RESEARCH ARTICLE

Rapid-warming tolerance correlates with tolerance to slow warming but not growth at non-optimal temperatures in zebrafish

Eirik R. Åsheim[1,2,*], Anna H. Andreassen[1], Rachael Morgan[1] and Fredrik Jutfelt[1]

ABSTRACT

Global warming is predicted to increase both acute and prolonged thermal challenges for aquatic ectotherms. Severe short- and medium-term thermal stress over hours to days may cause mortality, while longer sub-lethal thermal challenges may cause performance declines. The inter-relationship between the responses to short, medium and longer thermal challenges is unresolved. We asked if the same individuals are tolerant to both rapid and slow warming challenges, a question that has so far received little attention. Additionally, we investigated the possibility of a thermal syndrome where individuals in a population are distributed along a warm-type to cold-type axis. We tested whether different thermal traits correlate across individuals by acclimating 200 juvenile zebrafish (*Danio rerio*) to sub- or supra-optimal temperatures for growth (22 and 34°C) for 40 days and measuring growth and thermal tolerance at two different warming rates. We found that tolerance to rapid warming correlated with tolerance to slow warming in the 22°C treatment. However, individual tolerance to neither rapid nor slow warming correlated with growth at the supra-optimal temperature. We thus find some support for a syndrome-like organisation of thermal traits, but the lack of connection between tolerance and growth performance indicates a restricted generality of a thermal syndrome. The results suggest that tolerance to rapid warming may share underlying physiological mechanisms with tolerance to slower heating, and indicate that the relevance of acute critical thermal tolerance extends beyond the rapid ramping rates used to measure them.

INTRODUCTION

Climate change is imposing a range of different thermal challenges on organisms. At the end of the century, the mean global temperature is projected to increase by 1.5–5°C compared with pre-industrial time (Pachauri et al., 2014). Additionally, weather is becoming more extreme and variable, with heatwaves predicted to increase in both frequency and severity (Perkins et al., 2012). In this context, ectothermic animals may be especially vulnerable as their body temperature often directly follows that of their environment (Angilletta and Angilletta, 2009).

There are two main views on how aquatic ectotherms may be directly affected by a warming climate. The acute upper thermal tolerance view proposes that survival during short-duration transient heatwaves (hours to days) is the dominant determinant of fitness during warming. This view has support from observations that global distribution patterns of species match acute upper thermal tolerance measurements (Sunday et al., 2012), from mass mortality in nature during warming (Wegner et al., 2008) and from findings that populations can function and perform well up to very close to their lethal temperature (Morgan et al., 2019; Sandblom et al., 2016). The upper thermal performance view focuses on the level of functioning of important traits such as growth, fecundity and locomotion over longer time scales at temperatures above optimal but below lethal. The support for this view comes from medium- and long-term laboratory experiments, as well as field observations (Pörtner and Knust, 2007), where sub-lethal fitness effects such as reduced growth and fecundity occur at supra-optimal temperatures (Gräns et al., 2014; Morgan, 2020; Pörtner et al., 2001; Rogers et al., 2011). It is unknown if these two views of thermal effects can be united by any common principles of thermal physiology. That is, do traits for survival during acute warming correspond to traits for higher thermal performance? Currently, knowledge is lacking on both the causes of variation in thermal traits (Schulte, 2015; Somero, 2010) and whether different thermal traits are independent, or linked by shared underlying physiological mechanisms (Jutfelt et al., 2018; Clark et al., 2013). A potential linkage between different thermal traits would not only give clues to the underlying physiological mechanisms, but would also have major implications for how selection on these traits occurs under climate change.

The critical thermal maximum (CT_{max}), a form of acute upper thermal tolerance, is one of the traits most commonly used to test the thermal biology of a species. CT_{max} is the temperature at which some specified endpoint occurs as the organism's body temperature is steadily ramped upwards from its acclimation temperature (Becker and Genoway, 1979; Lutterschmidt and Hutchison, 1997; Morgan et al., 2018, 2019). In fishes, two commonly used endpoints are the onset of muscle spasms and the loss of equilibrium (LOE), the latter being a state where the fish loses the ability to maintain an upright swimming position. The endpoint represents a state where the animal, while still alive, could be considered ecologically dead as in nature it would be unable to escape its condition. Measurement of CT_{max} has become a common measure of thermal tolerance, and it is increasingly being used to connect thermal physiology to the consequences of climate change (Comte and Olden, 2017; Deutsch et al., 2008; Sandblom et al., 2016; Sunday et al., 2012).

Despite its frequent use, knowledge is lacking on what the CT_{max} tells us about the overall thermal physiology of an ectotherm (Kovacevic et al., 2019), and whether or not it can predict warming tolerance in nature. While having been linked with geographical distributions of species, few attempts have been made to link this trait with other thermal performance traits, like growth, fecundity or locomotion. Additionally, the recommended warming rate for

[1]Department of Biology, Norwegian University of Science and Technology, 7491 Trondheim, Norway. [2]Organismal and Evolutionary Biology Research Programme, Institute of Biotechnology, University of Helsinki, 00014 Helsinki, Finland.

*Author for correspondence (eirik.asheim@helsinki.fi)

E.R.Å., 0000-0002-8171-9732; A.H.A., 0000-0003-0208-1812; R.M., 0000-0001-9589-6388; F.J., 0000-0001-9838-3991

CT_{max} assays of fish is 0.3°C min^{-1} (Becker and Genoway, 1979), but warming events in the wild may occur over longer time frames. To differentiate CT_{max} at 0.3°C min^{-1} from tolerance to other warming rates we use the terms rapid-warming tolerance and slow-warming tolerance. It is a well-established pattern that the rate of warming affects the temperature where LOE occurs (Mora and Maya, 2006) and that this relationship varies between species and taxa (Kovacevic et al., 2019), but it remains unknown if individuals with a high rapid-warming tolerance are also more tolerant to slow warming (Fig. 1A). Generally, thermal tolerance is reduced when the warming rate is slower, and it has been hypothesised that this is because the slower warming rates increasing the time spent at each successive temperature exhaust the animal before a higher critical temperature is reached (Morgan, 2020; Rezende et al., 2014). Another possibility is that different warming rates affect different physiological mechanisms, meaning that slow- and rapid-warming tolerances should be considered disparate traits.

