a heating unit (titanium heaters with thermostats or glass heaters). The tanks were separated into three climate rooms. In each room the air temperature was set slightly below the temperature of the aquarium with the lowest in this specific room. This system makes it easy to heat up every aquarium separately to the exact temperature needed and spare on energy as well as costly and complicated cooling.

The light was turned on from 08:00 to 20:00 every day and feeding took place once in the morning (also on weekends) and once in the afternoon (not on weekends).

The temperatures were changed over an interval of eleven days after fish were accommodated in the climate rooms. Aquaria that had to be adjusted to lower temperatures than 28°C were decreased by 2°C daily. The warming process to higher temperatures followed the same approach until 34°C, from there on the temperatures were increased by only 1°C per day. The temperatures were hold constant over an interval of 35 days after final temperatures were reached, stretching the whole experiment to a span of 46 days. CT_{max} and final growth measurements were performed on four days at the end of the period, all fish were recorded in the growth measurement, while only up to 20 fish of each aquarium were used in the CT_{max} experiment.

Experiment 2: F1 zebrafish (n=300) were hatched in early September 2017 out of the existing wild Zebrafish population in the lab. Also, lab-strain zebrafish (WT-AB line; n=300) were raised from eggs supplied by the Yaksi group. Each 300 juveniles were separated into 15 aquaria (total of 30 aquaria, 15 for wild and 15 for lab-strain fish, fig 3.3 right), which were changed to different temperatures ranging from 10°C to 38°C with a difference of 2°C in between, during the first part of the acclimation process (fig 3.3 left). The smaller temperature range was a result of the experience gained in the Experiment 1. As a result, 20 fish of each population were accommodated at one temperature. Male and female individuals were in every experimental unit. Every aquarium contained environmental enrichment, a foam filter and a corner filter that keep the water clean and facilitate water mixing, a thermocouple to record the water temperature, and a heating unit consisting of a thermostat and a titanium heater. The tanks were separated into three climate rooms. In each room the air temperature was set slightly below the temperature of the two aquaria with the lowest in this specific room.

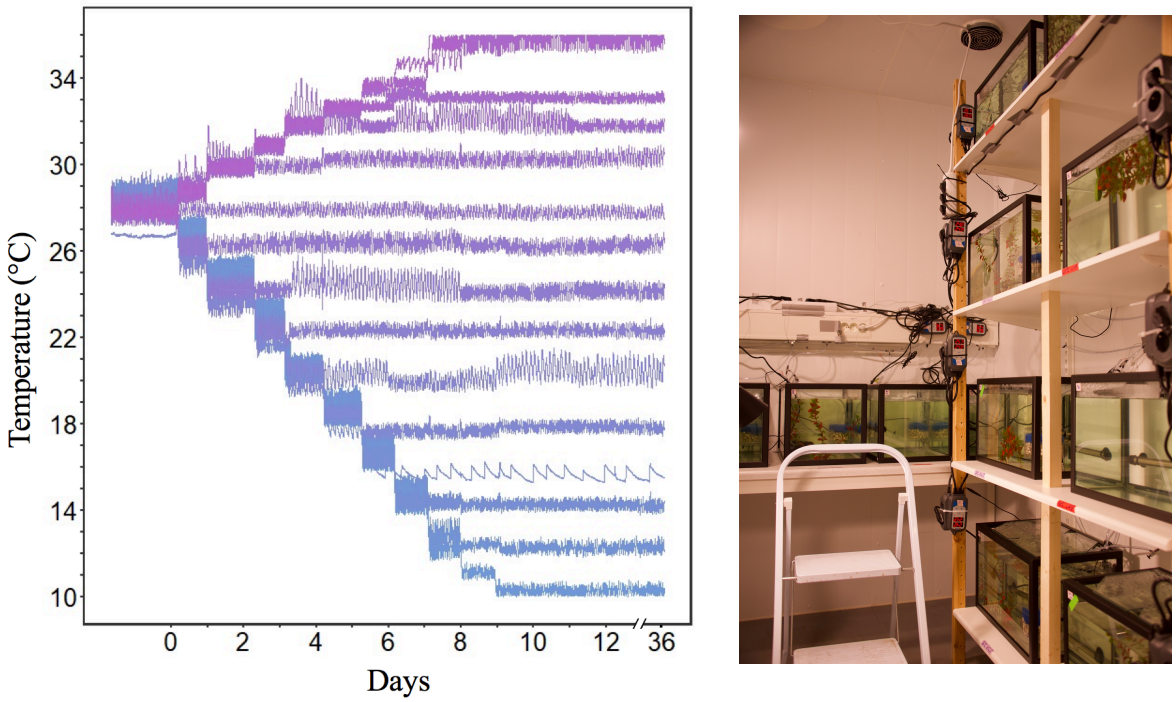

Figure 3.3 Acclimation setup **Left** Temperature development during Experiment 2 in all 14 measured aquaria (38°C excluded due to mortality). The acclimation started at with all aquaria around 28°C, temperatures were changed by 1°C/day to the warmer end, and 2°C/day to colder temperatures until reaching 12°C, from there on 1°C/day until 10°C was reached **Right** One of the acclimation rooms containing the aquaria with temperatures from 16°C to 26°C

Aquaria positions in the rooms were randomized in respect to impact of light coming from opening of the door and varying light conditions from the light source located on the ceiling. The light was turned on from 07:00 to 21:00 every day and feeding took place once in the morning (also on weekends) and once in the afternoon (not on weekends). Over the time of 10 days, the temperature of the aquaria was changed to reach their final acclimation temperature starting at 28°C. All the aquaria that had a lower destination were turned down by 2°C each day until 12°C, the last tank destined for 10°C was changed by 1°C for two days. On the other hand, the aquaria that had to be warmer were heated up by 1°C each day. After the final temperatures were reached, the acclimation temperature was held constant for another 26 days ensuring long term exposure.

For sampling all mentioned performances, it was necessary to sample for twelve to 13 days in a row. This is the reason why the acclimation for both lines was offset, starting with the wild population first and the lab-strain 13 days later; the protocol for both lines was the same. After 14 days of acclimation eight fish per line and acclimation temperature participated in three

days of activity and behaviour measurements. Following, four of these eight identified fish were used during up to ten days of measurements for startle response, standard metabolic rates, hypoxia tolerance, maximal metabolic rates, and swim speed. Thereby, the first measurements overlay with the activity and behaviour measurements. Fish were not fed 24 hours before entering metabolic measurements and isolated in a beaker when back in their aquarium. At last, there were two to three days of final sampling, e.g. CT_{max} and growth. Acclimation temperatures were mixed from high to low through the days of measurement, and were also measured on all times of the day.

3.3 Performance measurements

All measurements were performed at the respective acclimation temperature of the animals and if needed the water temperature was stabilised by a water bath.

Anaesthesia: Buffered Tricaine Methanesulfonate (MS222) with a concentration of 110mg/L was used as anaesthesia (Hohn & Petrie-Hanson 2013). The fish were placed in a glass with the anaesthetic and lost equilibrium in a timescale of one to three minutes. Anaesthesia was in full when the loss of equilibrium is completed and the fish do not respond to physical touch. The animals were placed in normal tank water after the given procedure and regain consciousness and equilibrium after thirty seconds to a few minutes. The fish was monitored until it is completely conscious again. The concentration of the anaesthetic was decreased by a magnitude up to three when used on earlier life stages.

Euthanasia: In case of killings after experiments or in emergency incidents to reduce stress, pain and suffering the euthanasia technique of choice was a sharp blow to the head of the animal with a small metal rod or an overdoses of MS222.

Tagging: The tag itself consists of fluorescent colour, so called elastomers, and was injected with a small needle distal next to either side of the backbone or on the ventral side between the ventral and caudal fin (Hohn & Petrie-Hanson 2013). The fish was anaesthetized beforehand and was placed on a sponge. A notch was cut into the sponge to help fixating the fish while tagging. Using different colours on ventral and dorsal tagging location, together with left and right tagging allow a high number of different tag variation. Thirty different tags were used in Experiment 1 and twenty different ones in Experiment 2 (Appendix fig. 1).

