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Physiological mechanisms of rapid and long-term thermal acclimation in a fish

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ABSTRACT

To better cope with changing water temperatures, most aquatic ectotherms undergo physiological acclimation which often alters their thermal tolerance. The timeframe and relative contribution of various underlying mechanisms remain unclear, but are crucial to understand for predictions of climate change impacts. We investigated three candidate mechanisms involved in thermal acclimation in fish: heat shock protein expression (specifically HSP70), mitochondrial density (citrate synthase [CS] expression), and membrane fluidity (via spectroscopic fluorescence polarization). Zebrafish were acclimated to cold (20 °C), control (28 °C), or warm (35 °C) temperatures for two weeks, followed by half of each group receiving a 3-h heat shock to observe their potential for additional rapid acclimation (heat hardening). As expected, acute warming tolerance (CT_{max}) increased with long-term acclimation temperature. CT_{max} also increased by ~0.5–1 °C following the 3-h heat shock, which is surprisingly fast. Expression of HSP70 in the brain was strongly upregulated after both long-term and rapid warm acclimation, aligning with the CT_{max} results. CS expression was not affected by thermal acclimation at these time scales. Membrane fluidity in muscle decreased for long-term warm-acclimated fish when measured at a common temperature (21 °C), whereas the 3 h heat shock had no effect. Collectively, this suggest that adjustments in membrane fluidity are slow while the heat shock response with HSP expression can act sufficiently fast to respond to rapid temperature fluctuations and is likely involved in determining thermal tolerance in fish.

1. Introduction

Heatwaves pose a major threat to the performance and persistence of aquatic ectothermic animals in both marine and freshwater ecosystems, and climate change is already increasing their frequency and severity (Till et al., 2019; Genin et al., 2020; Smith et al., 2023; Calvin et al., 2023). Furthermore, many shallow water habitats such as coastal marine areas, lakes, and rivers, can experience rapid heating (several °C per hour) and large daily fluctuations in temperature that can reach the upper thermal limits of resident populations (Wood et al., 2016; Andersen et al., 2017; Leeuwis and Gamperl, 2022; Ern et al., 2023; Desforges et al., 2023). Such heat wave-induced fish mass mortality events are becoming increasingly common (Braz-Mota and Luis Val, 2024), yet our ability to predict their impacts is partly hampered by a lack of knowledge on the potential for mitigation through thermal acclimation.

To cope with high temperatures and promote long-term survival in a new thermal environment, many aquatic ectotherms undergo thermal acclimation, which involves the alteration of physiological and biochemical processes (Loeb and Wasteneys, 1912; Fry, 1958; Jutfelt et al., 2024; Ern et al., 2023). It is well recognised that ectotherms can adjust many traits of their thermal performance and tolerance through acclimation (Angilletta, 2009; Norin et al., 2013; Seebacher et al., 2015). Thermal acclimation is usually a long-term process that can start immediately (within minutes) upon thermal exposure, but can take many weeks to complete depending on species and temperature (De Bonville et al., 2025). In fish, warm acclimation generally increases the acute warming tolerance (commonly measured as the critical thermal maximum; CT_{max}). For example, zebrafish (*Danio rerio*) acclimated to 10 °C for one month have a CT_{max} of ~30 °C, while 36°C-acclimated fish

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have a CT_{max} of ~42 °C (Morgan et al., 2019, 2022). Between those two extremes, CT_{max} increases with acclimation temperature at a diminishing rate towards the maximum acclimation temperature (Morgan et al., 2020, 2022).

At the cellular level, the mechanisms behind warm acclimation are poorly known, and they are likely dependent on several factors, such as the study species and timeframe of the acclimation period (Jutfelt et al., 2024; Somero et al., 2017). Three important mechanisms of thermal acclimation (Fig. 1) have been proposed: 1) the heat shock response, 2) mitochondrial function, and 3) membrane fluidity (Jutfelt et al., 2024; Johnston and Dunn, 1987; Hochachka and Somero, 2002).

Heat shock proteins (HSPs) are highly conserved families of chaperone proteins participating in the folding and conservation of the three-dimensional structure of other proteins (Iwama et al., 1998). Heat stress often induces the expression of HSPs, which likely protects the organism from thermal injury (Lindquist and Craig, 1988; Dietz and Somero, 1993; Kregel, 2002). There are several different HSPs present in cells, with HSP70 being the most abundant family in eukaryotes (Radons, 2016). HSP70 has been shown to be particularly important for protecting against heat stress and can exhibit an elevated expression with higher temperatures in fish (Dietz and Somero, 1992; Basu et al., 2002; Metzger et al., 2016; Lewis et al., 2016).

Thermal acclimation often involves changes in mitochondrial function, as the mitochondrial density is often optimised for the thermal environment of the organism. This acts as a compensation mechanism for changes in enzymatic rates caused by the temperature sensitivity of biochemical processes. The cellular mitochondrial density can be altered through biogenesis, fission, fusion and degradation (Chan, 2020), and is typically increased at colder temperatures compared to warmer temperatures where it is decreased (Egginton and Sidell, 1989; Guderley and St-Pierre, 2002; White et al., 2012). Although the exact time-course of this mechanism is not well studied in fish, it is likely a more time consuming response compared to the heat shock response. A common marker for mitochondrial density is the activity or content of the enzyme citrate synthase (CS). Although CS is not a direct measure of mitochondrial density, a strong correlation between CS activity and mitochondrial content has been reported (Larsen et al., 2012). There are no known isoforms of animal CS, and studies found no variation in functional properties of CS across different fish species, concluding that CS activity likely reflects CS content (Dalziel et al., 2005).

Acute temperature fluctuations are associated with changes in the fluidity of biological membranes, whereby cooling leads to more viscous membranes, while warming increases membrane fluidity (Jutfelt et al., 2024). If the fluidity is above or below the optimum, it can threaten the function of the organism. Thermal acclimation can therefore include alterations of the lipid composition of membranes (i.e., homeoviscous adaptation). In fish, membrane cholesterol content and the relative amount of saturated fatty acids are increased in various tissues during warm acclimation (Somero et al., 2017; Robertson and Hazel, 1995; Logue et al., 2000). Such changes reduce fluidity and likely counter the direct effects of elevated temperature on the membranes. This means that if successfully acclimated membranes are measured at a common temperature, one would expect a decreased fluidity for warm-acclimated membranes compared to colder acclimation groups. The decreased fluidity signifies a thermal compensation for those membranes at the warm temperature.

