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RESEARCH ARTICLE

Selection for upper thermal tolerance in rainbow trout (*Oncorhynchus mykiss* Walbaum)

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ABSTRACT

Rainbow trout (*Oncorhynchus mykiss* Walbaum) in southern Western Australia have undergone passive selection for over 19 generations to survive high water temperatures. Based on the conceptual model of 'oxygen- and capacity-limited thermal tolerance', we measured critical thermal maximum (CT_{max}), maximum heart rate ($f_{H,max}$) and aerobic scope to test the hypothesis that these rainbow trout can maintain aerobic scope at high temperatures through a robust cardiac performance supporting oxygen delivery. Across five family groups CT_{max} averaged 29.0±0.02°C. Aerobic scope was maximized at 15.8±0.3°C (T_{opt}), while the upper pejus temperature (T_{pej}, set at 90% of maximum aerobic scope) was 19.9±0.3°C. Although aerobic scope decreased at temperatures above T_{opt}, the value at 25°C remained well over 40% of the maximum. Furthermore, pharmacologically stimulated $f_{H,max}$ increased with temperature, reaching a peak value between 23.5±0.4 and 24.0±0.4°C (T_{max}) for three family groups. The Arrhenius breakpoint temperature (T_{AB}) for $f_{H,max}$ was 20.3±0.3 to 20.7±0.4°C, while the average Q_{10} breakpoint temperature (T_{QB}, when the incremental Q_{10}<1.6) for $f_{H,max}$ was 21.6±0.2 to 22.0±0.4°C. Collectively, $f_{H,max}$ progressively became less temperature dependent beyond 20°C (T_{AB} and T_{QB}), which coincides with the upper T_{pej} for aerobic scope. Although upper thermal performance indices for both aerobic scope and $f_{H,max}$ were compared among family groups in this population, appreciable differences were not evident. Compared with other populations of rainbow trout, the present assessment is consistent with the prediction that this strain has undergone selection and shows the ability to tolerate higher water temperatures.

KEY WORDS: Aerobic scope, Critical thermal maximum, Heart rate, Metabolic rate, Temperature tolerance, Thermal adaptation

INTRODUCTION

Thermal adaptation of many fish species in the northern hemisphere has been the result of range expansion since the last glaciation period (Bernatchez and Wilson, 1998), which is no more evident than within the genus *Oncorhynchus*. This genus is generally regarded as a post-glaciation invader that is naturally distributed between the Arctic and the Northern Tropic. However, many species have been introduced to areas throughout the world because of their high value for fisheries and aquaculture. Despite a preference for temperatures well below 20°C, *Oncorhynchus* species are remarkably adaptable. Indeed, recent studies suggest that there may have been local intraspecific adaptation to the thermal regimes experienced by adult sockeye salmon (*Oncorhynchus nerka*) during the spawning migration (Lee et al., 2003; Eliason et al., 2011). Similarly, the redband trout (*Oncorhynchus mykiss gairdneri*) appears to have uniquely adjusted its physiology to tolerate temperatures that may reach 29°C in the desert environment (Rodnick et al., 2004), a sign of thermal adaptation that has been supported by genetic evidence (Narum et al., 2010). In a more rapid and directed fashion, artificially transplanting animals to a new environment may also lead to selection by accumulating favourable heritable adaptations to create strains that are better suited to the new habitat. Here, rainbow trout (*Oncorhynchus mykiss* Walbaum) introduced into the warm environment of Western Australia were used to study their thermal performance and help understand the adaptive capacity for this species.