Growth measurements: Body weight and length were obtained at beginning and end of the acclimation experiments. The results of the growth conditions will be presented without presenting any condition factors due to the high correlation between weight and length (appendix fig. 3). Specific growth rate was used as a measurement that is independent of the length of acclimation, and is calculated as followed:

Specific growth rate = $((\ln(W_F) - \ln(W_0)) / \text{time interval}) \times 100$

Where W_F is the final weight measurement, and W_0 is the initial measurement. The time interval was the days of acclimation in between both measurements.

Acute Thermal Tolerance / Critical thermal maxima (CT_{max}) test: The fish were placed in a container filled with water. The container is separated in two segments; the bigger one is for the fish and contains the sensor for the thermometer (see fig. 3.2). The other compartment contains the heat pump, which heats up 9 litres of water by 0.3°C min^{-1}. The permanent, but slow rise in temperature allows an exact measurement of CT_{max}, which is the temperature when the fish lose equilibrium. This procedure enabled a use of up to ten fish at once, thus reduced number of needed CT_{max} cycles and therefore time as well as resources. The fish is placed in water temperate to acclimation temperature and is monitored until it is completely conscious again (Experiment 1), or euthanized in ice water mixed with MS222 after reaching the CT_{max} (Experiment 2). The CT_{max} in differently acclimated zebrafish lab strains start around 39°C and can reach up to 42°C and higher (Morgan et al., 2018). It was estimated that this procedure confronts the fish with around 15 minutes of discomfort during the top five degrees of heat exposure. Survival in these trials is generally close to 100% like in this study or other experiments performed in the Jutfelt Fish Ecophysiology lab (Morgan et al., 2018; Morgan et al., unpublished). In this study, the procedure started at the given acclimation temperature and could due to this fact enlarge the discomfort for individuals that have been acclimated to lower temperatures.

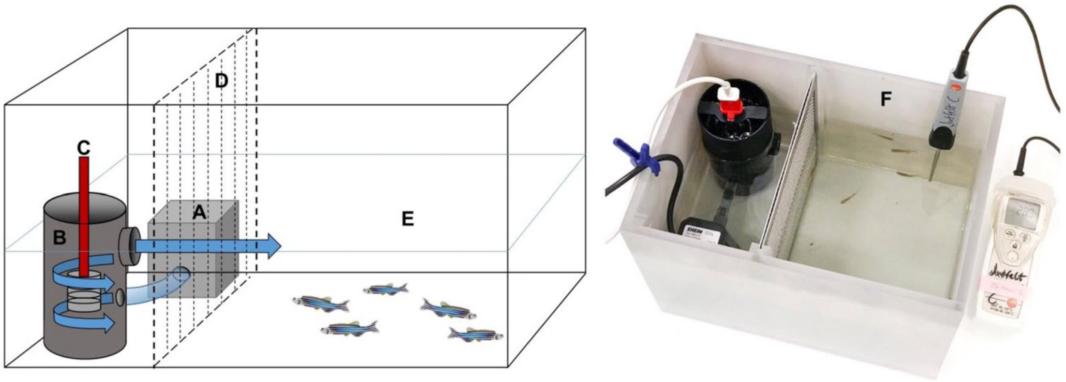

Figure 3.2 CT_{max} experimental setup, ensuring homogenous water temperature and consistent hearting rate for all trials. **A** Water pump **B** custom-made cylindrical steel heating case **C** 300W coil heater **D** mesh (preventing fish swimming into the heating compartment **E** fish compartment with a thermometer (Morgan et al., 2018)

Metabolic scaling and swim speed: Metabolic scope is an elegant way to combine the measurements of minimal and maximal oxygen consumption and can give insight into an organism's metabolic performance and how this metabolism is reflected in the phenotype of an organism. Therefore, the measurement of aerobic scope was planned to be included in this thesis. Aerobic scope is measured through the oxygen consumption rate (Mo_2) and is defined as the difference between minimum (Mo_{2min}) and maximum oxygen consumption rate (Mo_{2max}). Techniques to measure Mo_2 vary wildely from species to species, given by different body size and different life style. It is always important to consider which technique and circumstances fit the best for the animal and the most reliable data. Zebrafish as a small species can be measured in small respirometers that use optodes that are not particularly pressure or temperature sensitive and don´t consume oxygen during measurements. In general, 'swim tunnel' respirometers are used to measure Mo_{2max} and can give as well information about the swimming ability and speed of a single fish. They contain a propeller that circulates the water and can be adjusted to a given swim speed. Mo_{2min} can be measured with 'static' respirometers, which do not contain a propeller and should be constructed as so that the fish does not require to swim to maintain its position. Respirometers for zebrafish can be constructed in a small standard due to its modest body size.

Due to the small size of the used individuals, it was not possible to record sufficient data for Mo_{2max} in the swimming flume, which is why only swim speed data was recorded and will be presented in the results. Mo_{2max} data for whole groups was obtained in Experiment 2, but won´t be included because it would exceed the volume of this thesis.

The water speed of the tunnel was estimated through speed measurements with 200 μm fluorescent microspheres produced by Loligo systems at different speeds and temperatures (Appendix fig. 2).

Additional measurements and final sampling: In addition to the results presented in this thesis, there were many other measurements and samples obtained during the experiment. A group of ten people (the Jutfelt lab and assistants) participated in the final sampling period of three weeks, and the work was conducted under my supervision.
Activity was measured filming the fish in tall containers. The first five minutes were to calm the fish down, the next ten minutes were recorded for activity measurements and after this full 15 minutes a predator cue made of out zebrafish tissue was injected to measure if fleeing or hiding behaviour was induced or lost. Furthermore, a startle response was measured with a self-build set up. An object was dropped onto the water surface and the animal´s response was recorded with a high speed camera at 1000 frames per second. During the final growth and CT_{max} measurements a number of tissues were sampled for various measurements. Three fish from each tank were going through CT_{max} and were flash frozen on liquid nitrogen (after overdoses euthanasia) for gas chromatography. Up to six further fish were also flash frozen on liquid nitrogen for further chemical analyses. The remaining fish, normally up to six, were used for blood sampling combined with a following organ sampling. The results of these measurements and samples are still being processed and analysed by other participants of the experiment, and will not be further presented in this thesis.

3.4 Exemplarily sampling procedure

This paragraph will be an example of how a day of the final sampling was performed. The working space was prepared to the given procedures, example CT_{max} and body measurement. Performing both together saves time for the researcher and reduces stress for the fish because the overall handling time is decreased. Following these preparations, the CT_{max} container was filled with 9 litres of tank water, the MS222 anaesthetics were prepared, the camera or callipers were adjusted and put in place for the length (and also morphological) measurement, and the scale has to be accessible to secure an unhindered process from one measurement to the next which decreases handling time to a minimum. Smaller tanks for one fish each were filled with water to separate the fish after the CT_{max} measurement depending on the used number of fish (up to ten per run). An overdoses of anaesthesia was given into the smaller tanks in Experiment

2 to euthanize the animals before further measurements. During Experiment 1, another bigger container with some environmental enrichment was prepared to take up the fish after the procedure.

The fish were caught with the tank related nets (in Experiment 2 different for wild and lab-strain) and held in a tank with some plastic plant imitate before they were put into the CT_{max} container. The process started at the given acclimation temperature and proceeded until the last fish lost equilibrium, thereby the temperature increased by 0.3°C m^{-1}. The fish that loses equilibrium first is removed from the tank and placed in one of the smaller containers. CT_{max} was noted on a protocol sheet. Every fish was monitored until it recovers from the heat shock, but not in Experiment 2 due to termination at the end of the experiment.

The body measurement started with introducing one fish at a time to the anaesthetics in the prepared glass, if not already euthanized like after CT_{max} in Experiment 2. A modified tea spoon was used to remove the fish from the glass again when complete sedation was achieved. This is the case if the fish lost equilibrium and does not respond to physical touch with the spoon anymore. Following, the fish was weighed and then placed on the millimetre paper to take a photo or measured with a calliper. Results were noted after the fish was placed in the recovering container or given to further measurements. Zebrafish normally recover from the anaesthesia in the first minutes of exposure to normal water conditions, the factor on which the anaesthesia impacts each fish depends on the body size, too. After recovery in Experiment 1, the animals are placed back in their former aquaria.