Since many shallow-water species experience very rapid warming during heat waves and daily thermal fluctuations (Ern et al., 2023; Desforges et al., 2023), being able to quickly acclimate can be crucial. In certain ectotherms that are acutely exposed to increasing temperatures, the CT_{max} is rapidly increased (Chung, 1981; Fangue et al., 2014; Aslanidi and Kharakoz, 2021; Stewart et al., 2023). This rapid acclimation process has sometimes been referred to as “heat hardening”. It is unclear if rapid and long-term warm acclimation are distinct processes, or simply represent a continuum of phenotypic responses to warming but measured at different time scales. In this view, rapid acclimation constitutes the immediate responses (i.e., heat hardening), while long-term acclimation includes all additional slower physiological and biochemical adjustments that occur as a response to warming (Stewart et al., 2023; Maness and Hutchison, 1980; Morgan et al., 2018; Dallas and Warne, 2023). It is currently unknown how similar the physiological mechanisms behind rapid acclimation are to those underlying long-term warm acclimation.

In this study, we aimed to better understand which physiological mechanisms drive both rapid and long-term thermal acclimation.We therefore investigated the effect of cold and warm temperature exposure over an extended duration (14–18 days) and after an additional rapid heat shock (3 h) on the warming tolerance of zebrafish (*Danio rerio*). We measured CT_{max} in three groups of 26 fish (78 in total) acclimated to either 20 °C, 28 °C or 35 °C, where half of the fish at each acclimation temperature additionally experienced a 3-h heat shock. Previous studies suggest that many fish species can be fully warm acclimated within two

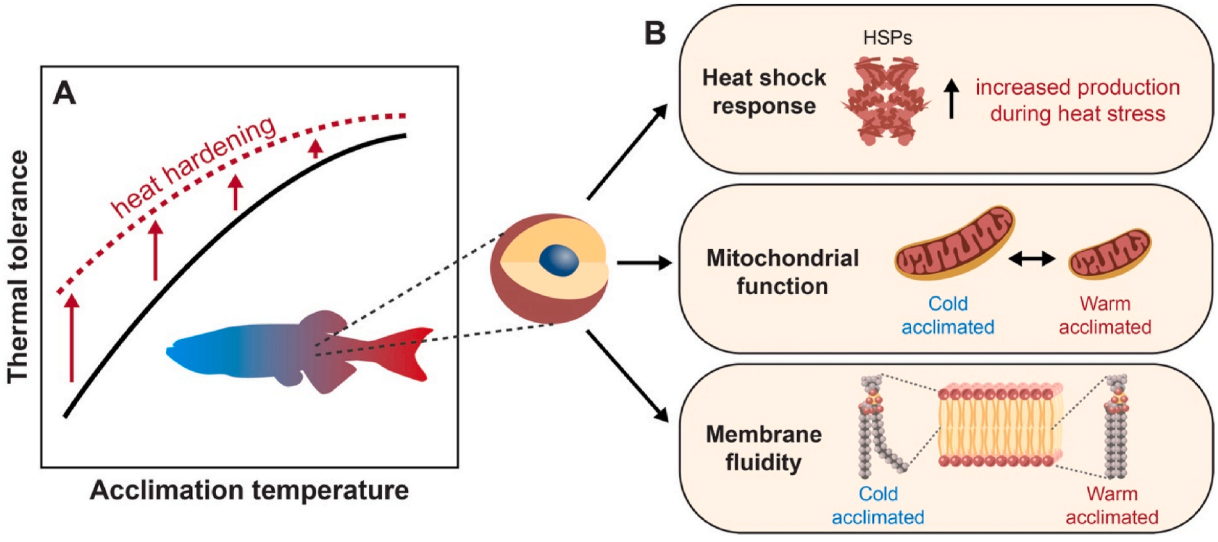

Fig. 1. Potential mechanisms for temperature acclimation of thermal tolerance. The solid line in A) shows how thermal tolerance typically increases with acclimation temperature and the red dashed line shows how the thermal tolerance might be further increased as a rapid acclimation response to acute heating, referred to as “heat hardening”. B) We focus on three main cellular mechanisms of thermal acclimation: the heat shock response (expression of heat shock proteins [HSPs]), mitochondrial function (density), and membrane fluidity (lipid composition).

weeks (De Bonville et al., 2025; Brett, 1946; Beitinger and Lutterschmidt, 2011). Although there is evidence for a rapid acclimation of CT_{max} in zebrafish (De Bonville et al., 2025; Morgan et al., 2018), it remains unclear how dependent that response is on the prior baseline acclimation temperature. We also collected tissues from an additional group of 78 fish (acclimated to the same three temperatures and with half additionally receiving a 3-h heat shock) to study three candidate mechanisms behind thermal acclimation: HSP expression, membrane fluidity, and mitochondrial density as estimated by CS expression. We chose to measure HSP70 because it is one of the most abundant HSPs in fish cells, and has previously been shown to be highly responsive to warm acclimation or heat shock (Liu et al., 2017). There are different isoforms of this HSP which we did not target individually, since we wanted to study the overall heat shock response of HSP70. We predicted that long-term warm acclimation would increase HSP70 expression, decrease mitochondrial density, and induce homeoviscous acclimation so that warm-acclimated fish have a lower membrane fluidity compared to cold-acclimated fish at a common temperature. We also predicted that the HSP70 expression would be sufficiently fast to respond within the 3 h heat shock.

2. Methods

2.1. Thermal acclimation of fish

The temperature exposures were conducted using three commercial zebrafish rack systems that automatically controlled temperature, pH, conductivity, and water replacement (ZebTEC, Tecniplast; Fig. 2). The photoperiod was set on a 14/10-h light/dark cycle, and fish were fed ad libitum with TetraPro energy flakes (Tetra, USA) three times a day. All experimental procedures were approved by the Norwegian Animal Research Authority (Food and Safety permit no. 29878) and were in accordance with the 2010/63/EU directive. We exposed a total of 156 adult zebrafish (weight ~250 mg, standard length ~25 mm, age ~7 months), initially maintained and reared at 28 °C from fertilisation, for two weeks to one of three different temperatures (52 fish per temperature): 20 °C, 28 °C, and 35 °C (also referred to as "slow acclimation" and the "baseline" treatment). Within each temperature group, the 52 fish were divided into four 3.5-L tanks (13 fish per tank) with flow-through, partially-recirculated water (Fig. 2). Prior to transfer into the racks, the fish for the cold and warm acclimation groups experienced an intermediate temperature (23 °C and 32 °C, respectively) for 3 h to reduce acute thermal stress during transfer and ensure survival. The control fish were treated the same way, but the intermediate temperature was equal to the control temperature (28 °C). All fish survived and were used in experiments. The two-week acclimation response ratio (ARR), representing the increase in CT_{max} per degree of acclimation, was determined by dividing the difference in CT_{max} between the acclimation groups by the difference in acclimation temperature:

$$ARR = \frac{\Delta CT_{max}}{\Delta Temperature} \quad \text{(Equation 1)}$$

2.2. Heat shock

After the two-week acclimation period, half of the of fish from each temperature treatment experienced a heat shock for 3 h, before being dissected for molecular analyses or used in CT_{max} experiments (Fig. 2). The heat shock was carried out by ramping up the water temperature of the tank, containing the fish, from the respective acclimation temperature (20 °C, 28 °C or 35 °C) to the corresponding heat shock temperature (31 °C, 35 °C or 39 °C) over a period of 15 min. Then, the temperature was kept constant (±0.2 °C) for 3 h with a digital controller (WH1436 Series, Willhi). The purpose of the ramping was to prevent immediate thermal shock and was done by heating a water bath with two titanium