Syndrome theory is a theoretical framework for studying systems of correlated traits (Sih et al., 2004). Syndrome theory has mostly been applied to animal personality research, but based on the tight relationship between temperature, metabolism and behaviour (Biro and Stamps, 2010), Goulet et al. (2017a) suggested that thermal physiology could be included in the pace-of-life syndrome hypothesis (POLS). In this hypothesis, consistent differences in behavioural traits are suggested to co-vary with life-history and physiological traits like growth and metabolism, placing individuals along a fast–slow life-history axis (Réale et al., 2010). Goulet et al. (2017a) proposed that the individual's thermal type would align with their behavioural and life-history types. According to this framework, thermal traits would be configured into a thermal syndrome with individuals distributed along a cold–hot axis, and their position in this continuum corresponding to different thermal types. The inclusion of thermal tolerance in this system has so far not been tested, and expanding this system to include this, cold-type individuals at one end of the axis would have left-shifted thermal performance curves, performing better at lower temperatures and having lower critical thermal limits; the opposite would be the case for hot-type individuals at the other end of the axis (Fig. 1C,D). Recent studies on delicate skinks (*Lampropholis delicata*) have revealed some interesting connections between thermal preference, thermal sprint performance, habitat selection, and traits related to boldness, exploration and social behaviour (Goulet et al., 2017a, 2017b; Michelangeli et al., 2018). These findings support individuals existing on a cold–hot axis with corresponding behavioural traits, but the role of thermal tolerance in such a system is so far unexplored.

This experiment had two aims. Aim 1 was to test the relevance of CT_{max} under rapid warming as a measure for predicting slow-warming tolerance as well as performance under supra-optimal temperatures. We predicted correlations between rapid- and slow-warming tolerance, measured as loss of equilibrium temperature at the warming rates 0.025 and 0.3°C min^{-1} as well as growth performance at a supra-optimal holding temperature (Fig. 1A,B). Aim 2 was to test the hypothesis that thermal traits are linked in such a way that they form a thermal syndrome where individuals are distributed along a cold-type to hot-type continuum. We predicted that thermal traits are correlated so that cold-type individuals have both lower thermal tolerance and a left-shifted thermal performance curve, giving them comparably higher growth at sub-optimal temperatures than hot-type individuals. This would be seen as a correlation between thermal tolerance and growth performance that
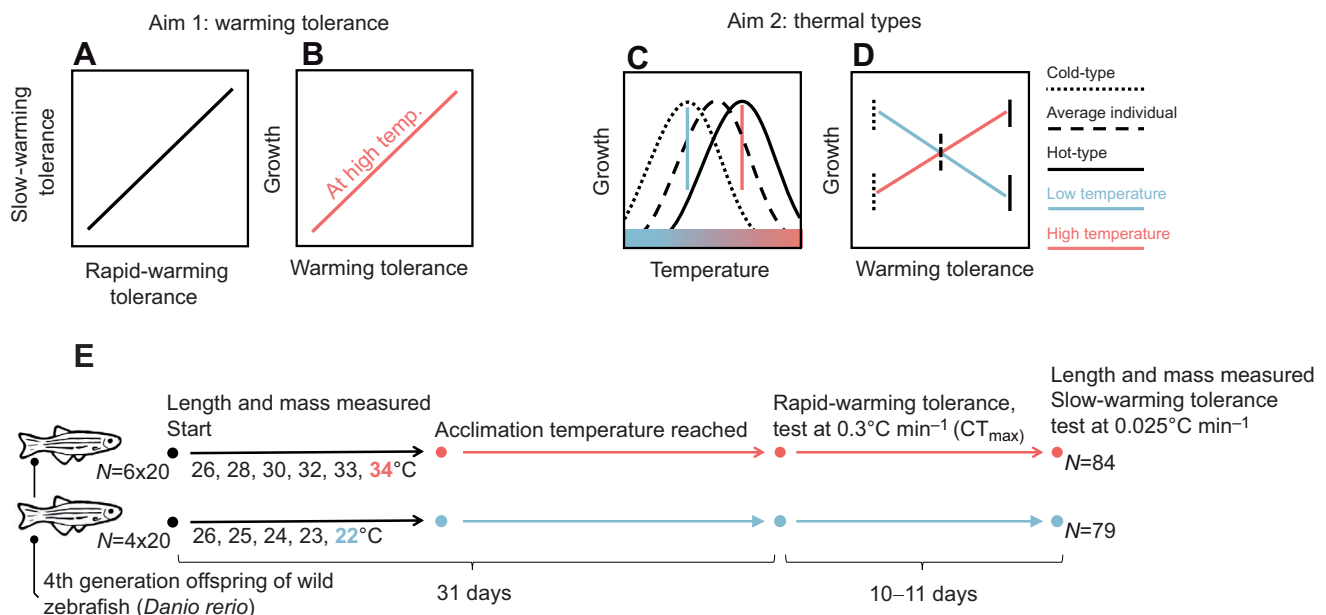
Fig. 1. Experimental aims and design. (A–D) Graphic representation of the predictions from the two experimental aims. Aim 1 was to test the hypothesis that tolerance to rapid warming also confers tolerance to slow warming and increased growth at supra-optimal temperatures. This would lead to (A) a correlation between rapid- and slow-warming tolerance, as well as (B) a correlation between warming tolerance and growth. Aim 2 was to test the hypothesis that thermal traits like thermal tolerance and performance are linked within individuals, placing individuals on a continuum from cold-types to hot-types. Specifically, we hypothesised that thermal traits are correlated so that cold-type individuals have a lower thermal tolerance and a (C) left-shifted thermal performance curve for growth. Given this, we predicted that (D) individuals with low warming tolerance have higher growth at sub-optimal temperatures than individuals with a high thermal tolerance, while the opposite would be true for hot-type individuals. (E) Time line of the experiment. A total of 200 individually tagged zebrafish, raised at 26°C, were divided into two treatments to be exposed to either 22 or 34°C. All fish were tested for thermal tolerance at a warming rate of 0.3°C min^{-1} (rapid-warming tolerance, CT_{max}) after 31 days of thermal exposure, and thermal tolerance at a warming rate of 0.025°C min^{-1} (slow-warming tolerance) 10 or 11 days after that. All individuals were measured for mass and length at the beginning and end of the experiment.

becomes negative when temperature is below optimal, and positive when above optimal (Fig. 1C,D).