3.5 Statistics and data analysis

The experimental unit in this experiment is a tank containing 20-30 zebrafish (depending on acclimation) on a given acclimation temperature (see 3.3 Experimental Setup). The selection of fish from the whole batch into the different temperature groups was randomly. Treatments like CT_{max} and body measurement are specifically ordered to avoid unnecessary stress and provide sufficient data.

The data were analysed with R, a program for statistical computing. Models were constructed to check the influence of the acclimation temperature and strain on the different performances. Linear models proved to be most fitting solution based on our data distribution. Models were tested against each other using the residual sum of squares (RSS). Lower values for RSS show that the predictions of the model are closer to the reality of the data itself. An ANOVA was run on the best model and followed by a Tukey as posthoc for growth performance and CT_{max}.

Swim speed data did not contain more than three to four data points per experimental unit/acclimation temperature and was therefore not big enough for sufficient statistical analyses. Still we ran an ANOVA on general effect, but posthocs led to divergent assumptions.

3.6 Ethical statement

The explained procedures are ethical acceptable because basic research serves a serious and important cause during times of changing climate. The existing knowledge about the main mechanisms underlying thermal performance, its evolution and the challenge that fish are confronted with is still insufficient. Ongoing experiments in the Jutfelt Fish Ecophysiology lab at the NTNU Trondheim will focus on two main questions:

What limits the thermal tolerance of ectothermic animals?

How rapid is the evolution of thermal tolerance in a population?

Zebrafish reproduce faster compared to bigger fishes, which have been used in other studies (Pörtner & Knust, 2007; Gräns et al., 2014). Research can be done in a faster pace with higher number of fish, and sufficient protocols and research descriptions will serve the cause to hopefully implement zebrafish as a key player in future thermoregulation and thermal performance-related studies.

The keeping of the zebrafish in the facility was based on previous knowledge, in case of feed, monitoring, tank equipment (e.g. environmental enrichment), and the 3Rs were included in the steps to provide best possible animal welfare. Replacement is not possible due to limited knowledge on which cells or organs are included in the limitations of thermal performance. If adequate data and resulting knowledge is gained it can potentially benefit to develop future relative or complete replacement strategies in thermal performance experiments. Reduction was taken into concern and the number of needed fish was calculated to a minimum with a sufficient number of sampled data points. Nevertheless, a high number of fish is needed to provide the necessary and natural high genetic variation for this project. A lot of refinement was integrated in the housing facilities, e.g. by promoting normal behaviour through an enriched environment and living feed or reduced stress through decreased handling times, based on recent information published on zebrafish handling.

Humane endpoints for the project are defined and were performed if the fish was displaying abnormal behaviour. The animals are treated if possible or euthanized immediately to prevent the animals from any exposure to unnecessary stress, pain or suffering.

4 Results

The results section is separated into different experiments. The insights and findings of the pilot study will be shortly explained, the paragraphs about both whole acclimation experiments will give information about mortality, growth, acute thermal tolerance, and for Experiment 2 swim speed as well.

4.1 Pilot study

The temperature exposure took place for 17 days in the cold room for young juveniles (below a 1cm in body length) and adult, 14 days for adults and 19 days for young juveniles in the warm room. We aimed to estimate the limits of thermal performance, and to detect stress signs like lowered appetite, altered body shape and colouration, and if not avoidable, mortality.

Activity and appetite was strongly decreased in the cold room during the whole process of changing temperatures, especially under 16°C. Some adult fish showed abnormal behaviour when swimming very close to the bottom, nearly lying on it. Even closer to the lowest temperatures, some young juveniles still ate while adult fish stopped eating nearly completely. Stomachs of the fish no longer looked round and were flat shaped, while the skin colouration maintained a good contrast. The lowest temperatures endured during the pilot were 8.8°C for adult fish and 8.5°C for the young juveniles.

Adult fish in the warm room showed lowered appetite when temperatures increased. Nevertheless, they endured temperatures up to 38.8°C and were taken out of the tank when specimens started to look pale and mortalities rose. Young juveniles equally resisted high temperatures up to 38.4°C, showed mortality compared to adults less, but the same signs of discomfort due to pale body colouration and unchanged feeding behaviour.

These insights led to further improvements of the following acclimation experiments and set the general boundaries.

4.2 Experiment 1

In accordance with the pilot study, some general unquantified observations were that activity was lowered in colder temperatures below 20°C and increased at higher temperatures compared to 28°C. Feeding behaviour stopped in temperatures below 16°C and was observed to return after several days of further acclimation. Unfortunately, deformities in the spinal column were observed in nearly all fish of this generation. We categorised the fish used in Experiment 1: 0 = no deformity; 1 = slight deformity; 2 = great deformity, and tested models for its effect.

Mortality: Recorded survival frequency was below one for all temperature groups after 46 days into the experiment. Especially temperatures on the extreme ends experienced high mortality, with 0.23 survival frequency at 10°C and 100% mortality below that and above 34°C (fig. 4.1). It has to be mentioned that all tanks marked as 100% mortality were stopped when survival frequency was approaching 0.0 before every single fish died to prevent a higher degree of unnecessary suffering. Fish were removed if dead or showing signs of ecological deaths (e.g. lost equilibrium), which was more often found in colder temperatures. Compared to the extremes, survival rates were a lot higher in the central areas from 12°C to 34°C with 0.6 survival or higher, e.g. 0.83 at 12°C, with three exceptions. Fish at 20°C and 30°C acclimation temperature displayed survival below 0.4. Furthermore, the fish at 32°C approached 100% mortality and was exchanged with a new tank and stocked up to 20 fish again from temperatures that were closest in the spectrum (24°C, 26°C, 28°C and 30°C) during the early acclimation process. In general, the largest amount of recorded mortality happened during the first two weeks while water temperatures in the aquaria were changed. It appears that high mortality was caused by both temperature stress (at the extremes) and potentially by poor water quality (e.g. at 20°C, 30°C, and 32°C).

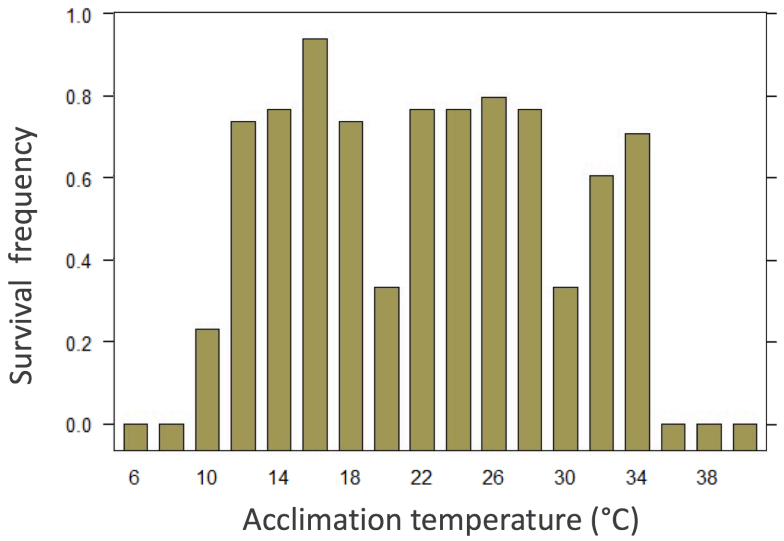

Figure 4.1 Survival frequencies (1.0 equals 100% survival) on different acclimation temperatures from 6°C to 40°C in 2°C intervals for zebrafish of Experiment 1. Survival frequency at 32°C displays only the frequency of the restocked fish after most of the initially used fish at this temperature died

Growth: Weight and length of the fish before and after the acclimation are positively correlated by linear regression and high adjusted coefficients of determination (Appendix fig. 3). Consequently, weight was used as the main parameter to estimate and discuss growth results of both Experiment 1 and 2.