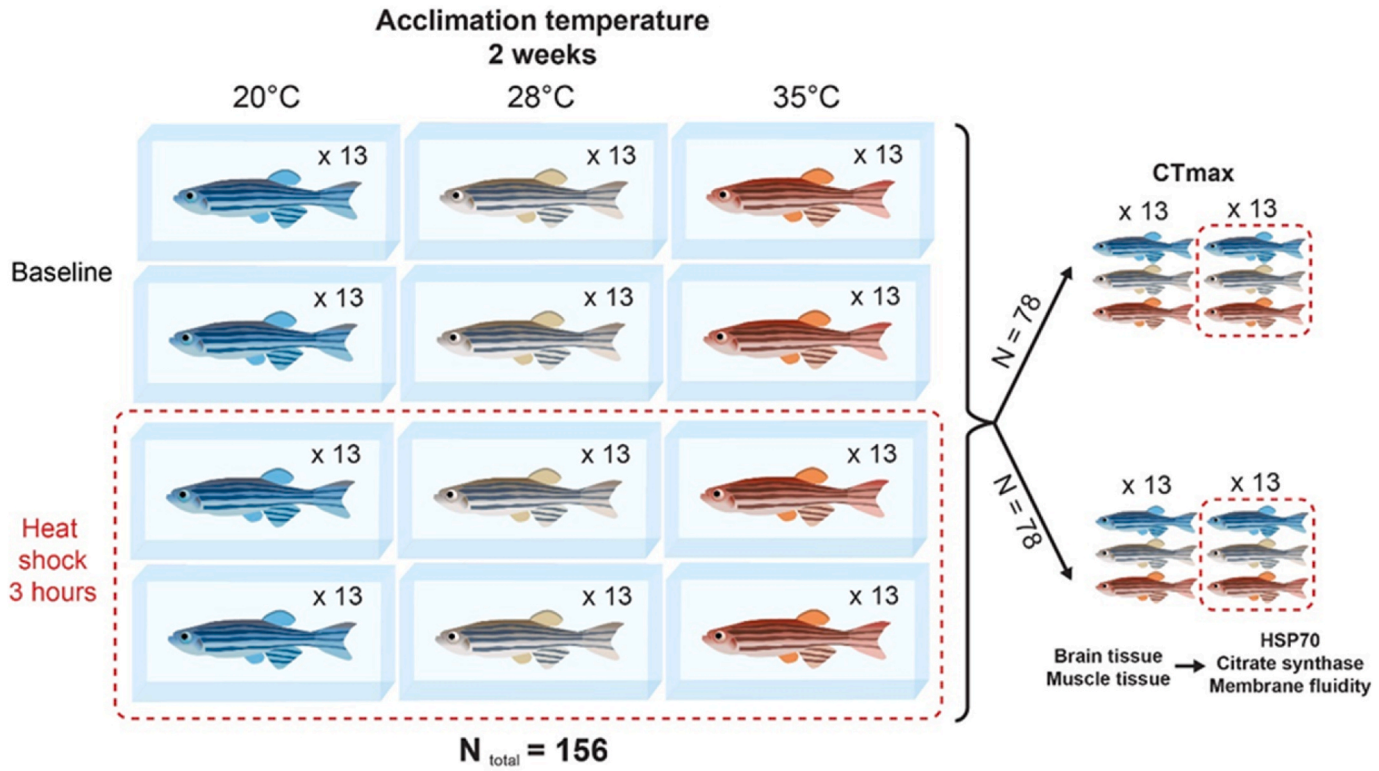

Fig. 2. Overview of the experimental design. In total, 156 fish were divided into three different acclimation groups, which were held for 2 weeks at 20 °C, 28 °C or 35 °C. After this, half of the fish from each acclimation group experienced a 3-h heat shock, while the other half did not experience a heat shock (baseline). The heat shocked and baseline fish were then equally divided into two groups, with one group used for CT_{max} measurements and the other for tissue sampling for HSP70, citrate synthase and membrane fluidity.

heaters (500 W and 100 W, Aqua Medic) and pumping the heated water to the tank through a hose connected to a small pump (170 L/h, Eheim). The heat shock temperatures were chosen so that all fish experienced approximately the same degree of increase in biological rates when warmed from starting (T_1) to end temperature (T_2), using the equation

$$T_2 = \frac{10R_2}{R_1 Q_{10}} + T_1, \quad \text{(Equation 2)}$$

where R is the metabolic rate and Q_{10} is estimated to be 2.5 based on teleost metabolic rate data (Watanabe and Payne, 2023). Although we standardised the duration of the ramping phase across treatments, this resulted in slightly different heating rates (during the 15 min ramp) between groups to reach the heat shock temperature. While this could contribute to minor variation in heat shock perception across groups, we consider this issue of minor relevance since there was zero mortality durng the heat shocks. Air supply via an air stone was used for oxygenation and mixing. Promptly following the heat shock, half of the individuals were transferred to CT_{max} experiments, and the other half were euthanised using buffered MS-222 solution (300 mg/L) and rapidly dissected (within 20 min) for molecular analyses. All fish survived the heat shock.

2.3. Acute thermal tolerance

The individual CT_{max} was measured in groups of 6–7 fish according to the method developed by Morgan et al., 2018, 2020. All 12 trials were carried out within 3 days. The trials were performed in tanks (25 x 22 × 18 cm) filled with 12 L of water, which were split into two compartments by a mesh to separate the fish from the water pump and heating element. The fish were added to the arena at their respective acclimation temperature (20 °C, 28 °C or 35 °C) and habituated to the arena for 15 min before thermal ramping began. During thermal ramping, the water was pumped (5 L/min, Eheim) through a cylindrical steel heating case with a 300 W coil heater, achieving a heating rate of 0.3 °C per min, which was monitored using a high precision digital thermometer (±0.1 °C accuracy; testo-112, Testo). To ensure a homogenous temperature in the entire arena, an additional pump (5 L/min, Eheim) was connected to a tube to pump water from the heating compartment directly into the test arena, bypassing the mesh. Oxygenation was maintained via an air stone. The fish were visually monitored and CT_{max} was determined as the temperature at which an individual lost equilibrium (LOE) for >3 s. All trials were performed by two people, whereby one recorded the temperature, and the other monitored for LOE and was blinded from the temperature during the trial to reduce the potential of any bias. When an individual reached CT_{max}, it was instantly removed from the arena and placed into a recovery tank, where recovery was monitored and recorded after 30 min. Finally, all fish were euthanised using MS-222, and wet weight and standard length were measured.