MATERIALS AND METHODS
Experimental procedure
The fish used in this experiment were third-generation offspring of wild-caught zebrafish (*Danio rerio* Hamilton 1822) from West Bengal, India, a strain brought into the laboratory in November 2016. We used a total of 200 juvenile zebrafish, about 40 days old, which were randomly divided into two treatment groups to be acclimated at 22±0.2°C (sub-optimal temperature, N=80) and 34±0.2°C (supra-optimal temperature, N=120). We chose temperature treatments at 22 and 34°C based on an earlier unpublished acclimation experiment (Morgan, 2020), where we observed an equally reduced growth at these two temperatures, being about 60% of what was observed at the optimal temperature for growth (28–30°C). A sample size of 80 was chosen based on a power calculation for linear models (although correlation tests were later found to be better suited for these data), given a power of 0.8, significance level of 0.05, and a small effect size f^2 of 0.10. Power calculations were done following Cohen (1988) using the package pwr (https://CRAN.R-project.org/package=pwr) in R. We used a higher sample size in the 34°C treatment to compensate for the possibility of increased mortality due to individuals reaching a higher CT_{max} in this treatment group. Individuals were divided into 10 tanks each containing 20 fish, with six tanks for the 34°C treatment and four tanks for the 22°C treatment. Before being distributed into their tanks, fish were tagged and measured while under anaesthesia [110 mg l^{-1} buffered tricaine methanesulfonate (MS-222)]. Visible implant elastomer tags (Northwest Marine Technologies, Shaw Island, WA, USA) were subcutaneously injected at the left and right side of their dorsal fin using a 0.5 mm syringe in different colour combinations (Hohn and Petrie-Hanson, 2013). During tagging, each individual was held in place in a groove cut into a moistened plastic sponge. Fish were then transferred to a piece of laminated paper for length measurement before being weighed. Mass was measured to the nearest microgram using a digital precision scale. To remove excess adherent water, the fish was lightly turned on both sides against the laminated paper, which removed most of the water. All measurements and tagging were done quickly without pause to limit air exposure, handling time and evaporative water loss. Standard length, defined as the distance from snout to base of tail, was measured to the nearest micrometre using a digital calliper. Initial holding temperature was 26±0.2°C and temperature acclimation started after 2 days of habituation to the holding tanks. The temperature was reduced by 1°C every day in the 22°C treatment until 22°C was reached. In the 34°C treatment, the temperature was increased by 2°C every day until 32°C, and 1°C every day until 34°C. Thus, final acclimation temperatures were reached after 6 days. The fish were tested for rapid-warming tolerance (CT_{max}; 0.3°C min^{-1}) after 22 days at their respective acclimation temperature, and slow-warming tolerance (0.025°C min^{-1}) 10 days after that (Fig. 1E). Each fish was tested in both protocols. The experiment was approved by the Norwegian Animal Research Authority (permit number: 8578). Experimental procedures and care of animals were done following all relevant local guidelines and policies.

Holding conditions
Holding aquaria of 45 litres (50×30×30 cm) were environmentally enriched with a red and green plastic ornamental plant, and had sponge biofilters used for filtration, air bubbling and water circulation (Fig. S1). Temperature was controlled using a thermostat (ITC-310 T, Inkbird, Shenzen, China) and one titanium heater (TH-100, Aqua Medic, Bissendorf, Germany) in each tank. Tanks in the 34°C treatment had an extra titanium heater installed, as well as an air stone for improving water circulation over the heaters. Lighting was set on a 12 h:12 h dark:light cycle. Salinity was kept at 0.3 ppt using natural sea salt. Fish were fed ground-up TetraPro energy flakes *ad libitum* twice a day (Tetra, Blacksburg, VA, USA). Water was replaced after 13 and 15 days for the 22 and 34°C tanks, respectively.

Thermal tolerance measurements
Two separate procedures were used to test thermal tolerance, one with a 0.3°C min^{-1} warming rate and one with a 0.025°C min^{-1} warming rate. In both procedures, LOE was used as the test endpoint (Becker and Genoway, 1979). We defined the LOE as the state where the fish had, for more than 3 s, been unable to right itself and maintain an upright swimming position. Water temperature at LOE was recorded using a high precision digital thermometer with ±0.1°C precision (Testo-112, Testo, Lenzkirch, Germany).

The rapid-warming tolerance test (CT_{max}; 0.3°C min^{-1}) was conducted using a heating tank (25 cm×22 cm×18 cm) filled with 9 litres of water at the acclimation temperature for each treatment. The tank had a heating element and a pump for circulation and a detailed description of this CT_{max} set-up can be found in Morgan et al. (2018). Ten individuals were tested simultaneously in the same heating tank, and tolerance was defined as the temperature where LOE occurred for each individual. Each individual was immediately removed from the heating tank after LOE and put in a small holding tank at its respective acclimation temperature to recover before it was relocated to its holding tank.

The slow-warming tolerance test (0.025°C min^{-1}) was conducted in the holding tanks on the last day of the experiment. The water level was reduced to 10 cm (15 litres), filters and ornamental plants were removed, and the titanium heater was placed horizontally and close to the water surface on each tank's longest wall with the air stone placed underneath to provide circulation over the heater. The thermometer for recording water temperature at LOE was placed on the opposite side of the tank. A thermostat (ITC-310 T) was used to control the titanium heater while gradually heating the water. The thermostat's thermal probe was placed close to the air stone to keep it close to the water flow but underneath the heater. Temperature was recorded as each individual reached LOE, and the individual was immediately euthanized (MS222 overdose), weighed and measured.

Statistical analysis
All analyses were done using the R 3.5.1 software environment (https://www.r-project.org/). Growth was defined as the percentage increase in body mass during the experiment, calculated using the initial and final mass. Growth rates accounting for time in the experiment (assuming an equal growth rate each day) were calculated as percentage growth in mass per day and are thus useful for comparing between experiments:

$$\text{Growth rate} = \left(\left(\frac{\text{Initial mass}}{\text{Final mass}} \right)^{\frac{1}{\text{No. of days}}} - 1 \right) \times 100. \quad (2)$$

Only individuals that survived the entire experiment were included in the analysis. All comparisons on growth and thermal tolerance between the acclimation treatments were tested using two-tailed Welch's *t*-tests (for unequal variance). Any tank effect on

thermal tolerance or growth was tested using an ANOVA on a linear model with holding tank as the independent variable against slow-warming tolerance, rapid-warming tolerance or growth as the dependent variable. Holding tank was found to significantly affect both types of thermal tolerance, but not growth (Table S1). Small variation in tank temperature is a likely reason for causing differences in acclimated temperature. To account for tank effects on the inter-individual differences these data were mean centred, which re-defined each measurement as its difference from its respective tank mean. The mean-centred values for growth and thermal tolerance at both warming rates have the same variance as the raw values and the mean within each tank is centred on 0 (Fig. S2). For transparency, results in Table 1 show correlations using both raw and mean-centred values. We also chose to use mean-centring on the growth measurements for consistency, even though tanks did not affect the growth results. Correlations were tested using Pearson's product moment correlation between all three measurements (rapid-warming tolerance, slow-warming tolerance and growth). Two separate sets of correlations were tested, using either raw uncorrected values or mean-centred values (Table 1). Outliers were defined as values being over five times the interquartile range beyond either the upper or lower quartile. After all data were collected, one individual from the 34°C treatment with a slow-warming tolerance at 38.5°C was removed, being a lower-range outlier and the cause of a likely false correlation between rapid-warming and slow-warming tolerance. Normal distributions were confirmed visually using histograms. After a few borderline distributions were found, the robustness of the correlations was tested using the non-parametric Spearman's rank correlation coefficient, which produced results very similar to Pearson's correlation (Table S2; Table 1). The ANOVA analysis assumptions of normally distributed residuals and equal variance among groups were visually confirmed using R's diagnostic 'Normal Q-Q' and 'Residuals vs Fitted' plots for linear models.

RESULTS
Mass and growth
Mean mass of all individuals was 0.078±0.019 g (mean±s.d.) before acclimation and 0.118±0.024 g at the end of the experiment, equivalent to a 56.85±43.74% increase, or a growth rate of 1.02± 0.05% mass increase per day. There was no significant difference in growth between the acclimation treatments (t=0.80, P=0.42) (Fig. 2B).