Average specific growth rate was positive but low in all groups (figure 4.2), especially 10 and 12°C that even showed negative specific growth rates. Growth rate of the fish at these two low temperatures were significantly different to fish with an acclimation temperature of 22°C or higher (posthoc tukey: $p<0.05$). There were some individual fish with negative specific growth rates found on nearly all acclimation temperatures. It is possible to see a trend of increasing specific growth rate from 20°C to an optimal rate at 28°C and a following decrease with higher temperatures, despite low and similar growth rates from 14°C to 18°C and high performance on 32°C. A significant effect on the fish´ growth by the acclimation temperature in each group was found (ANOVA, $p<0.001$).

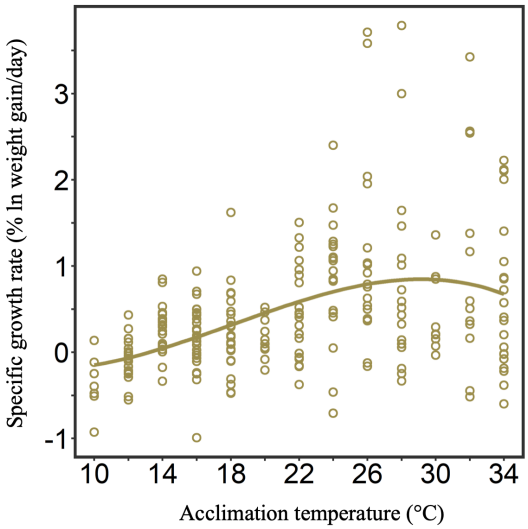

Figure 4.2 Growth in specific growth rate (% ln weight gain/day) at different acclimation temperatures from 10°C to 36°C in 2°C intervals for zebrafish of Experiment 1

Acute thermal tolerance (CT_{max}): There was a positive relationship between acclimation temperature and measured CT_{max}, with every increase in acclimation temperature the mean CT_{max} increases as well (fig 4.3 Left). Therefore, CT_{max} was lowest in the coldest acclimated fish with around 32°C at 10°C acclimation temperature and nearly up to 43°C at 34°C acclimation temperature. Even though the trend line shows an increase in CT_{max}, the difference between acclimation temperature and CT_{max} is decreasing. This is visualised with the thermal safety margin (TSM), which is decreasing with increasing acclimation temperatures from 12°C upwards (fig. 4.3 Right). It has to be mentioned that 10°C had a lower TSM than 12°C.
Mortalities during this experimental procedure were low with only two out of 125 individuals that didn´t recover after measurement. Acclimation temperature had a significant impact on CT_{max} (ANOVA, $p<0.001$). Furthermore, models for the effect of size and the level of deformity were tested and were excluded (both with higher RSS).

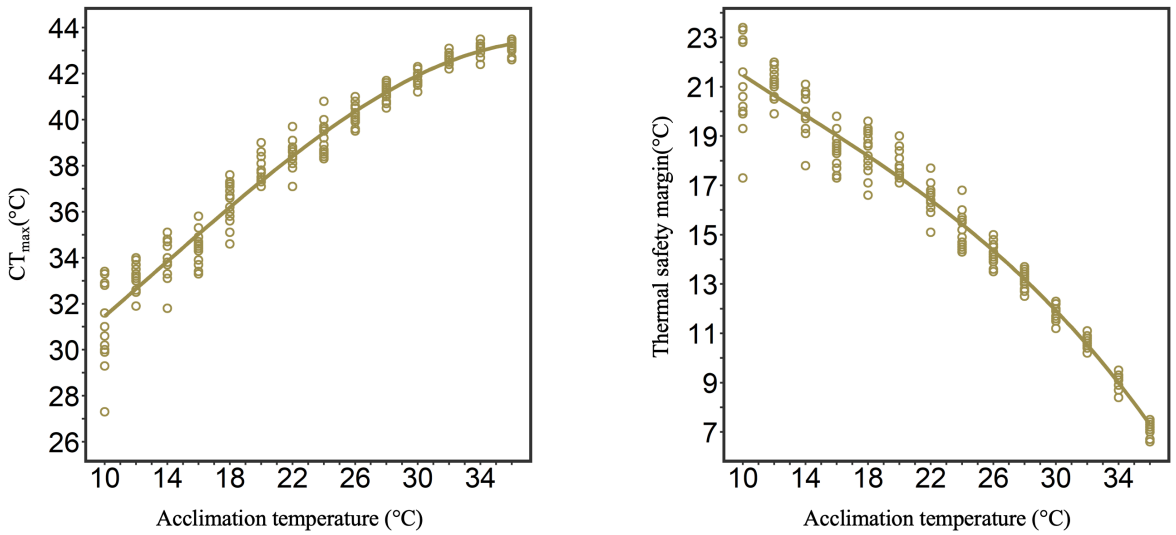

Figure 4.3 Upper thermal tolerance on acclimation temperatures from 10°C to 36°C for zebrafish of Experiment 1. **Left** Acute thermal tolerance (CT_{max}) **Right** Thermal safety margin, the difference between CT_{max} and acclimation temperature

4.3 Experiment 2

Unquantified subjective activity was lowered in temperatures below 20°C and increased at those above 28°C. As seen in Experiment 1, feeding stopped in fish that were acclimated below 16°C and was observed to return after several days of further acclimation. Deformities like observed in Experiment 1 were not found in more than 2 individuals of the total 600 zebrafish used, one in each strain. Lab-strain fish seemed to be less disturbed by people entering the experimental rooms, and less stressed during feeding sessions (unquantified observation). Temperature development in the aquaria worked as planned and showed in the methods (fig. 3.3).

Mortality: Survival frequencies were high in all experimental tanks. Only the zebrafish of both strains at 38°C showed high mortality, which was followed by stopping the experiment for this temperature to avoid further suffering. Besides that, survival frequency of wild fish acclimated to 36°C sunk to only 0.75. Except for one, all other fish populations showed survival rates between 0.9 to 1.0 between 12°C and 34°C.

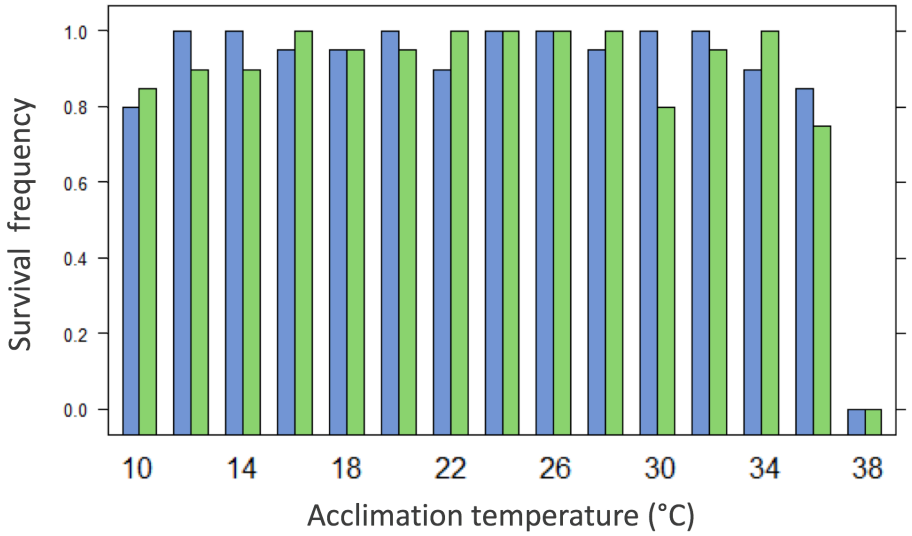

Figure 4.4 Survival frequencies (1.0 equals 100% survival) on different acclimation temperatures from 10°C to 38°C in 2°C intervals for zebrafish of Experiment 2. Lab-strain fish are shown in blue and wild fish in green

Growth: Both strains showed positive average growth rates at all measured temperatures (figure 4.5). There were single individuals with negative specific growth rates found at some temperatures. Growth rates for the wild (green circles) fish lie close together in the temperatures below 16°C, there was a clear and slow increase from there on until the optimal temperatures for growth from 26°C to 30°C, which was followed by a sharper decrease to the higher temperature extreme. The estimated curve for the lab-strain growth (blue triangles) showed a similar trend but nearly doubles the performance of the wild fish on every temperature. Also, the increase in growth at lower acclimation temperatures seemed to happen more pronounced. Variation inside each temperature was high, but there was not much overlap between both strains.