2.4. Tissue sampling

To study potential mechanisms behind acclimation of thermal tolerance, we dissected brain and white muscle tissue from the remaining half of the fish (Fig. 2). We used brain tissue because it appears to be a heat sensitive organ which might limit thermal tolerance in some fish (Morgan et al., 2019; Andreassen et al., 2022). Muscle tissue was used to get enough mass for isolation of cell membranes. The fish were euthanised using MS-222 followed by measurements of wet weight and standard length. The dissection equipment was cleaned and disinfected with 70 % ethanol between each fish. Tissues were immediately flash frozen in liquid nitrogen and stored at −80 °C until further processing.

2.5. Western blot for HSP70, citrate synthase and β-actin

Protein was isolated by homogenising the tissue samples in RIPA lysis buffer (Thermo Scientific™) using a plastic pestle and centrifuging at 500 RCF for 5 min at 4 °C. The supernatant (lysate) was transferred into a new set of tubes (1.5 mL) and the total protein concentration of each lysate was determined through a Bicinchoninic acid assay (Walker, 1994; Pierce™ BCA Protein Assay Kit, Thermo Scientific™). The volume of lysate was adjusted to achieve a final protein concentration of 1 μg/μL in each sample after mixing with 4x Laemmli buffer (375 mM Tris-HCl, 9 % SDS, 50 % glycerol, 9 % β-mercaptoethanol, 0.03 % bromophenol blue; Thermo Scientific™) and deionised water. Samples were denatured at 100 °C for 5 min, then stored at −80 °C.

We used 10 % precast polyacrylamide gels (Bio-Rad), loaded 20 μg of protein from each sample, and ran samples at 150 V and 200 mA for 55 min in a vertical electrophoresis cell (Bio-Rad, USA) containing running buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS, pH 8.3). The separated protein was transferred to a polyvinylidene fluoride membrane at 25 V, 1.3 A for 7 min (Trans-Blot Turbo Transfer System, Bio-Rad) with a transfer buffer (25 mM Tris, 192 mM glycine, 20 % methanol, pH 8.3). The membranes were stained with Ponceau S (Thermo Scientific™) for 3 min and photographed to visually confirm equal lane loading (Syngene).

Nonspecific site binding of each membrane was blocked in 15 ml TBS-T (Tris buffered saline, 0.1 % Tween) with 5 % non-fat milk powder for 1 h at room temperature. After washing with TBS-T, membranes were incubated for 4 h at 4 °C with primary antibody for HSP70, citrate synthase or β-actin diluted 1:1000 in 15 mL TBS-T with 5 % BSA (Table S6 for antibody information). The membranes were then incubated for 1 h at room temperature with HRP-conjugated secondary antibody diluted 1:2000 in 15 mL TBS-T with 5 % BSA. Each membrane was imaged by adding enhanced chemiluminescence substrate (SuperSignal™ West Pico, Thermo Scientific™) and using an exposure time of 10 s for HSP70 and 1 min for CS and β-actin. The β-actin was intended as a housekeeping reference protein for normalisation and has previously been used successfully in studies with fish including zebrafish (Lewis et al., 2016; McCurley and Callard, 2008). The expression levels of each protein were quantified in ImageJ (version 1.53) by measuring integrated density of the blot bands.

2.6. Membrane fluidity

Membrane fluidity was measured using the spectroscopic fluorescence polarization method (Shinitzky and Barenholz, 1978). The principle of this technique is to stain isolated cell membranes with an elongated fluorescent probe that is oriented along with the membrane spacer groups. By exciting the sample with polarized light at a common temperature (21 °C), and measuring the polarization of the emitted light, the dynamic properties of the membrane can be quantified. Specifically, the anisotropy (r) describes how a property (in this case light polarization) of an object (the probe in the membrane) changes according to its motion. In this case, a high r is indicative of a rigid membrane, whereas a lower value of r indicates a more fluid membrane. As the anisotropy was measured at a common temperature (21 °C), potential membrane remodelling compensations could be detected between groups.

We homogenised dissected white muscle tissue (approximately 40 mg per individual) in 700 μL ice-cold homogenisation buffer (50 mM Tris-HCl, pH 7.4) for 2 min using a tissue lyzer (Qiagen, Germany). The homogenates were centrifuged at 700 RCF at 4 °C for 10 min to remove nuclei debris and the supernatant was collected in a new tube and centrifuged at 10000 RCF for 1 h at 4 °C. The pellet, containing the crude membrane fraction (including plasma membrane, sarcoplasmic reticulum, mitochondria and Golgi), was washed once and resuspended in 300 μL homogenisation buffer. Total protein concentration was determined through a BCA assay and samples with an equal protein

concentration of 100 μg/ml were prepared. Membrane preparations were flash frozen in liquid nitrogen and stored at −80 °C.

For spectroscopic analyses, the membrane preparations were stained with the fluorescent probe 1,6-Diphenyl-1,3,5-hexatriene (DPH). Due to the light sensitivity of DPH, all handling of this chemical was conducted under low light (Plásek and Jarolím, 1987). Each membrane preparation was mixed in a 1:1 ratio with working solution of 4 μM DPH in PBS (phosphate buffered saline), made from a stock solution of 200 μM DPH in acetone. Prior to incubation, the DPH working solution was flushed with nitrogen gas for 10 min to evaporate the remaining acetone that might interfere with the membrane. The final DPH and protein concentrations (as a proxy for membrane concentration) were 50 μg/ml and 2 μM, respectively. The samples were wrapped in aluminium foil and incubated at 37 °C for 1 h to ensure intercalation of DPH.