Mortality and outliers
In the 22°C treatment, there was a mortality of 1% through the entire experiment, while the 34°C treatment had a mortality of 30% after the rapid-warming test, leaving the final number of individuals tested under both slow and rapid warming at 79 in the 22°C treatment and 84 in the 34°C treatment. One individual from the 34°C treatment was removed as a lower-range outlier having a slow-warming tolerance at 38.5°C, causing a likely false correlation between rapid-warming and slow-warming tolerance (Fig. 2A). Given the high mortality, future experiments should avoid CT_{max} testing on zebrafish acclimated to high temperatures.

Correlations
Rapid-warming tolerance and slow-warming tolerance correlated significantly in the 22°C acclimation group (r=0.390, P=0.0004). In the 34°C group, this correlation was positive, but non-significant (r=0.200, P=0.071) (Fig. 3A). Growth and rapid-warming tolerance (CT_{max}) did not correlate in any of the treatments (Fig. 3B). Growth and slow-warming tolerance only correlated significantly in the 22°C treatment when using uncorrected raw values (r=0.240, P=0.033), but this correlation was non-significant when the relationship was tested using Spearman's rank correlation coefficient (Table S2). Correction using mean centred values adjusting for tank effects resulted in a positive but non-significant correlation (r=0.205, P=0.070) (Table 1, Fig. 3C).

Thermal tolerances
Acclimation temperature significantly affected thermal tolerance at both rapid (t=−55.91, P<0.0001) and slow warming (t=−41.00, P<0.0001) (Fig. 2A). Rapid-warming tolerance was 38.83±0.62 and 42.99±0.23°C in the 22 and 34°C treatments, respectively. In the same order, slow-warming tolerance was 40.36±0.33 and 42.07±0.16°C. In the 22°C treatment, rapid-warming tolerance was significantly lower than slow-warming tolerance (t=−19.21, P<0.0001), while in the 34°C treatment the rapid-warming tolerance was significantly higher than the slow-warming tolerance (t=29.96, P<0.0001) (identity line; Fig. 2A). The non-surviving individuals in the 34°C group were among those reaching the highest temperature during the rapid-warming tolerance test, reaching a mean of 43.17±0.03°C. This suggests temperature-dependent mortality, although that was not directly tested. Average duration of the slow-warming tolerance test was 743 and 322 min in the 22 and 34°C treatments, respectively. Average duration of the rapid-warming tolerance test was 56 and 30 min.

Table 1. Correlations between rapid-warming tolerance, slow-warming tolerance and growth in *Danio rerio*

| Relationship | Acclimated temperature (°C) | Raw values | | Mean centred (m.c.) | |
| | | Correlation I | | Correlation II | |
		r	P	r	P
Growth–slow-warming tolerance	22	0.240	0.033*	0.205	0.070‡
	34	−0.021	0.849	−0.054	0.629
Growth–rapid-warming tolerance	22	0.117	0.306	0.050	0.662
	34	−0.019	0.863	−0.040	0.720
Rapid-warming tolerance–slow-warming tolerance	22	0.416	0.0001*	0.390	0.0004*
	34	0.060	0.588	0.200	0.071‡

The table includes Pearson's correlation coefficient (r) and corresponding P-values for correlations between all combinations of growth, rapid-warming tolerance [measured as the temperature where loss of equilibrium (LOE) occurs at a warming rate of 0.3°C min^{-1} (also known as CT_{max})] and slow-warming tolerance (LOE at a warming rate of 0.025°C min^{-1}) at two acclimation temperatures (22 and 34°C). To correct for tank effects, mean centring was done by redefining each value as its deviance from the tank mean. Correlations were tested using both raw values and mean-centred values. *Significant P-values below 0.05; ‡near-significant P-values below 0.1. An alternative analysis of the same relationships using Spearman's rank correlation coefficient is found in Table S2.
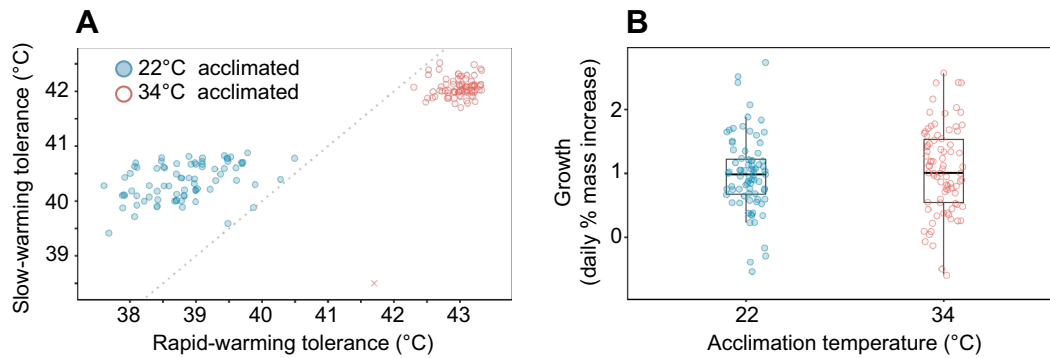
Fig. 2. Temperature, growth and rapid- and slow warming tolerance. Results are for two acclimation treatments of juvenile zebrafish at 22°C (*N*=79) and 34°C (*N*=80). (A) Individuals' rapid-warming tolerance, measured as the temperature where loss of equilibrium (LOE) occurs at a warming rate of 0.3°C min^{-1} (also known as critical thermal maximum, CT$_{max}$) and slow-warming tolerance (LOE at a warming rate of 0.025°C min^{-1}) for both treatments. Fish were tested for rapid-warming tolerance after 22 days of acclimation and slow-warming tolerance after 32 days. The identity line is drawn with grey dots. The orange '×' indicates a removed outlier from the 34°C treatment. (B) Growth (displayed as daily percentage mass increase) for all included individuals in the two treatments. Points are displaced in both A and B to reveal overlapping points, but only horizontally in B, and no more than 0.02°C in A.

DISCUSSION

Upper thermal tolerance under rapid warming correlated significantly with upper thermal tolerance under slow warming, demonstrating that individuals with high tolerance to rapid warming (minutes) are also individuals tolerant to slow warming (hours). This suggests that rapid CT$_{max}$ measurements can be used as a quick and practical proxy for estimating thermal tolerance. The finding thus extends the importance of CT$_{max}$ measurements from a being a laboratory test of acute thermal tolerance to a potentially ecologically relevant metric for estimating tolerance to heatwaves in nature.

At the 34°C acclimation treatment, the correlation between slow- and rapid-warming tolerance was not significant. This is probably due to the reduced variance as the acclimation temperature

approaches upper long-term thermal limits (Morgan et al., 2019; Pintor et al., 2016) and increasing the relative measurement error, making correlations difficult to detect. This could mean that CT$_{max}$ measurements have lower predictive power at higher temperatures. Additionally, the mortality following the first rapid-warming tolerance test selectively removed individuals that reached very high temperatures before losing equilibrium. This may have further increased the uncertainty at the higher end of thermal tolerance (in the 34°C treatment).