Acclimation temperature together with strain had a significant effect on growth performance (ANOVA, $p<0.001$). In lab-strain and wild fish there was a significant difference found between acclimation temperatures lower than 16°C to 20°C and above (posthoc tukey: $p<0.05$). Moreover, the central temperatures of 26°C and 28°C were significantly different from the highest temperature 36°C and every temperature below 20°C (posthoc tukey: $p<0.05$).

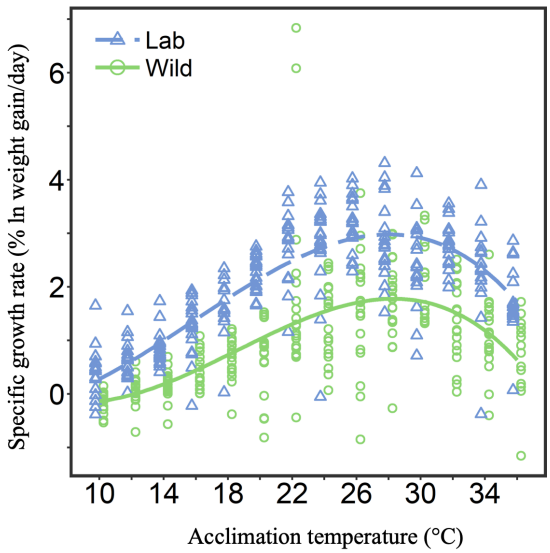

Figure 4.5 Growth in specific growth rate (% ln weight gain/day) at different acclimation temperatures from 10°C to 36°C in 2°C intervals for zebrafish of Experiment 2. Lab-strain fish are shown in blue and wild fish in green

Acute thermal tolerance (CT_{max}): In both strains, a positive relationship between acclimation temperature and measured CT_{max} was found, with every higher step in acclimation temperature the mean CT_{max} increased (fig 4.6 Left). CT_{max} was lowest in the coldest acclimated fish with around 31°C and 32°C in lab-strain and wild fish at 10°C acclimation temperature, respectively. At the colder temperatures, the CT_{max} increased with acclimation temperature at a rate close to 1:1, while at higher temperatures the effect of acclimation on CT_{max} decreased. Above temperatures around 28/30°C, the CT_{max} only increased by about 1/3 of a degree for each degree of increasing acclimation temperature. Both strains reached the same maxima at 43°C at 36°C acclimation temperature. While CT_{max} increased with acclimation temperature, TSM is decreased (fig. 4.6 Right). Compared to wild fish, lab-strain fish showed a slightly decreased CT_{max} performance and TSM on lower acclimation temperatures below 18°C. Strain significantly impacted CT_{max} (ANOVA, $p<0.01$), as well as acclimation temperature (ANOVA, $p<0.001$). A model to test if body size had an influence on CT_{max} was inferior to all other models tested (RSS values were higher). There was a significant difference between fish at the acclimation temperatures 10°C and 12°C, as of all temperatures below 16°C to 18°C and above (posthoc tukey: $p<0.05$).

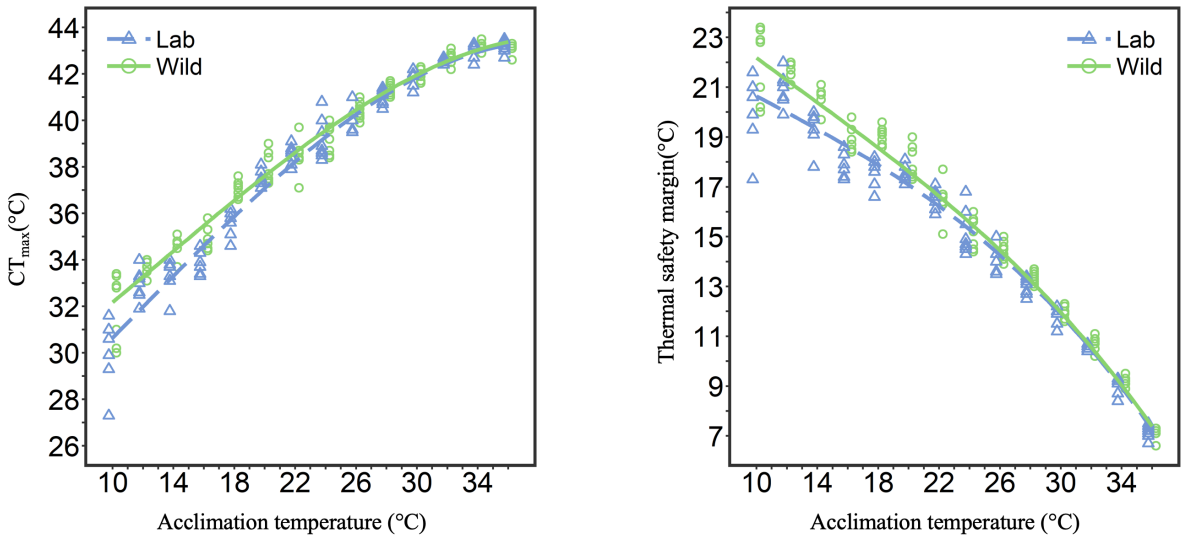

Figure 4.6 Upper thermal tolerance on acclimation temperatures from 10°C to 36°C for zebrafish of Experiment 2. Lab-strain fish are shown in blue and wild fish in green. **Left** Acute thermal tolerance (CT_{max}) **Right** Thermal safety margin, the difference between CT_{max} and acclimation temperature

Swim speed: This performance was measured in metres per second (figure 4.7 Left) and translated into a body-size independent measurement by transforming it into body length/s (figure 4.7 Right). In both visualizations lab fish show a better performance closer to the middle of the thermal spectrum around 26°C. The performance dropped to both ends, nearly down to half at 10°C compared with 26°C. Performance of the wild strain increased similarly from 10°C up to 26°C. From there on the swim speed in m/s and body length/s exceeded the lab-strain performance and did not decrease with higher temperature, reaching the best performances at 34°C and 36°C. In all temperature groups sample size was low (3-4/unit) and variation was high. It was found that acclimation temperature had an effect on swim speed in body length/s (ANOVA, $p<0.001$), but no effect of strain (ANOVA, $p>0.1$).

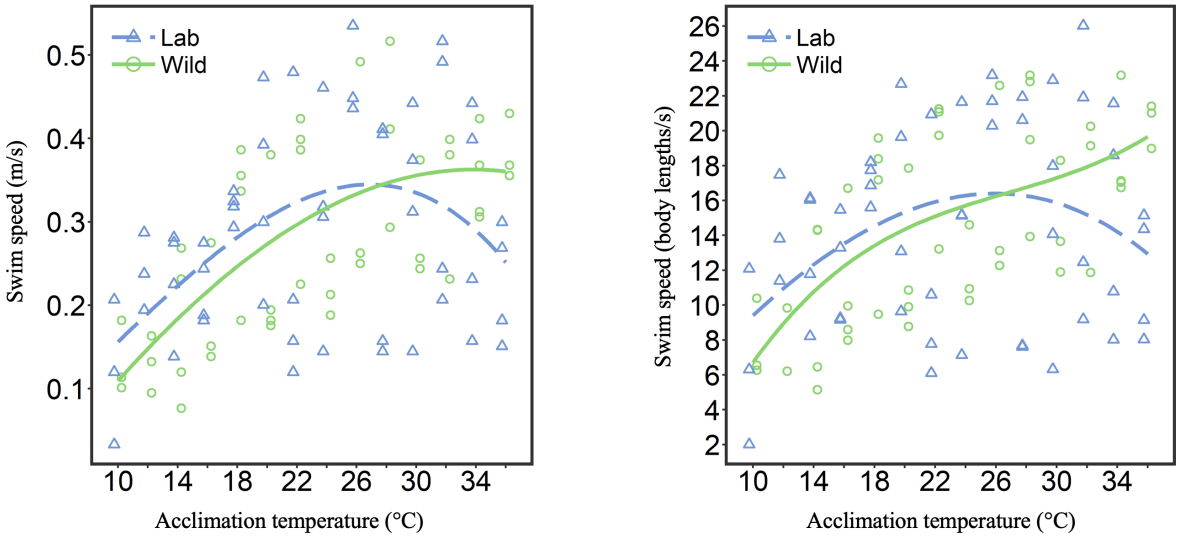

Figure 4.7 Swim speed in accordance to sampled acclimation temperatures from 10°C to 36°C for zebrafish of Experiment 2. Lab-strain fish are shown in blue and wild fish in green. **Left** Swim speed in m/s **Right** Swim speed in body length/s