Since the early 1890s, rainbow trout have been translocated to New Zealand and Australia from their native habitat in the Pacific northwest of North America (Ward et al., 2003). Inevitably, these introductions exposed animals to many new environmental challenges, which led to either extirpation or possibly natural selection of the surviving population. Indeed, the establishment of rainbow trout in Western Australia has proved to be difficult (Molony, 2001) with an extremely hot climate being one of the critical limiting factors (Morrissy, 1973). Stocking locations in Western Australia also have to be carefully selected to balance the economic and recreational fishing outcomes while limiting negative impacts on native fish species. Despite these challenges, a rainbow trout strain has been successfully maintained at the Pemberton Freshwater Research Centre (PFRC) for several decades and is used to annually stock the dams and creeks in southern Western Australia. During hatchery culture, the PFRC rainbow trout population experienced several bouts of severe mortality (>90%) due to exceptionally hot summers in the 1980s (Molony et al., 2004). Consequently, the PFRC strain of rainbow trout (termed the H-strain) is a particularly good candidate to study thermal adaptation, given the strength, form and duration of selection.

Both genetic and phenotypic evidence exists to demonstrate thermal selection in PFRC H-strain rainbow trout. Compared with a self-sustaining wild-type population (S-strain) in the deep Serpentine Dam in Western Australia, which was stocked by PFRC prior to the 1960s, the PFRC H-strain of rainbow trout has considerably less genetic variation in microsatellite loci (Ward et al., 2003), an expected result if the population had experienced a

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List of symbols and abbreviations

CT_{max}	critical thermal maximum
$f_{H,max}$	maximum heart rate
Hb	haemoglobin
Hct	haematocrit
MMR	maximum metabolic rate
PFRC	Pemberton Freshwater Research Centre
RMR	routine metabolic rate
RVM	relative wet ventricle mass to body mass
T_{AB}	Arrhenius breakpoint temperature
T_{AR}	arrhythmic temperature
T_{crit}	critical temperature
T_{max}	temperature at which $f_{H,max}$ is maximum
T_{opt}	optimum temperature
T_{pej}	pejus temperature
T_{QB}	breakpoint temperature for Q_{10} of $f_{H,max}$

selection-driven bottleneck in the past. Phenotypically, the PFRC H-strain has been shown to tolerate the extreme temperature of 27°C for twice as long as the wild-type S-strain from Serpentine Dam (Molony et al., 2004). More broadly, an earlier study showed the longer survival time at high temperatures (25–29°C) of PFRC rainbow trout than their counterparts from New South Wales and Victoria, where the PFRC rainbow trout directly originated (Morrissy, 1973). These results suggest the possibility that poorly adapted individuals have been eliminated during hatchery rearing because the water temperature cannot be operationally maintained below 25°C (Fig. 1), whereas similar selection pressures were avoided by wild fish in the dam that had access to cool water at depth (<20°C). Therefore, to better elucidate the underlying physiological changes of PFRC H-strain rainbow trout that renders them more heat tolerant, we undertook a comprehensive physiological phenotypic assessment of their upper thermal performance. Specifically, the ability of PFRC H-strain rainbow trout to tolerate acute warming was characterized by measuring critical thermal maximum (CT_{max}), aerobic scope [the absolute difference between maximum metabolic rate (MMR) and routine metabolic rate (RMR)] and the response of maximum heart rate ($f_{H,max}$) to acute warming. The experiments tested the hypothesis that: (1) PFRC H-strain rainbow trout maintain aerobic scope and $f_{H,max}$ at a high temperature; and (2) phenotypic variation has been reduced among individuals as a result of intensive selection.

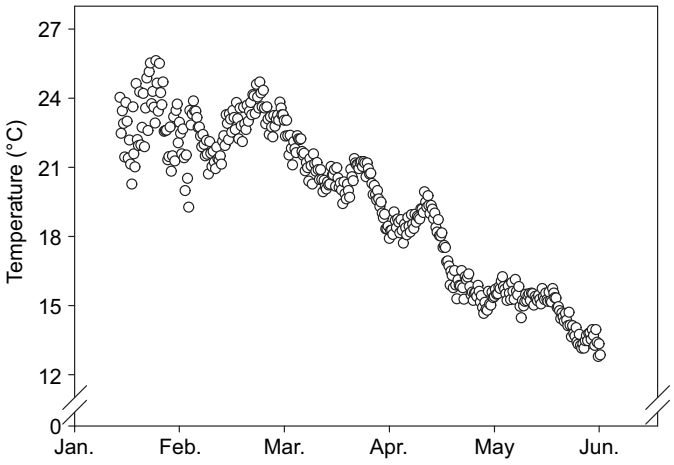

Fig. 1. Temperature of water passing through Pemberton Freshwater Research Centre (PFRC) ponds from January to May, 2014.