Zebrafish is a species where acute upper thermal tolerance could be central to its population survival under climate change. Historically, the peak temperature of heatwaves in the north-east of India (a central part of the zebrafish range) has been in the range
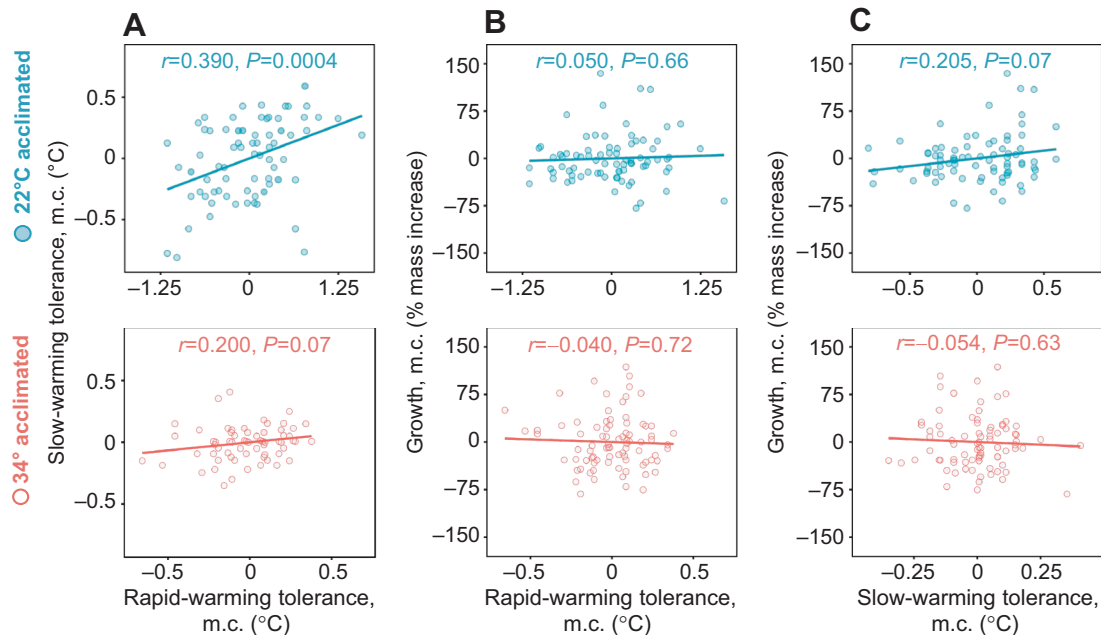
Fig. 3. Correlations between rapid-warming tolerance, slow-warming tolerance and growth. The figure shows correlations including Pearson's correlation coefficient (*r*) and respective *P*-values between all combinations (A,B,C) of rapid-warming tolerance, measured as the temperature where loss of equilibrium (LOE) occurs at a warming rate of 0.3°C min^{-1} (also known as critical thermal maximum, CT$_{max}$); slow-warming tolerance (LOE at a warming rate of 0.025°C min^{-1}) and growth (percentage mass increase over 32 days) for two acclimation treatments of zebrafish at 22°C (*N*=79) and 34°C (*N*=80). Measurements are corrected for tank effects by mean centring (m.c.) all values on their respective tank means. Lines are fitted using least squares regression for each plot's values and are for illustrative purposes only.

of 40–45°C (air temperature), with a duration around 1–4 days and a frequency of one to two occurrences per season (Murari et al., 2015). With some scenarios of carbon emissions (Pachauri et al., 2014), the peak temperature, duration and frequency are likely to increase in this region (Murari et al., 2015). Zebrafish are often found in shallow, low-flow freshwater habitats (Engeszer et al., 2007; Sundin et al., 2019), making them naturally exposed to rapid thermal fluctuations. Survival of zebrafish in these areas is thus depending on the ability to survive higher temperatures and longer heatwaves. An increase in the severity of thermal challenges may thus select for more thermally tolerant individuals. The results of this experiment suggest that slow- and rapid-warming tolerance could be co-selected under these circumstances as closely connected traits.

A potentially important although unquantified observation during our thermal challenges tests was that the nature of the LOE changed between the two warming rates. At the standard, rapid warming rate, zebrafish display a distinct form of disorganised swimming, characterised by fast, erratic swimming coupled with an inability to remain upright. Under slow warming, however, it was more common for the fish to lose equilibrium from what appeared to be exhaustion. That is, instead of swimming without a righting response (as in the rapid-warming test), the fish simply stopped swimming, and thus also lost their ability to remain upright. These different responses leading to LOE suggest different underlying mechanisms ultimately causing the LOE at the two warming rates. The almost instant LOE and disorganised swimming during rapid warming suggest an immediate failure of some vital mechanism, like cardiac (Sidhu et al., 2014) or neurological malfunctioning (Jutfelt et al., 2019; Miller and Stillman, 2012; Robertson, 2004), while the slow exhaustion-like response during the slow warming may suggest a gradual build-up of some malfunction, metabolic waste products, or the exhaustion of some system. Still, the correlation found in this experiment suggests that important links between these two traits exist. One source of this correlation could be a more fundamental mechanism that ultimately governs both long-term and acute thermal tolerance, for example membrane failure (Bowler, 2018), oxygen limitation (Pörtner and Knust, 2007), enzyme denaturation or a shared genetic or developmental component.

In the 34°C acclimation treatment, individuals reached higher temperatures during rapid warming than during slow warming, a pattern similar to that seen in other species (Kovacevic et al., 2019; Mora and Maya, 2006). The current explanation for this difference is that the higher cumulative stress of a prolonged thermal challenge makes the fish lose equilibrium before reaching high temperatures as during a shorter test using a more rapid warming rate (Rezende et al., 2014). Interestingly, this pattern was reversed in the 22°C acclimation treatment, with fish reaching higher temperatures during slow warming. One potential explanation for this could be that the total time of the slow-warming tolerance test in the 22°C acclimation treatment was over twice as long as in the 34°C treatment (742 versus 322 min), giving individuals in the 22°C treatment group more time to rapidly acclimate during the trial. It is, however, unclear which physiological or biochemical mechanisms would be amenable for adjustment over such short time scales. One possibility could be production of heat shock proteins. Alternatively, the exposure to a high temperature during the rapid-warming tolerance test, done 10 days before the slow-warming test, might also have caused a slight upwards temperature acclimation (heat hardening) in the individuals of the 22°C treatment (Morgan et al., 2018), whereas in the 34°C treatment, the individuals were already acclimated closer to their upper limit.