5 Discussion

5.1 Thermal optima of performances

A major aim of the thesis was to test if different performances display optima at different temperatures, of if all performances show matching optimal temperatures as predicted by the OCLTT hypothesis (Pörtner & Knust, 2007).

The swimming and growth performance of the lab-strain fish peaked around the same temperatures between 22°C and 26°C, supporting matching thermal optima and therefore not confirming the MPMO predictions. However, the wild fish showed a different response with increasing swimming performance up to the highest temperatures, creating a thermal mismatch between growth and swimming performance.

Aerobic scope could not be included in this thesis, but there are two main possibilities for the outcome of the measured metabolic data: (1) Aerobic scope peaks at central temperatures in acclimated animals (Healy & Schulte, 2012), or (2) aerobic scope continues to grow with increasing temperature until lethal thermal limits are reached (Claireaux et al., 2000, Gräns et al., 2014).

In scenario (1), the aerobic scope and growth would share thermal optima, but swimming performance would not peak in this central temperature range. This outcome would partially fit predictions of the OCLTT hypothesis.

In the second scenario, aerobic scope would potentially peak together with the swimming performance at higher temperatures and would leave the growth maximum aside at central temperatures. Again, the OCLTT hypothesis would be only partly supported. Another major prediction of the OCLTT hypothesis that was not supported was that thermal tolerance breadth breadths were the same for young juveniles and adults. It was hypothesised that aerobic limitations would be experienced earlier by larger than by smaller individuals (Pörtner, 2004; Pörtner & Farrell, 2008).

The present results suggest different thermal optima for different performances, as predicted by the MPMO model. Before a final conclusion can be drawn on the implications for the OCLTT vs. MPMO debate, more of the recorded measurements (e.g. activity, startle response, or behaviour performance) should to be analysed.

5.2 Reduced acclimation capacity in lab-strain fish

The second hypothesis was tested in Experiment 2. We predicted that lab-strain zebrafish have a reduced capacity to acclimate to non-optimal temperatures outside of the 25°C to 28°C range they were domesticated to in and potentially adapted to (fig. 2.5). Surprisingly, lab-strain fish appeared to have maintained the capacity to acclimate to the entire temperature range, and sustain a growth performance that even exceeded the wild population at all temperatures. This may not seem to fit the hypothesis, but it could possibly be explained by domestication favouring high growth rates in general. A physiological domestication effect of increased growth rate lab and wild zebrafish was shown before (Robison & Rowland 2005).

For the other performances, however, the pattern looks different. Lab-strain fish had lower thermal safety margin than wild fish below 16°C, which could be a sign of reduced acclimation capacity at the colder end of the temperature spectrum and potentially be an ecological disadvantage. Swimming performance for lab-strain fish declined when temperatures were increased, but not in the wild fish. This could also indicate reduced acclimation capacity in lab-strain fish, but for this performance it occurred at higher temperatures.

While the data is not presented in this thesis, a first glimpse into the metabolic measurements point into the same direction. They hint of a lower thermal acclimation capacity for the metabolic rates of the domesticated lab-strain zebrafish. Lastly, it can be said that our data mostly support this second hypothesis, that lab-strain zebrafish have lost some acclimation capacity through adaptation to optimal temperatures.

5.3 Mortality

As expected, mortality appeared highly temperature-dependent with higher rates especially closer to the extremes below 10°C to 12°C and above 34°C to 36°C where fish are shifted further from their preferred temperature.

Already the results of the pilot seem to explain many findings from the full sized acclimation experiments. While in Experiment 2 only two tanks completely collapsed on 38°C for both strains, Experiment 1 was plagued by definitely lower survival frequency (fig. 4.1). The limits set by the pilot lay at low temperatures with 8.8°C and higher at 38.8°C, it was expected that the fish could endure even a bit further treatment with better care and slower acclimation rate. This was proven to be wrong when the experiment had to be stopped at similar temperatures (≤10°C and ≥38°C) in both acclimations. Even though this could not be conducted, it proves the results of the pilot even more, with a short-term (days) tolerance range from around 9°C to

39°C, and long term (weeks) tolerance range of 10°C to 36°C. Another interesting result of the pilot is that young juveniles as well as adults had similar thermal limits (±0.3/0.4°C). This contradicts the theory presented in fig. 2.2 by Pörtner and Farrell (2008), which suggested different thermal windows for different life stages of organisms. More research on more species should be performed to settle this matter.

Experiment 2 shows the long-term acclimation window of both strains, with high survival from 10°C to 36°C. The lower thermal limit was found to be between 8°C and 10°C. However, zebrafish that were found in nature at 6°C, indicating an even lower thermal tolerance in some populations (Spence et al., 2008). A possible reason for this difference in tolerance could be the high number of different zebrafish sub populations that have a high genetic diversity (Brown et al., 2012; Patowary et al., 2013). The genetic diversity of the fish in this experiment could be subject to limitation on the lower end of the temperature span. It is unclear why zebrafish at 10°C in Experiment 1 performed much worse than in Experiment 2. The rate of temperature change could have been too high, because most mortality happened in this early stage of the experiment.

The fish dying at 38°C during Experiment 2 showed no decline in survival before the final temperature was reached and the aquaria were kept at 38°C for more than four days before mortality increased, showing that survival is time limited at this temperature. Zebrafish have been found in the wild at 38°C (Engeszer et al. 2007), and also our parental generation originates from waters with temperatures up to at least 37°C. Water quality can become an issue in aquaria at 34°C. High nitrite levels can be dangerous to fish held in aquaria and can be easily taken up from the environment through the gills because of its high affinity to the Cl^- /HCO_3^- exchanger. High uptake of nitrite leads then to iron oxidation of haemoglobin creating ferrihaemoglobin, which is incapable of reversible oxygen binding, causing further problems in oxygen supply (Kroupova et al., 2005).

5.4 Growth

The thermal profiles for growth rate appeared similarly in both Experiment 1 and 2, also for both zebrafish strains in Experiment 2, with maxima around 26°C and 28°C.

Lab-strain fish grew about twice as fast as wild fish at all temperatures. This was surprising but could potentially be due to four reasons: (1) Lab-strain fish have been domesticated in the lab for over fifty years and were potentially selected for fast growth and successful reproduction to perform experiments easier and more rapidly. (2) Wild F1 zebrafish show less

appetite and increased distraction and escaping behaviour when a person enters the room to feed the animals. (3) The age of the strains was slightly different and could have an impact on the performance shown. Both lines in Experiment 2 were chosen to start at roughly equal body size in disfavour of starting at the exact same age, while Experiment 1 was simply started when fish were considered big enough for tagging. (4) The water quality in Experiment 1 was worse than in Experiment 2 due to less maintenance. Thereby, it can be argued that 1st, 3rd, and 4th reason most certainly had a role in these results, while the 2nd guess is hard to prove without any concrete measurement of appetite.