The fluorescence polarization assay was performed employing a steady-state PTI Quantamaster 8075-22 (Horiba Scientific) equipped with Double Mono 300 spectrometer chambers for both excitation and emission. The OB-75X (75 W Xenon arc lamp) was used as the light source for continuous-wave measurements. Polarizers for UV/Vis (UV Polarizing Film 25–111, Edmund Optics) were mounted on goniometers produced by the local NTNU workshop by 3D printing (Fig. S1). All measurements were conducted at a room temperature of 21 °C. Typically, measurements were carried out by exciting each sample with vertically (V) or horizontally (H) polarized light at 360 nm and then recording the emitted (H) or (V) light spectrum of DPH in the wavelength range 370–670 nm (Fig. S2). Membrane fluidity is represented as the anisotropy of each sample obtained by analysing the emission spectra in Origin v.10.0 and SciDAVis v.2.3.0. The anisotropy was calculated using the formula

$$r = \frac{I_{VV} + GI_{VH}}{I_{VV} + 2GI_{VH}}, \quad \text{(Equation 3)}$$

where I is the integrated intensity of the emission spectra of DPH, where the excitation and emission were both vertically polarized (I_{VV}) or vertically and horizontally polarized, respectively (I_{VH}). Because the polarization sensitivity can differ between spectrometers and settings, the correcting G-factor (G) is incorporated. The G-factor is defined by the equation

$$G = \frac{I_{HV}}{I_{HH}}, \quad \text{(Equation 4)}$$

where I is the integrated intensity of the emission spectra of DPH, where the excitation and emission were both horizontally polarized (I_{HH}) or horizontally and vertically polarized, (I_{HV}), respectively. The G-factor is independent of the sample composition and was calculated as the mean of seven measurement conditions using the same spectroscopic settings. A small slope background signal was removed by fitting a line between the starting point and end point of the emission spectra where the signal was constant and close to zero to avoid systematic errors in the integration of the emission spectra.

2.7. Statistical analyses

All statistical analyses were conducted in R v.4.3.2 (R Core Team, 2023). We used linear mixed effects models (lmer) using the lme4 package (Bates et al., 2015). The mixed effects models tested the effect of the categorical variables acclimation temperature and treatment (heat shock vs. baseline), and their interaction (acclimation temperature x treatment), on each of the continuous response variables CT_{max}, HSP70 expression, CS expression or anisotropy. For the CT_{max} model, trial (two trials per acclimation temperature and treatment) was included as a random factor. For the HSP70 and CS models, gel number was included as a random factor to take gel variation into account. The tank number was initially included as a random factor for all models, but it had no significant effect and was removed from the final model. For each model, the 28 °C acclimation group was set as a reference, but to determine the p-value for specific pairwise comparisons of acclimation and treatment not shown in the original model, the reference temperature was changed to 35 °C or 20 °C. All figures in the results section show the raw data. Statistical outputs for all models are summarised in Tables S1–S5 in the supplementary material.

3. Results

3.1. Acute thermal tolerance

With 28 °C as a control temperature and reference point, the two-week cold (20 °C) and warm (35 °C) acclimations significantly affected CT_{max} in zebrafish ($p < 0.001$ and $p < 0.001$), with CT_{max} observed to increase with acclimation temperature (Fig. 3A). The 20°C-acclimated fish had the lowest mean CT_{max} ± S.E. (37.8 °C ± 0.19) followed by the 28 °C (41.3 °C ± 0.08) and 35°C-acclimated fish (42.1 °C ± 0.16). The ARR (Equation (1)) was 0.438 between 20 °C and 28 °C, and 0.114 between 28 °C and 35 °C. The additional 3-h heat shock further increased CT_{max} in all three acclimation groups. The heat shock had the largest effect in the 20 °C acclimation group, where the mean CT_{max} of fish increased by 0.95 °C ± 0.16 ($p < 0.001$). For 28 °C and 35°C-acclimated fish, mean CT_{max} increased by 0.42 °C ± 0.07 and 0.75 °C ± 0.08, respectively ($p = 0.029$ and $p < 0.001$) (Fig. 3B).

3.2. HSP70 expression

Two weeks of warm acclimation increased baseline protein expression of HSP70 in the brain threefold (2.87 ± 0.33, $p < 0.001$), whereas cold acclimation did not affect the baseline HSP70 expression ($p = 0.62$) relative to the control (Fig. 4). Heat shock had a significant effect on HSP70 expression within all acclimation groups (20 °C: $p = 0.001$, 28 °C and 35 °C: $p < 0.001$) (Fig. 4). There was also a significant interaction where the heat shock had a smaller effect on the absolute increase of HSP70 for the 20°C-acclimated fish ($p < 0.001$). The 3-h heat shock increased HSP70 expression in the 20°C-acclimated fish by a factor of 2.3 ($p < 0.001$), a factor of 3.7 in the 28°C-acclimated fish ($p < 0.001$), and a factor of 2.2 in the 35°C-acclimated fish ($p < 0.001$).

3.3. Citrate synthase expression

Neither a two-week warm nor cold acclimation significantly changed the baseline CS expression in the brain relative to the control (20 °C: $p = 0.45$, 35 °C: $p = 0.82$). However, CS expression increased by a factor of 1.6 after the 3-h heat shock for the 20°C-acclimated fish ($p = 0.02$) (Fig. 5A). There was no significant difference in CS expression between the 28 °C and 35°C-acclimated fish and those that additionally experienced a heat shock (28 °C: $p = 0.54$, 35 °C: $p = 0.52$) (Fig. 5A).

3.4. β-Actin expression

When quantifying HSP70 and CS expression through western blot, β-actin was initially intended as a housekeeping protein for loading control and taking gel variation into account. Unexpectedly, however, β-actin expression was strongly affected by the heat shock treatment and the expression increased in all acclimation groups (20 °C: $p = 0.004$, 28 °C: $p = 0.003$, 35 °C: $p < 0.001$) (Fig. 5B). There was also a significant interaction where the heat shock had a larger effect on β-actin expression in the 35°C-acclimated fish ($p < 0.001$). This made us unable to use β-actin to account for any potential within-gel loading variation, although the exact same protein amount (20 μg) was loaded on each gel. As samples from all acclimation groups and treatments were spread out on all gels, we could instead account for the between gel variation by incorporating the variation in the statistical model as a random effect, as described in the methods.