The approaches used here stem from the pioneering physiological studies of thermal performance in fishes by Dr Fred Fry (1947) and the more recent concept of 'oxygen- and capacity-limited thermal tolerance' (Pörtner and Knust, 2007; Pörtner and Farrell, 2008), as well as the recent finding that rate transition temperatures for heart rate reveal much about the upper thermal tolerance that governs the biogeographical distribution of a species (Tepolt and Somero, 2014). Indeed, recent physiological studies of thermal optima in fish range from warm water temperature species such as coral reef fishes (Nilsson et al., 2009) and *Danio* (Sidhu et al., 2014), through several temperate salmonid species (Casselman et al., 2012; Anttila et al., 2014; Chen et al., 2013; Verhille et al., 2013) and goldfish, *Carassius auratus* (Ferreira et al., 2014), to polar species such as the Antarctic notothenid fish *Pagothenia borchgrevinki* (Franklin et al., 2007) and Arctic cod, *Boreogadus saida* (Drost et al., 2014). Limitations to this physiological approach have been questioned in general (Clark et al., 2013) and rebutted (Pörtner and Giomi, 2013; Farrell, 2013). More specifically, the questioning was for a fish species (Norin et al., 2014), an amphibian species (Overgaard et al., 2012) and a tropical shrimp (Ern et al., 2014). Yet, aerobic scope, $f_{H,max}$ and CT_{max} have been satisfactorily compared through the studies in goldfish (Ferreira et al., 2014), *Danio* (Sidhu et al., 2014) and salmonids (Casselman et al., 2012; Chen et al., 2013). Therefore, the present study determined the optimum temperature for aerobic scope (T_{opt}) and the extent to which aerobic scope diminished at supraoptimal temperatures up to 25°C. In addition, the rate transition temperatures, when $f_{H,max}$ failed to keep up with a steady Q_{10} during acute warming, were characterized because of the clear evidence in salmonids that the primary response to deliver more oxygen to tissues is increasing f_H (Farrell, 2009; Eliason et al., 2011).

RESULTS

CT_{max}

The mean (±s.e.m.) CT_{max} among all individuals in this study was 29.0±0.02°C. The individual variance in CT_{max} was very small, with CT_{max} values for the 375 fish from the 25 families spanning just 1.6°C and ranging from 28.1 to 29.7°C. Minor CT_{max} differences could be statistically distinguished among some family groups: family group 1 had a significantly higher CT_{max} (29.3±0.02°C, P<0.01) than all others, while family groups 2 and 5 had a significantly lower CT_{max} (28.9±0.03 and 28.8±0.03°C, respectively, P<0.01) compared with all the others (Fig. 2).

Metabolic rates and aerobic scope

In family groups 1, 2 and 5, RMR increased by about 6-fold with acute warming from 8 to 25°C (Fig. 3A). The RMR values for family groups 1 and 5 were significantly different (P=0.049). Family group 1 had the lowest Q_{10} (2.9) and the highest CT_{max}, whereas family group 5 had the highest Q_{10} (3.1) with the lowest CT_{max}. No statistically significant difference was found among the three family groups for MMR at any common test temperature (P>0.13). Before reaching its maximum value, MMR increased with a similar average Q_{10} of 1.6 for the three family groups (Fig. 3A). According to the Gaussian functions, MMR had its maximum value at 20.0°C for group 1 (753.0 mg O_2 kg^{-1} h^{-1}), 19.2°C for group 2 (747.2 mg O_2 kg^{-1} h^{-1}) and 20.6°C for group 5 (775.8 mg O_2 kg^{-1} h^{-1}) (Fig. 3A).