The growth rates observed in this experiment were close to that observed previously at 22 and 34°C, and about half of the growth rate at optimal temperature (Morgan, 2020), showing that these temperatures had a strong negative effect on growth rates. We predicted a relationship between thermal tolerance and ability to grow in non-optimal temperatures. However, only a near-significant positive correlation was found between these traits, and only in the 22°C treatment under slow warming. The lack of clear correlations between thermal growth performance and rapid-warming thermal tolerance suggests that acute thermal tolerance has little mechanistic connection with the ability to maintain growth performance outside optimal temperatures. Whichever mechanism allows some individuals to have a higher CT_{max} does not give them a considerable advantage or disadvantage in growth when acclimated to temperatures outside their optimum. If, for example, oxygen limitation is reducing growth at high temperatures (Pörtner and Knust, 2007), it probably does not play a significant role during acute temperature increases such as during a CT_{max} trial. The results suggest that variation in thermal tolerance and growth performance (under supra-optimal temperatures) are governed by disparate mechanisms.

In terms of a thermal syndrome, the results suggest that the scope of such a syndrome may be more limited than we predicted. Although a correlation was found between tolerance to slow and rapid warming, no significant correlation was found between warming tolerance and growth. The results show, contrary to our predictions, that the ability to grow at non-optimal temperatures is not connected to the ability to survive acute thermal challenges. However, other thermal traits not tested may yet be organised in some form of thermal syndrome, similar to what has been found in reptiles (Goulet et al., 2017a, 2017b; Michelangeli et al., 2018).

Conclusions

Tolerance to rapid warming correlates with tolerance to slow warming across individuals. This means that the measure of rapid-warming tolerance also predicts tolerance to slower warming challenges at the scale of what can be experienced during daytime under a heatwave. This suggests that CT_{max} tests may be useful for predicting impacts of climate change in a broader context than what is given by the rapid warming rate usually used to measure it.

We did not find support for a thermal syndrome that links growth performance at non-optimal temperature and thermal tolerance measures, suggesting that these traits may be selected for independently in thermally stressed populations.

Author contributions
Conceptualization: E.R.Å., A.H.A., R.M., F.J.; Methodology: E.R.Å., A.H.A., R.M., F.J.; Formal analysis: E.R.Å.; Investigation: E.R.Å., A.H.A., R.M.; Resources: F.J.; Data curation: E.R.Å.; Writing - original draft: E.R.Å.; Writing - review & editing: E.R.Å., A.H.A., R.M., F.J.; Visualization: E.R.Å.; Supervision: R.M., F.J.; Project administration: E.R.Å., R.M., F.J.; Funding acquisition: F.J.

Data availability
The complete dataset and the R-script used for analysing the data is publicly available from figshare: https://doi.org/10.6084/m9.figshare.12311102.v2

Supplementary information
Supplementary information available online at
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References
Angilletta, M. J., Jr and Angilletta, M. J. (2009). *Thermal adaptation: a theoretical and empirical synthesis*. Oxford University Press.
Becker, C. D. Genoway, R. G. (1979). Evaluation of the critical thermal maximum for determining thermal tolerance of freshwater fish. *Environ. Biol. Fishes* **4**, 245. doi:10.1007/BF00005481
Biro, P. A. and Stamps, J. A. (2010). Do consistent individual differences in metabolic rate promote consistent individual differences in behavior? *Trends Ecol. Evol.* **25**, 653-659. doi:10.1016/j.tree.2010.08.003
Bowler, K. (2018). Heat death in poikilotherms: is there a common cause? *J. Therm. Biol.* **76**, 77-79. doi:10.1016/j.jtherbio.2018.06.007
Clark, T. D., Sandblom, E. and Jutfelt, F. (2013). Aerobic scope measurements of fishes in an era of climate change: respirometry, relevance and recommendations. *J. Exp. Biol.* **216**, 2771-2782. doi:10.1242/jeb.084251
Cohen, J. (1988). *Statistical power analysis for the behavioral sciences*, 2nd edn. Hillsdale, NJ: L. Erlbaum Associates.
Comte, L. and Olden, J. D. (2017). Climatic vulnerability of the world's freshwater and marine fishes. *Nat. Clim. Change* **7**, 718-722. doi:10.1038/nclimate3382
Deutsch, C. A., Tewksbury, J. J., Huey, R. B., Sheldon, K. S., Ghalambor, C. K., Haak, D. C. and Martin, P. R. (2008). Impacts of climate warming on terrestrial ectotherms across latitude. *Proc. Natl. Acad. Sci.* **105**, 6668-6672. doi:10.1073/pnas.0709472105
Engeszer, R. E., Patterson, L. B., Rao, A. A. and Parichy, D. M. (2007). Zebrafish in the wild: a review of natural history and new notes from the field. *Zebrafish* **4**, 21-40. doi:10.1089/zeb.2006.9997
Goulet, C. T., Thompson, M. B. and Chapple, D. G. (2017b). Repeatability and correlation of physiological traits: do ectotherms have a 'thermal type'? *Ecol. Evol.* **7**, 710-719. doi:10.1002/ece3.2632
Goulet, C. T., Thompson, M. B., Michelangeli, M., Wong, B. B. and Chapple, D. G. (2017a). Thermal physiology: a new dimension of the pace-of-life syndrome. *J. Anim. Ecol.* **86**, 1269-1280. doi:10.1111/1365-2656.12718
Gräns, A., Jutfelt, F., Sandblom, E., Jönsson, E., Wiklander, K., Seth, H., Olsson, C., Dupont, S., Ortega-Martinez, O. and Einarsdottir, I. (2014). Aerobic scope fails to explain the detrimental effects on growth resulting from warming and elevated CO_2 in Atlantic halibut. *J. Exp. Biol.* **217**, 711-717. doi:10.1242/jeb.096743
Hohn, C. and Petrie-Hanson, L. (2013). Evaluation of visible implant elastomer tags in zebrafish (*Danio rerio*). *Biol. Open* **2**, 1397-1401. doi:10.1242/bio.20136460
Jutfelt, F., Norin, T., Ern, R., Overgaard, J., Wang, T., McKenzie, D. J., Lefevre, S., Nilsson, G. E., Metcalfe, N. B., Hickey, A. J. R., et al. (2018). Oxygen- and capacity-limited thermal tolerance: blurring ecology and physiology. *J. Exp. Biol.* **221**, jeb169615. doi:10.1242/jeb.169615
Jutfelt, F., Roche, D. G., Clark, T. D., Norin, T., Binning, S. A., Speers-Roesch, B., Amcoff, M., Morgan, R., Andreassen, A. H. and Sundin, J. (2019). Brain cooling marginally increases acute upper thermal tolerance in Atlantic cod. *J. Exp. Biol.* **222**, jeb208249. doi: 10.1242/jeb.208249
Kovacevic, A., Latombe, G. and Chown, S. L. (2019). Rate dynamics of ectotherm responses to thermal stress. *Proc. R. Soc. B* **286**, 20190174. doi:10.1098/rspb.2019.0174
Lutterschmidt, W. I. and Hutchison, V. H. (1997). The critical thermal maximum: history and critique. *Can. J. Zool.* **75**, 1561-1574. doi:10.1139/z97-783
Michelangeli, M., Goulet, C. T., Kang, H. S., Wong, B. B. and Chapple, D. G. (2018). Integrating thermal physiology within a syndrome: locomotion, personality and habitat selection in an ectotherm. *Funct. Ecol.* **32**, 970-981. doi:10.1111/1365-2435.13034
Miller, N. A. and Stillman, J. H. (2012). Neural thermal performance in porcelain crabs, genus Petrolisthes. *Physiol. Biochem. Zool.* **85**, 29-39. doi:10.1086/663633
Mora, C. and Maya, M. F. (2006). Effect of the rate of temperature increase of the dynamic method on the heat tolerance of fishes. *J. Therm. Biol.* **31**, 337-341. doi:10.1016/j.jtherbio.2006.01.005