There are more certain explanations concerning the lower performance at colder temperatures. In other fish species, like Gilt-head bream *Sparus aurata,* or Atlantic halibut *Hippoglossus hippoglossus,* growth was reduced through the induction of colder temperatures (Ibarz et al., 2005; Gräns et al., 2014). Different reasons were proposed for these observations: (1) Loss of appetite (Ibarz et al., 2005) that could be a symptom of an underlying physiological issue, (2) decreased activity and metabolism as an energy conserving strategy (Pörtner & Farrell, 2008), (3) constrained enzyme kinetics (Angilletta et al., 2010). The 3rd explanation is given by thermodynamic laws in which biological rates slow down with decreasing temperature, it is also possible that organisms account for this with acclimation through increased enzyme numbers or enzyme isoforms with different thermal optima. The first two explanations can be discussed based on some general observations during the three distinctive experiments. Loss of appetite was initially displayed by all fish that were exposed to temperatures below 16°C, and was not recovered before several days had passed, sometimes even a week or longer. During acclimation for temperatures below 16°C, this results in an effective loss of up to ten days for food digestion and growth. Moreover, activity was positively correlated with temperature according to our unquantified observations, which gives fuel to the 2nd explanation.

In contrast, it is harder to explain for the decline in growth performance at higher temperatures. Oxygen transport limitation leading to tissue hypoxia and reduced tissue growth has been suggested as a mechanism (Pörtner & Knust, 2007; Pörtner, 2010). However, this has been contested by some experiments (Gräns et al., 2014; Li et al., 2015). Reduced cell functions through lowered aerobic scope (and consequently tissue hypoxia) might be an explanation at the colder end of the spectrum, but would not be explanation enough on the higher end. It was reported by Gräns et al. (2014) that a mismatch of growth and aerobic scope was found for the two highest acclimation temperatures used. Here, aerobic scope was at its highest, while growth was decreased, showing that oxygen transport was not limiting growth (Gräns et al., 2014). It

will be interesting to see if this mismatch occurs also in our experiment when metabolic measurements are thoroughly analysed. Appetite could again be a possible reason for reduced growth at high temperatures in our study, because it was observed to decrease at increased temperatures during the pilot study. Therefore, we suggest that a measurement for appetite should be used for future experiments, since this variable could be a potential explanation for many unresolved questions especially on lower acclimation temperatures.

5.5 Acute thermal tolerance

The acute tolerance to an acute temperature increase was tested with the critical thermal maxima (CT_{max}) test, which is commonly used and an integral part of several studies conducted in our lab (Morgan et al., 2018). Data obtained from both of our experiments go in line with previous studies that recorded CT_{max} values at similar acclimation temperatures (Cortemeglia and Beitinger, 2005; Schaefer and Ryan, 2006). Also, a clear positive relationship in several different fish species between acclimation temperature and CT_{max} has been observed before (Beitinger et al., 2000). Comparison of the wild zebrafish performance in Experiment 1 and 2, and comparison of the performances of both strains used in Experiment 2, both show that acute thermal tolerance is relatively consistent (Aroújo et al., 2013; Grigg & Buckley, 2013), because two sets of offspring from the same parental generation showed nearly equal ranges of acute upper thermal limit in separate experiments.

Based on Morgan et al. (2018), the CT_{max} measured in our acclimation experiment only displays the innate CT_{max}. This means that fish undergo an initial heat shock with the first CT_{max} trial that can only be experienced once in life and that initiates physiological mechanisms that activate warm acclimation, e.g. by increased heat shock protein production (Lindquist, 1986; Sørensen et al., 2003). Repeating the same test on the same fish would lead to a result that can be considered as an acclimated CT_{max} result, which may differ from the innate CT_{max} value. It was shown that these results differ from one another and that repeatability between trials increase in the trials performed after the first initial CT_{max} test (Morgan et al., 2018). This process was already described as “heat hardening” (Hutchison, 1961; Maness & Hutchison, 1980; Hutchison & Maness, 1979) and questions how our results would look like when displaying the acclimated CT_{max}. It can be assumed that the heat hardening response would be more pronounced in the zebrafish acclimated to lower temperatures, because heat hardening could have already taken place to a certain degree in fish that were acclimated to higher temperatures.

As explained in the results section, the TSM (the difference between acclimation temperature and CT_{max}) was decreasing with increasing acclimation temperature. Such shrinking end of the TSM at the higher end between T_{opt} and upper T_{crit} has already been found in other fish species (Sandblom et al., 2016). It also matches the heat tolerance curve of Payne and Smith (2017) with a higher TSM for animals with a lower T_{opt} (fig. 1.1 Right). Payne and Smith´s curve may be a result of different studies and species, but the TSM data presented in this study shows a very similar pattern in both strains. It might be questionable if Antarctic stenothermic ectotherms can be integrated into this idea, since these animals have low thermal tolerance and low TSM (Somero, 2010), but would according to Payne and Smith (2017) have the largest TSM. It is possible that a combination of the temperature variability hypothesis (adaptation to the occurring temperature range) and biological rate hypothesis governs the patterns we observe in nature.

The differences in CT_{max} between the strains were not as pronounced as in growth rates, which indicates that size does not seem to have an impact on CT_{max} as also reported by others (Recsetar et al., 2012; Anttila et al., 2013). This was as well confirmed by our statistical analysis. This suggest that the used heating rate (0.3°C min^{-1}) in this setup is appropriate, because it is not too fast to create an offset between body and water temperature, which could have been the case if body size had an effect on CT_{max}.

The most pronounced difference between lab-strain in CT_{max} and wild fish in Experiment 2 happened between the cold-acclimated fish below 16°C, which were significantly different from higher acclimation temperatures. Here, the CT_{max} of the wild fish was higher on average, which indicates two things: (1) Lab-strain fish may have acclimated better to lower acclimation temperatures by shrinking their upper thermal limit to improve other performance, e.g. growth. (2) Wild fish acclimated better, because they are better prepared for rapid temperature changes in their environment. Both approaches have their validity, but the 2^{nd} seems more reasonable when facing the environment of this species. Zebrafish live in different streams with high variable temperatures from site to site as well as over time. If fish are migrating from one river to another, it would be an advantage to be prepared for a greater shift in temperature. There are expectations for transient warm episodes to develop with higher frequencies and intensity caused by climate change (Meehl & Tebaldi, 2004; Seneviratne et al., 2014), which could be harmful for local zebrafish populations.

CT_{max} is only an acute measure and may therefore have limited value for predicting thermal performance in the wild. Measurements of CT_{max} and equivalents often display a higher thermal limit than actually found in the wild and often species are able to survive short-term,

but not long-term acclimation to these temperatures or are unable to reproduce and sustain the population (Sunday et al 2012; Peck et al. 2009; Peck et al 2014; Richard et al., 2012; Payne & Smith, 2017; Richter & Kolmes, 2005; Clark et al., 2011).

Zebrafish as an eurythermal species have not been considered threatened by climate change, even though there are higher temperatures to be expected and more frequent warm episodes predicted (Pörtner et al., 2014; Meehl & Tebaldi, 2004; Seneviratne et al., 2014). Still, the potential for local extinctions (Pörtner & Knust, 2007) cannot completely be excluded considering marked seasonal and daily variations in temperature in the habitat of wild zebrafish, as well as the fact that this species already lives close to its thermal limits (Payne and Temple, 1996; Spence et al., 2008). The measured TSM at higher temperatures is below 6°C, which is a small buffer against heat waves or gradually rising temperatures.

In these experiments we have shown the maximum temperature the fish strains can tolerate is 38°C over the time of a few days, even though we had to stop the experiment when fish started to die in greater numbers. These 38°C are lower than the acute CT_{max} measurements, which goes in line with claims that acute thermal tolerance measurements often overestimate maximal thermal limits (Sunday et al 2012; Peck et al. 2009; Peck et al 2014).

Even though of perhaps less relevance in the current threat of climate change, it would have been interesting to measure CT_{min} to evaluate the full acute thermal breadth of the used zebrafish strains. Because of time constraints CT_{min} could not be included in these experiments.