Although the regression equations for aerobic scope were based on the group data (Fig. 3B), all the rate transition temperatures were based on a fitted function for each individual. According to the

The Journal of Experimental Biology (2015) 218, 803-812 doi:10.1242/jeb.113993

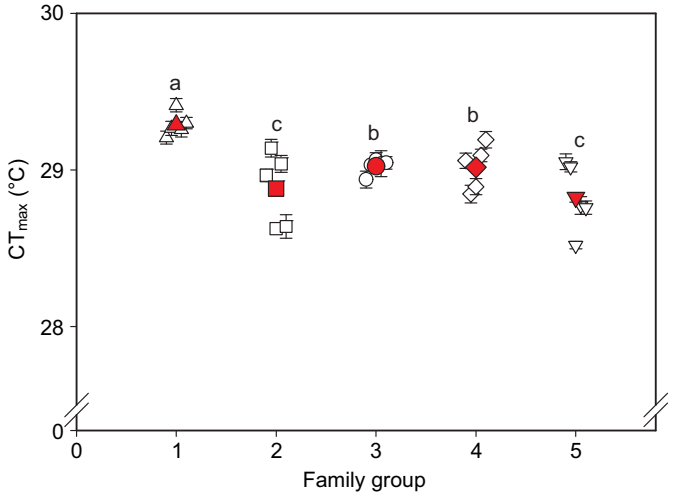

Fig. 2. Critical thermal maximum (CT_{max}) of five family groups of H-strain rainbow trout (*Oncorhynchus mykiss*), each of which contained five sub-families that shared the same sires. The individual mean values (±s.e.m.) for the half-sibling families (white symbols; *N*=15 per half-sibling family) and the mean value for the family groups (red symbols; *N*=75 per group) are separated from each other on the *x*-axis for clarity. Letters above the symbols denote a statistically significant difference of means among family groups (P<0.05).

Gaussian functions, aerobic scope reached a maximum value for the three family groups around 16°C (550.0±25.6 mg O_2 kg^{-1} h^{-1} at 16.5±0.6°C for group 1, 559.7±22.0 mg O_2 kg^{-1} h^{-1} at 15.3±0.2°C for group 2 and 541.2±24.3 mg O_2 kg^{-1} h^{-1} at 15.8±0.7°C for group 5) (Table 1). Both the maximum aerobic scope values and the rate transition temperatures were not significantly different among the family groups (Table 1). Pejus temperatures (T_{pej}) were arbitrarily assigned at 90% of maximum aerobic scope (Eliason et al., 2011). The lower T_{pej} values were clustered between 11.5±1.0 and 12.4±0.4°C, while the upper T_{pej} values were clustered between 19.0±0.4 and 20.5±0.8°C (Table 1). Upper critical temperature (T_{crit}) values were between 31.6±1.7 and 36.0±1.6°C. The factorial aerobic scope (i.e. MMR/RMR) was greatest at 8°C (5.9–6.4 among groups), decreased with temperature but remained an impressive 1.4–1.8 even at the highest test temperature (25°C). Family group 1 had the highest factorial aerobic scope. At 25°C, aerobic scope was 29–43% of the maximum aerobic scope at T_{opt}.

$f_{H,max}$

All three family groups increased $f_{H,max}$ with incremental warming until a maximum value for $f_{H,max}$ was reached and a cardiac arrhythmia followed with further warming (Fig. 4A). The peak value for $f_{H,max}$ occurred between 23.5±0.4 and 24.0±0.4°C (T_{max}) among families, which was above the upper T_{pej} determined from aerobic scope (Table 2). The temperature at which cardiac arrhythmia (T_{AR}) first developed averaged around 25°C for the three family groups. No fish maintained a regular heart beat beyond 27°C, with 23°C being the lowest T_{AR} in all three family groups (Fig. 4A). At 25°C, which was the highest test temperature used to measure aerobic scope, 50% of the fish tested displayed cardiac arrhythmias. Across all test temperatures, family group 2 maintained the highest $f_{H,max}$ across all temperatures (Fig. 4A).