Morgan, R. (2020). Physiological plasticity and evolution of thermal performance in zebrafish. PhD thesis, NTNU.
Morgan, R., Finnøen, M. H. and Jutfelt, F. (2018). CT_{max} is repeatable and doesn't reduce growth in zebrafish. *Sci. Rep.* **8**, 7099. doi:10.1038/s41598-018-25593-4
Morgan, R., Sundin, J., Finnøen, M. H., Dresler, G., Vendrell, M. M., Dey, A., Sarkar, K. and Jutfelt, F. (2019). Are model organisms representative for climate change research? Testing thermal tolerance in wild and laboratory zebrafish populations. *Conserv. Physiol.* **7**. doi:10.1093/conphys/coz036
Murari, K. K., Ghosh, S., Patwardhan, A., Daly, E. and Salvi, K. (2015). Intensification of future severe heat waves in India and their effect on heat stress and mortality. *Reg. Environ. Change* **15**, 569-579. doi:10.1007/s10113-014-0660-6
Pachauri, R. K., Allen, M. R., Barros, V. R., Broome, J., Cramer, W., Christ, R., Church, J. A., Clarke, L., Dahe, Q. and Dasgupta, P. (2014). *Climate change 2014: synthesis report. Contribution of Working Groups I, II and III to the fifth assessment report of the Intergovernmental Panel on Climate Change*. IPCC.
Perkins, S. E., Alexander, L. V. and Nairn, J. R. (2012). Increasing frequency, intensity and duration of observed global heatwaves and warm spells. *Geophys. Res. Lett.* **39**, L20714. doi:10.1029/2012GL053361
Pintor, A. F., Schwarzkopf, L. and Krockenberger, A. K. (2016). Extensive acclimation in ectotherms conceals interspecific variation in thermal tolerance limits. *PLoS One* **11**, e0150408. doi:10.1371/journal.pone.0150408
Pörtner, H. O. and Knust, R. (2007). Climate change affects marine fishes through the oxygen limitation of thermal tolerance. *Science* **315**, 95-97. doi:10.1126/science.1135471
Pörtner, H. O., Berdal, B., Blust, R., Brix, O., Colosimo, A., De Wachter, B., Giuliani, A., Johansen, T., Fischer, T., Knust, R. et al. (2001). Climate induced temperature effects on growth performance, fecundity and recruitment in marine fish: developing a hypothesis for cause and effect relationships in Atlantic cod (*Gadus morhua*) and common eelpout (*Zoarces viviparus*). *Cont. Shelf Res.* **21**, 1975-1997. doi:10.1016/S0278-4343(01)00038-3
Réale, D., Garant, D., Humphries, M. M., Bergeron, P., Careau, V. and Montiglio, P.-O. (2010). Personality and the emergence of the pace-of-life syndrome concept at the population level. *Philos. Trans. R. Soc. B Biol. Sci.* **365**, 4051-4063. doi:10.1098/rstb.2010.0208
Rezende, E. L., Castañeda, L. E. and Santos, M. (2014). Tolerance landscapes in thermal ecology. *Funct. Ecol.* **28**, 799-809. doi:10.1111/1365-2435.12268
Robertson, R. M. (2004). Thermal stress and neural function: adaptive mechanisms in insect model systems. *J. Therm. Biol.* **29**, 351-358. doi:10.1016/j.jtherbio.2004.08.073
Rogers, L. A., Stige, L. C., Olsen, E. M., Knutsen, H., Chan, K.-S. and Stenseth, N. C. (2011). Climate and population density drive changes in cod body size throughout a century on the Norwegian coast. *Proc. Natl. Acad. Sci.* **108**, 1961-1966. doi:10.1073/pnas.1010314108
Sandblom, E., Clark, T. D., Gräns, A., Ekström, A., Brijs, J., Sundström, L. F., Odelström, A., Adill, A., Aho, T. and Jutfelt, F. (2016). Physiological constraints to climate warming in fish follow principles of plastic floors and concrete ceilings. *Nat. Commun.* **7**, 1-8. doi:10.1038/ncomms11447
Schulte, P. M. (2015). The effects of temperature on aerobic metabolism: towards a mechanistic understanding of the responses of ectotherms to a changing environment. *J. Exp. Biol.* **218**, 1856-1866. doi:10.1242/jeb.118851
Sidhu, R., Anttila, K. and Farrell, A. P. (2014). Upper thermal tolerance of closely related *Danio* species. *J. Fish Biol.* **84**, 982-995. doi:10.1111/jfb.12339
Sih, A., Bell, A. and Johnson, J. C. (2004). Behavioral syndromes: an ecological and evolutionary overview. *Trends Ecol. Evol.* **19**, 372-378. doi:10.1016/j.tree.2004.04.009
Somero, G. N. (2010). The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine 'winners' and 'losers'. *J. Exp. Biol.* **213**, 912-920. doi:10.1242/jeb.037473
Sunday, J. M., Bates, A. E. and Dulvy, N. K. (2012). Thermal tolerance and the global redistribution of animals. *Nat. Clim. Change* **2**, 686-690. doi:10.1038/nclimate1539
Sundin, J., Morgan, R., Finnøen, M. H., Dey, A., Sarkar, K. and Jutfelt, F. (2019). On the observation of wild zebrafish (*Danio rerio*) in India. *Zebrafish* **16**, 546-553. doi:10.1089/zeb.2019.1778
Wegner, K. M., Kalbe, M., Milinski, M. and Reusch, T. B. (2008). Mortality selection during the 2003 European heat wave in three-spined sticklebacks: effects of parasites and MHC genotype. *BMC Evol. Biol.* **8**, 124. doi:10.1186/1471-2148-8-124