5.6 Swim speed

Measuring swimming performance can be valuable to study, as it can display the physiological status of a fish (Plaut, 2001). A high performance in the wild helps avoiding predators, can increase the uptake of food, or even help finding a mating partner (Drucker, 1996; Watkins, 1996). Drawing ecological relevance from lab to the wild has to be done carefully (Plaut, 2001). Even though we intended to reflect a natural environment for the zebrafish with time to acclimate to the new situation, a barrier between the fish and the observer, and the placement of a mirror right next to the flume to simulate other individuals swimming with the measured fish, a lab situation can not completely represent the situation in the wild.

Overall the swim speed measured in this study was quite variable. A limitation was the low sample size, with only three to four fish measured per temperature and strain, which reduces the power of statistical analysis. The sample size per temperature was low in order to sample the full range of 2°C temperature intervals. However, the total number of fish was considerably

large (wild: n=49; lab-strain: n=53).

There is evidence from the common killifish (*Fundulus heteroclitus*) that no statistical difference was found in swimming performances of fish acclimated to temperatures between 10°C and 34°C (Fangue et al., 2008). This is not comparable to our data since an effect of acclimation temperature on swimming performance was found in our study, but no sufficient statistical evidence concerning the trends of the different strains. While the performance of lab-strain fish showed a bell-shaped performance curve, the highest performances was found with the highest temperatures in the wild population. This hints of a potential deficit in thermal acclimation capacity for the lab-strain population, and is in line with the predictions of the second hypothesis of this thesis.

A positive correlation between acclimation temperature and swimming performance has been found for several species, where swim performance increased until an optimum was reached, and then plateaued before declining with even higher temperatures (O'Steen & Bennett, 2003; MacNutt et al., 2004). This could be the case for both of our strains, even though the wild fish showed the highest performance at 36°C.

5.7 Conclusions

We show that zebrafish of both strains, lab and wild, are able to acclimate and survive an impressively wide temperature range from 10°C to 36°C. We also note that the optimal temperature for growth is 28°C for both wild and lab-strain zebrafish, which affirms that the use of that temperature in laboratory settings. It also suggests that the lab fish have maintained the optimal temperature through the domestication process and the 50 to 500 generations in laboratory conditions.

Concerning our first hypothesis, it can be said that ecological valuable measurements, e.g. growth rate and swimming performance, have different optima in the wild zebrafish population. These observations better fit the predictions by the MPMO model than the OCLTT hypothesis, yet more performances are needed before final conclusions can be drawn.

For our second hypothesis, the experimental support was clearer. Lab-strain zebrafish have maintained high thermal acclimation capacity, as shown by high survival and growth over a wide range of temperatures, even after decades of domestication to optimal temperatures. However, wild zebrafish tolerate abrupt temperature increases (CT_{max}) when acclimated to low temperatures better than lab-strain fish. This can happen frequently in the natural environment of zebrafish when migrating from one river to the next and displays an ecological advantage.

Additionally, wild zebrafish swim faster at higher acclimation temperatures, matching our prediction of higher acclimation capacity.

Appendix

Fish tagging schemes

Fish	Left Back	Right Back	Bottom
1			
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3			
4			
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Fish	Left Back	Right Back	Bottom
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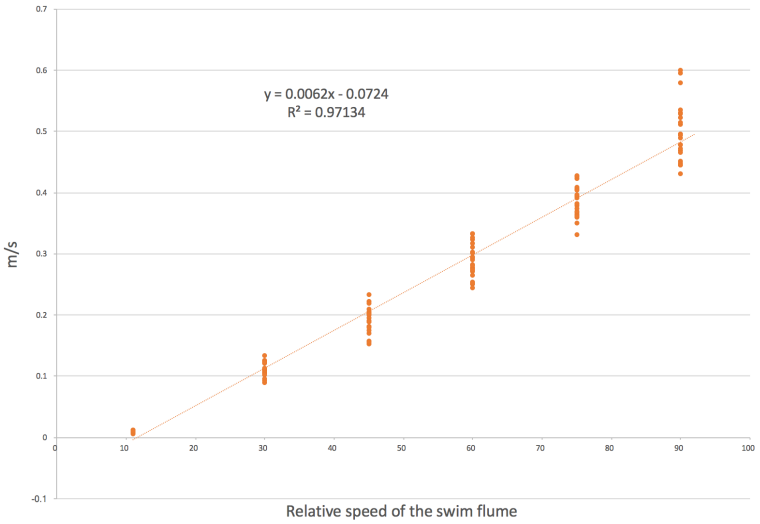
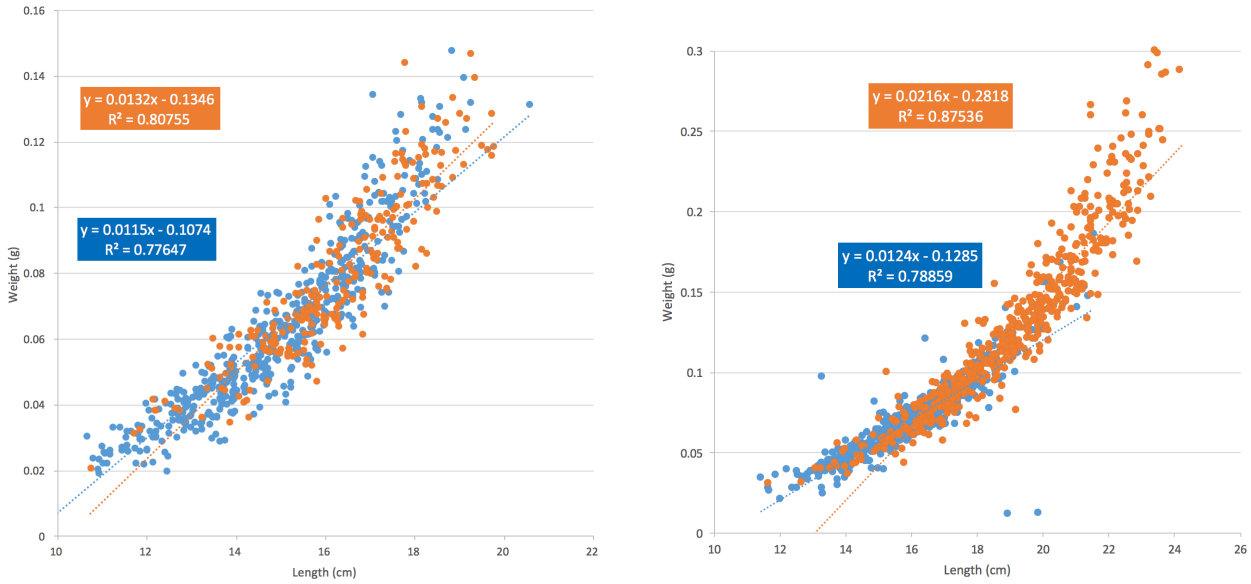
Appendix figure 1 Fish tagging schemes. Zebrafish can be tagged on three different locations, two on the dorsal and one on the ventral side of the animal **Left** 30 different tags used in Experiment 1 **Right** 20 different tags used in Experiment 2

Swim flume: Calculation of the real water speed



Appendix figure 2 Calculation of the real swim speed that the swimming flume of Loligo system is able to perform. Data points were recorded on different temperatures, but no pattern was discovered. Therefore, all data points were included in one equation.

Relationship between length and weight



Appendix figure 3 Measured weight in relation to measured length. Initial values at the beginning of the experiment is displayed in blue, end values are shown in orange **Left** Experiment 1 **Right** Experiment 2

List of abbreviations

CT_{max}	Critical thermal maxima
Mo_2	Oxygen consumption rate
Mo_{2min}	Minimal oxygen consumption rate
Mo_{2max}	Maximal oxygen consumption rate
MPMO	Multiple performances multiple optima
MS222	Tricaine Methanesulfonate
OCLTT	Oxygen- and capacity-limited thermal tolerance
RSS	Residual sum of squares
T_{optAS}	Temperature for optimal aerobic scope
T_{opt}	Temperature for optimal performance
T_{crit}	Critical temperature for reaching lethal limits
TSM	Thermal safety margin
TTB	Thermal tolerance breadth

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