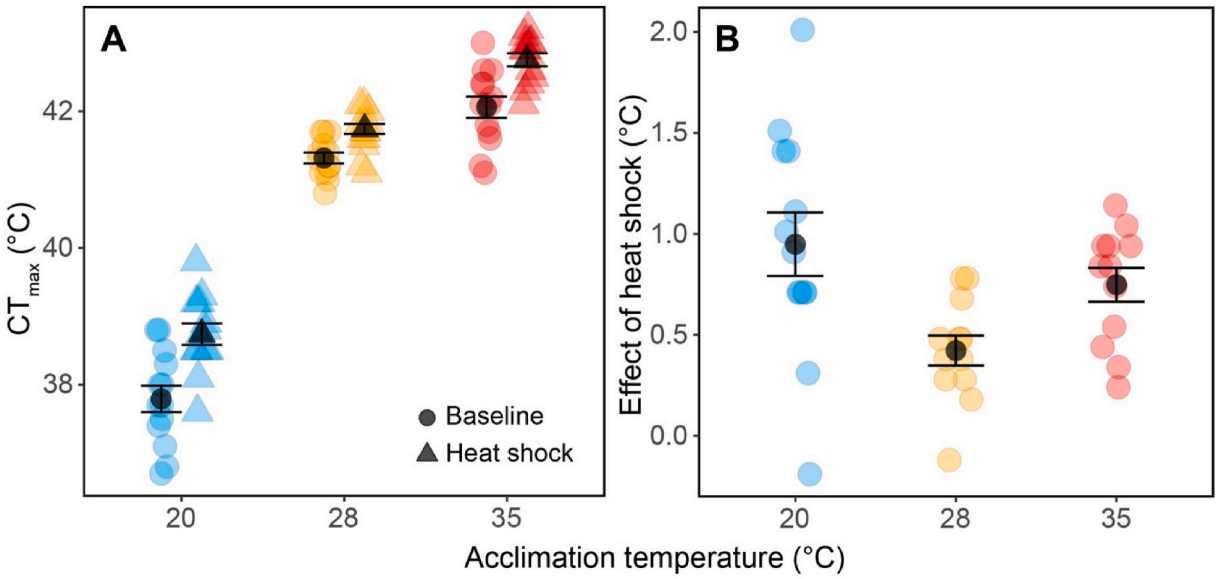

Fig. 3. A) Recorded individual CT_{max} for each acclimation group (blue = 20 °C, yellow = 28 °C, red = 35 °C). Acclimation temperature refers to the temperature at which fish were acclimating for two weeks, prior to the heat shock treatment. The treatment within the groups is denoted as circle = baseline and triangle = 3-h heat shock. B) The effect of heat shock shown as the difference in temperature between heat shocked fish and the mean for the baseline thermal tolerance. Black symbols and error bars represent the mean value and the standard error, respectively.

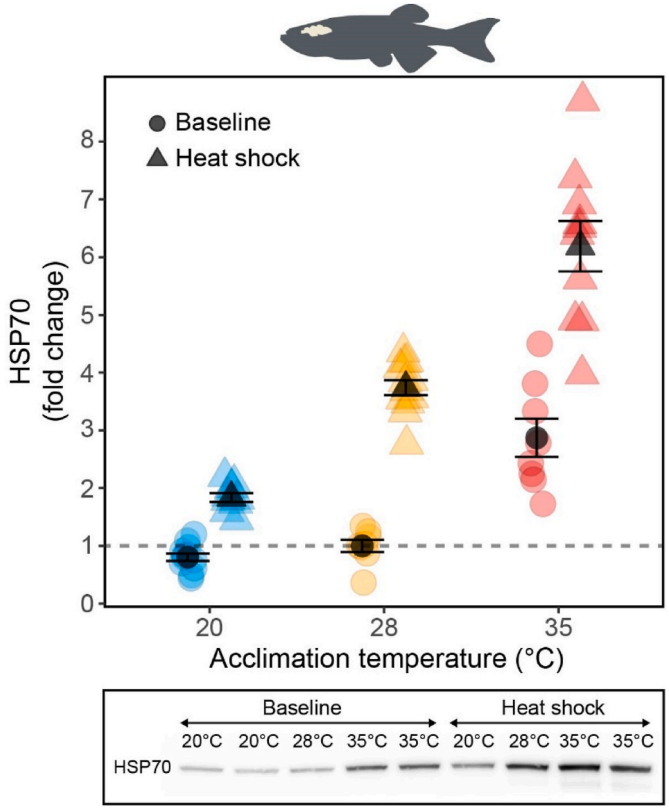

Fig. 4. Fold change in HSP70 expression in zebrafish brain between individuals acclimated to three different temperatures (blue = 20 °C, yellow = 28 °C, red = 35 °C) and exposed to two different treatments (circle = baseline, triangle = 3-h heat shock). Black points and error bars represent the mean and the standard error. The fold change in protein expression is relative to the baseline of the 28°C-acclimated fish (dashed line). Below the plot is a representative western blot. All blots differed in the number of samples from each treatment.

3.5. Membrane fluidity

Membrane fluidity was measured as the anisotropy of the fluorescent probe DPH when intercalated into isolated membrane from white muscle tissue (Fig. 6A). The anisotropy was significantly affected by acclimation temperature, but only when the fish were warm-acclimated (35 °C) ($p = 0.02$) (Fig. 6B). The heat shock treatment had no significant effect on the anisotropy in any acclimation group (20 °C: $p = 0.32$, 28 °C: $p = 0.73$, 35 °C: $p = 0.35$) (Fig. 6B).

4. Discussion

This experiment demonstrates that the acute upper thermal limits can be increased following a 3-h heat shock, indicating a capacity for a rapid acclimation response (heat hardening) that has rarely been studied in fish. For ectotherms, this capacity for rapid thermal acclimation over timescales of hours might be a particularly important trait in highly variable environments and during heatwaves. Rapid acclimation may also be used during daily cycles, such as those reported in intertidal arrow gobies (*Clevelandia ios*) (Kraskura et al., 2024). In the present study, the effect of the heat shock on CT_{max} was present in all three long-term acclimation groups. This suggests that even at high acclimation temperatures (Morgan et al., 2022), zebrafish are not maximally warm acclimated and they maintain some additional capacity for increasing CT_{max}. This maintained capacity for rapid acclimation might therefore represent a crucial trait for fish facing acute warming.

While CT_{max} clearly differed across acclimation treatments, the values observed in the cold-acclimated group may not reflect a fully stabilised acclimation state due to slower physiological rates. Nonetheless, our CT_{max} estimates align with those reported for zebrafish after longer cold acclimation durations (Morgan et al., 2022; Åsheim et al., 2020), suggesting that these fish were at least close to reaching a steady state of their thermal acclimation.

This study additionally set out to investigate which physiological mechanisms may contribute to the thermal acclimation response. Our results suggest that the heat shock response appears to be involved in both rapid and long-term warm acclimation. HSP70 expression in the brain increased with acclimation temperature (Fig. 4), indicating that HSP70 might play a role in modulating the acute upper thermal