Fig S1. Experimental setup. The picture shows the tanks used in this experiment. Red labels indicates 34°C treatment and white label indicates 22°C treatment. The temperature in each tank is controlled with a thermostat (seen on the shelves columns) connected to one or two titanium heaters (seen on tank's left side). Each tank was equipped with one red and green ornamental plastic plant and two sponge biofilters for filtration, aeration and circulation. The 34°C tanks had an extra air stone installed to increase circulation over their heaters, resulting in a more even and stable temperature in their tanks.
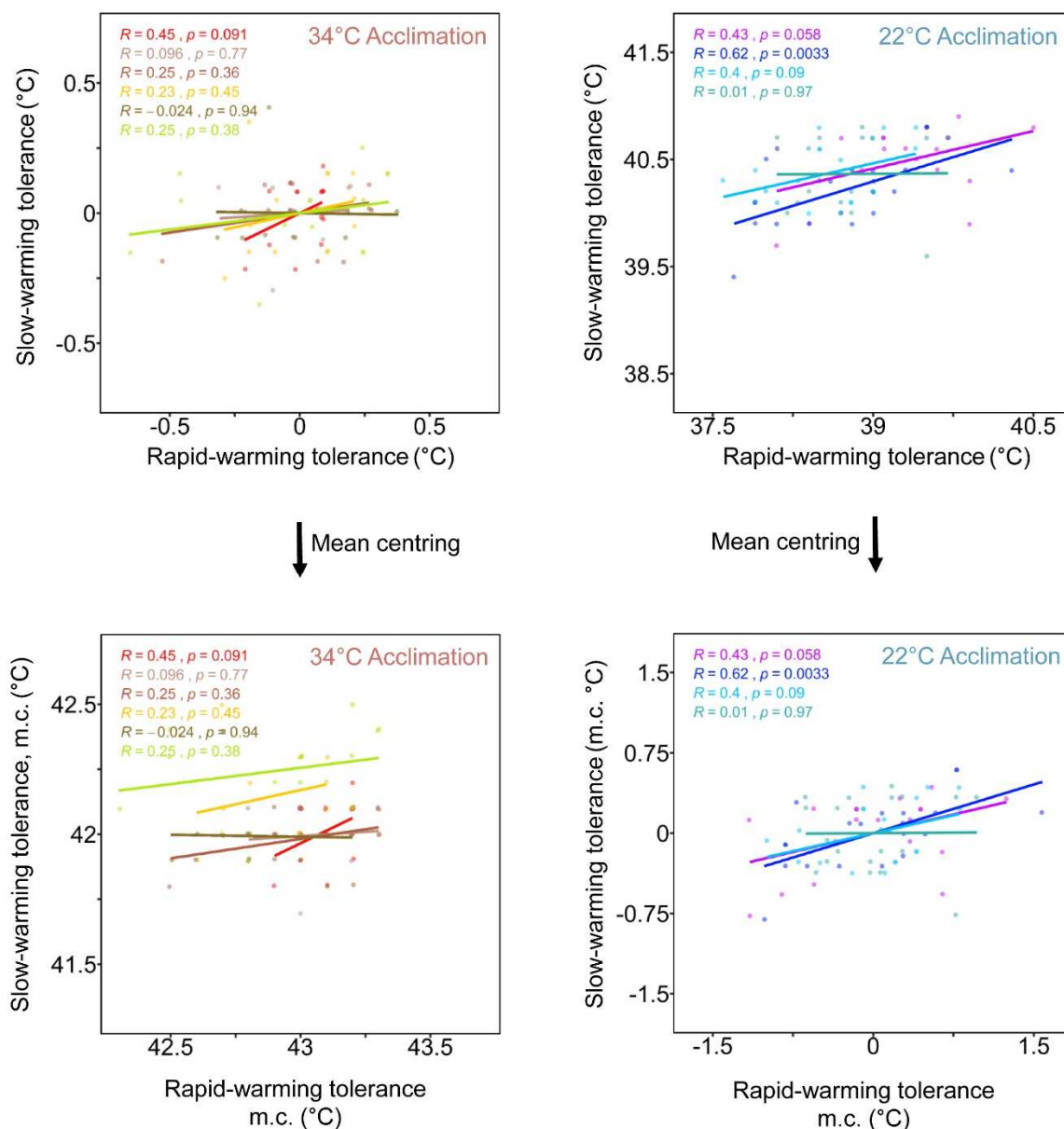
Fig S2. Mean centring adjusting for tank effects. Shows rapid-warming tolerance, measured as the temperature where loss of equilibrium (LOE) occurs at a warming rate of 0.3°C min^{-1} (also known as CT$_{max}$; Critical thermal maximum) and slow-warming tolerance (LOE at a warming rate of 0.025°C min^{-1}) for both acclimation treatments before and after mean centring (m.c.) of values. Mean centring redefines each value by subtracting the mean of its respective holding-tank from it, centring all tank-means on zero. Lines are fitted using least-square regression and are for illustrative purpose only.

Table S1. Tank effects. Results of analysis on linear models modelling either rapid-warming tolerance, measured as the temperature where loss of equilibrium (LOE) occurs at a warming rate of 0.3°C min^{-1} (also known as CT$_{max}$; Critical thermal maximum); slow-warming tolerance (LOE at a warming rate of 0.025°C min^{-1}) or growth as the response variable against holding tank as the predictor variable. *Significant P-values below 0.05.

Relationship	Accl temp (°C)	SSq	F_{df}	p
Slow-warming tolerance ~ Tank	22	0.717	$F_{3,75} = 2.247$	0.090
	34	0.774	$F_{5,76} = 8.915$	1.07e-06*
Fast-warming tolerance ~ Tank	22	5.105	$F_{3,75} = 5.071$	0.003*
	34	0.974	$F_{5,76} = 4.548$	0.001*
Growth ~ Tank	22	13060	$F_{3,75} = 1.313$	0.276
	34	6003	$F_{5,76} = 1.385$	0.239

Table S2. Spearman correlations. The table includes an alternative analysis of the correlations in table S1, using Spearman's rank correlation coefficient (ρ) and corresponding p-values for correlations between all combinations of growth; rapid-warming tolerance, measured as the temperature where loss of equilibrium (LOE) occurs at a warming rate of 0.3°C min^{-1} (also known as CT$_{max}$; Critical thermal maximum) and slow-warming tolerance (LOE at a warming rate of 0.025°C min^{-1}) at two acclimation temperatures (22 and 34°C). To correct for tank-effects, mean-centring (m.c.) was done by redefining each value as its deviance from tank mean. Correlations were tested using both raw values and mean-centred values. *Significant P-values below 0.05; ‡near-significant P-values below 0.1. This alternative analysis gives the same main results as the first analysis using Pearson's correlation coefficient, but removes the near-significant (p<0.1) relationship between growth and slow-warming tolerance in the 22°C treatment.

Relationship	Acclimated temp (°C)	Raw values Correlation I ρ	Raw values Correlation I p	Mean centred (m.c.) Correlation II ρ	Mean centred (m.c.) Correlation II p
Growth - slow-warming tolerance	*22*	0.169	0.136	0.145	0.201
	34	0.031	0.783	0.006	0.960
Growth - rapid-warming tolerance	*22*	0.109	0.338	0.085	0.454
	34	-0.015	0.892	0.017	0.877
Rapid-warming tolerance - slow-warming tolerance	*22*	0.445	0.0004*	0.366	0.001*
	34	0.139	0.209	0.200	0.070‡