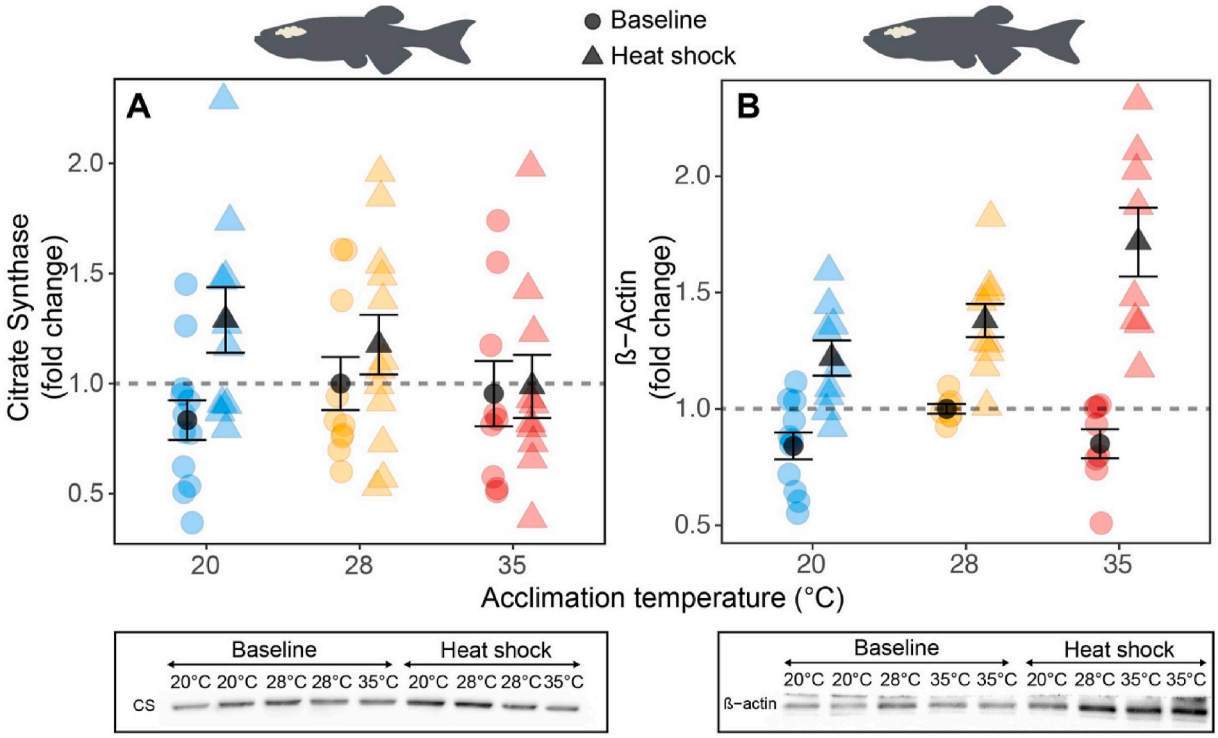

Fig. 5. Fold change in A) CS and B) β-actin expression in zebrafish brain between individuals acclimated to three different temperatures (blue = 20 °C, yellow = 28 °C, red = 35 °C) and assigned to two different treatments (circle = baseline, triangle = 3-h heat shock). Black symbols and error bars represent the mean and the standard error. The fold change in protein expression is relative to the baseline of the 28°C-acclimated fish (dashed line). Below the plots are representative western blots for each protein. All blots differed in the number of samples from each treatment.

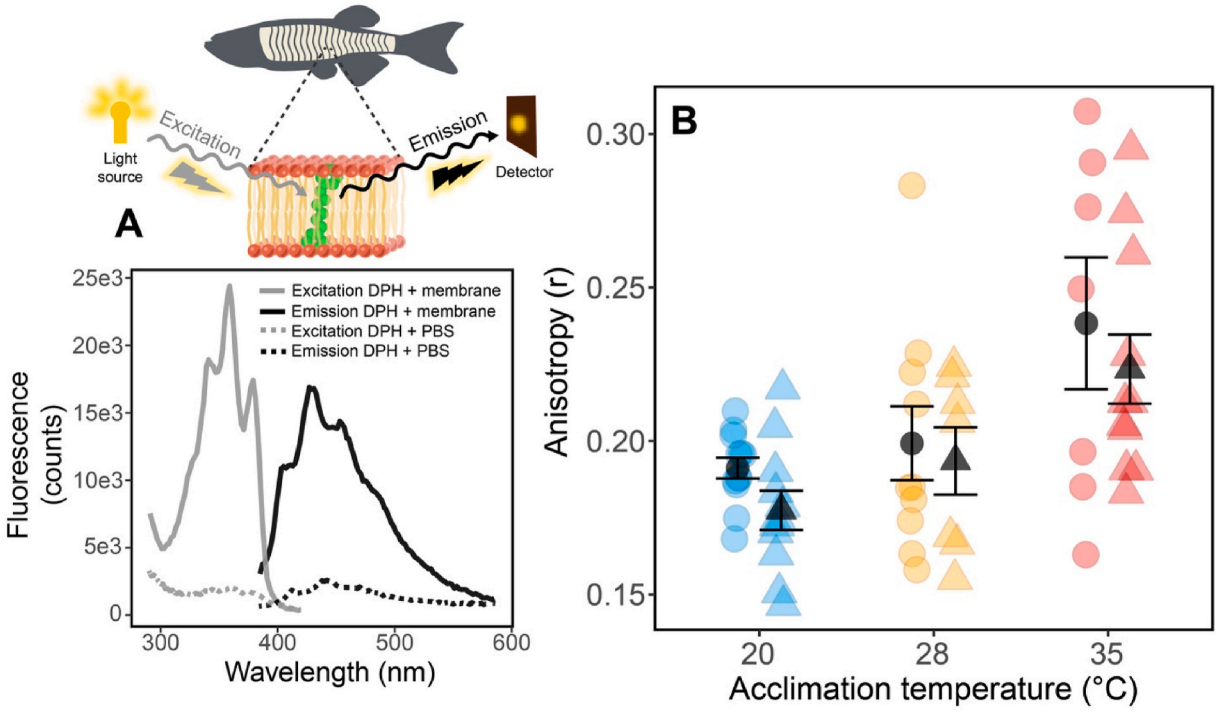

Fig. 6. A) Validation of the method for measuring the anisotropy of membranes from zebrafish white muscle tissue. DPH was excited with light at 290–420 nm (grey lines) and the emitted light was recorded at 385–585 nm (black lines). DPH shows a typical excitation-emission spectra when intercalated into the membrane (solid lines), while free DPH in PBS buffer shows a negligible signal (dashed lines). The peaks of excitation and emission are at 360 nm and 430 nm, respectively. B) The anisotropy (r) of crude membrane fractions in white muscle tissue from fish acclimated to three different temperatures (blue = 20 °C, yellow = 28 °C, red = 35 °C) and exposed to two different treatments (circle = baseline, triangle = 3-h heat shock. Black symbols and error bars represent the mean and the standard error. All measurements of the anisotropy were conducted at a common temperature of 21 °C.

tolerance in fish. HSP70 expression also appears to contribute to rapid acclimation, as it was the only measured cellular parameter that responded in the predicted direction to heat shock. Although our HSP70 antibody likely detected both inducible and constitutive isoforms, we consider this acceptable for the purposes of capturing the overall HSP70 response, while acknowledging this as a potential source of variation (or reduced magnitude of thermal response) in expression patterns.

Previous studies have demonstrated that HSP70 mRNA expression can increase as quickly as after 30 min of heat shock in arctic charr (*Salvelinus alpinus*) (Lewis et al., 2016) and in killifish (*Fundulus heteroclitus*) (Healy et al., 2010). It is worth mentioning that mRNA expression does not always correlate with protein expression (Liu et al., 2016). However, cold and warm-acclimated lake whitefish (*Coregonus clupeaformis*) exhibited a pattern of HSP70 mRNA expression at baseline and after a 1-h heat shock that resembled, albeit less strongly, the changes in protein expression observed in our study (Manzon et al., 2022). The arctic charr showed a time lag of 2–4 h between onset of increases in HSP70 mRNA and tissue HSP70 protein (Lewis et al., 2016). These studies used gill, liver and muscle tissue while fewer studies focus on the brain. Since the brain appears to be a heat sensitive organ and might limit thermal tolerance in some fish (Morgan et al., 2019; Andreassen et al., 2022), it is important to understand the role of HSPs in the brain. We show that zebrafish are capable of a three-fold increase in HSP70 expression following a 3-h heat shock, which could be fast enough to help this species cope with warming caused by a heatwave or daily temperature cycles in nature.

When fish experience changes in water temperature, it is important for the function of many cellular processes to maintain a specific fluidity of plasma and organelle membranes (Ern et al., 2023; Hochachka and Somero, 2002). By measuring fluidity at a common temperature following acclimation, we could observe if thermal compensation by lipid remodelling had occurred. Two weeks of warm acclimation affected the membrane fluidity, whereby the fluidity decreased (as shown by the increased anisotropy), which is the anticipated response (Fig. 6). However, there was no corresponding increase in membrane fluidity in the cold-acclimated fish, which was unexpected. A potential explanation is that membrane remodelling is more important for warm acclimation in zebrafish. Alternatively, the membrane remodelling process, involving changes in lipid biosynthesis, may require more time at cold temperatures, with two weeks of cold acclimation being insufficient to detect a change in anisotropy. The literature on membrane fluidity and acclimation is sometimes conflicting and does not show one uniform pattern. For example, there was a lag of 20 days before fluidity of goldfish (*Carassius auratus*) synaptical membranes increased during cold acclimation (Cossins et al., 1977), while carp (*Cyprinus carpio)* liver membranes rapidly increased fluidity with no further changes after four days (Wodtke and Cossins, 1991). While the study in goldfish also showed a rapid decrease in fluidity after only a couple of days of warm acclimation, no membrane lipid remodelling was observed in several antarctic nothothenioids in liver and muscle membranes after one month of warm acclimation (Malekar et al., 2018; Gonzalez-Cabrera et al., 1995). Taken together, the capacity for membrane remodelling seems to vary profoundly between species. It has been suggested that the membrane cholesterol response might be faster than membrane fatty acid composition changes and may thus provide a rapid short-term solution for adjustments of membrane fluidity (Podrabsky and Somero, 2004). Nevertheless, we did not detect rapid changes after the 3-h heat-shock, which may imply that change in membrane fluidity is a slow response in zebrafish compared to the rapid expression of heat shock proteins.

In contrast to our prediction that CS protein expression (a proxy for mitochondrial density) would decrease with acclimation to warmer temperatures as a compensation mechanism for increasing biological rates, we did not observe an effect of the long-term thermal acclimation on CS expression. This is surprising, because this form of thermal compensation has been found in various fish species such as killifish (Dhillon and Schulte, 2011), perch (*Perca fluviatilis*) (Pichaud et al., 2019), threespine stickleback (*Gasterosteus aculeatus*) (Orczewska et al., 2010), goldfish (*Carassius auratus*) (Dos Santos et al., 2013) and eelpout (*Zoarces viviparus*) (Lucassen et al., 2003). The acclimation time for the fish in these studies ranged from three weeks to eight months, but for the eelpout, two-fold increases in CS mRNA and enzyme activity were observed after just four and seven days, respectively. This indicates that changes in mitochondrial volume has the potential to happen quickly. On the contrary, a lack of thermal acclimation of CS activity has been reported several times, for instance in Atlantic salmon (*Salmo salar*) (Gerber et al., 2020) and in certain populations of killifish (Dhillon and Schulte, 2011). We found that CS expression increased in the cold-acclimated zebrafish after a heat shock, which is in the opposite direction of a predicted thermal compensation effect (Jutfelt et al., 2024). Taken together, there appears not to be a simple and consistent response in CS expression and enzyme activity to thermal acclimation. As CS expression was not consistently affected following long-term acclimation or heat shock, mitochondrial density may not be a major contributor to thermal acclimation of the zebrafish brain. Instead, there might be other aspects of mitochondrial function, such as morphology, that are more involved in thermal acclimation.

During the western blot measurements to quantify HSP70 and CS expression, we discovered that our intended housekeeping protein, β-actin, was strongly affected by the heat shock within all acclimation temperatures. This observation was unexpected, although changes in actin following heat stress have previously been reported from *in vitro* studies (Welch and Suhan, 1985; Glass et al., 1985). The increase in β-actin in the zebrafish brain during heat shock might be indicative of impacts on cell integrity and motility during acute heating, due to increased cellular shear stress or higher motor function. An implication, however, is that β-actin might not always be a suitable housekeeping protein for temperature physiology studies in zebrafish and should be validated before use.

5. Conclusions

The aim of this study was to better understand the physiological mechanisms underlying rapid and long-term thermal acclimation, and their role in determining acute upper thermal tolerance. We found that zebrafish acclimated to three different temperatures can rapidly increase their thermal tolerance after an acute heating event, which may become an increasingly important trait as climate change increases the frequency and intensity of heat waves. Moreover, heat shock proteins appear to be involved in the rapid acclimation response to acute heating, as well as in long-term acclimation during chronic heating. Mitochondrial density, measured as CS expression, did not appear to be a mechanism of thermal acclimation in the zebrafish brain, while remodelling of biological membranes is likely a slow, long-term acclimation mechanism. The large increase in HSP70 expression after only 3 h of acute heat exposure indicates that the heat shock response may be a sufficiently fast mechanism for many species to better cope with fluctuating temperatures encountered in the wild. Specifically, the additional rapid heat in already warm-acclimated fish may help wild populations survive the hottest hours of the day during severe heat waves. This rapid acclimation response is likely especially important for species living close to their thermal maximum, such as tropical shallow water species like zebrafish.

CRediT authorship contribution statement

Moa Metz: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Zara-Louise Cowan:** Writing – review & editing, Methodology, Investigation. **Robine H.J. Leeuwis:** Writing – review & editing, Methodology, Investigation. **Kang Nian Yap:** Writing – review & editing, Methodology. **Mikael Lindgren:**

Writing – review & editing, Methodology. **Fredrik Jutfelt:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Data accessibility statement

The data that support the findings of this study are openly available in Dryad at http://datadryad.org/stash/share/y4qAa2QlH1OHpsoN7uRZ_s368wArNULSl7GfvnGdLKo.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2025.104171.

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