The rate transition temperatures (T_{QB}) for $f_{H,max}$ are compared for the three family groups in Table 2, but no significant differences were discovered. The incremental Q_{10} for $f_{H,max}$ decreased progressively with acute warming (Fig. 4B). The T_{QB} values ranged from 21.6±0.2 to 22.0±0.4°C and were statistically indistinguishable from the Arrhenius

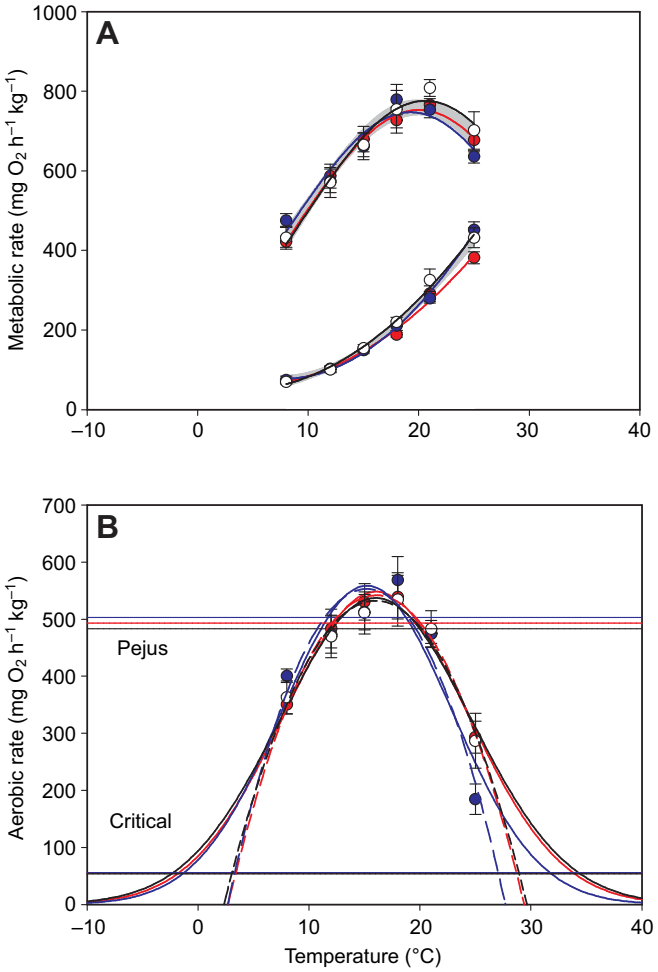

Fig. 3. Routine (RMR) and maximum (MMR) metabolic rate, and aerobic scope for three family groups of rainbow trout. (A) RMR and MMR of three groups of rainbow trout (means±s.e.m.; *N*=6 for each group). RMR was fitted with a polynomial quadratic function [family group 1, red, $y=74.7+(-6.8)x+0.8x^2$, R^2=0.99, P<0.01; group 2, blue, $y=149.7+(-18.9)x+1.2x^2$, R^2=0.99, P<0.01; group 5, white, $y=59.2+(-6.3)x+0.9x^2$, R^2=0.99, P<0.01). MMR was fitted with a Gaussian three-parameter function (group 1, red, $y=753.0e^{-0.5[(x-20.0)/11.2]^2}$, R^2=0.99, P<0.01; group 2, blue, $y=747.2e^{-0.5[(x-19.2)/11.1)^2}$, R^2=0.93, P=0.019; group 5, white, $y=775.8e^{-0.5[(x-20.6)/11.3]^2}$, R^2=0.98, P<0.01). The grey area represents the ±95% confidence interval of the aforementioned fittings for the overall means in three groups. (B) Aerobic scope (absolute difference between MMR and RMR) calculated for the three family groups. Solid lines represent a Gaussian three-parameter function fitted for each group to generate a Fry aerobic scope curve (family group 1, red, $y=548.1e^{-0.5[(x-16.0)/8.3]^2}$, R^2=0.98, P<0.01; group 2, blue, $y=558.5e^{-0.5[(x-15.2)/7.6]^2}$, R^2=0.84, P=0.062; group 5, white, $y=537.1e^{-0.5[(x-16.0)/8.6]^2}$, R^2=0.95, P=0.012). The horizontal lines represent 90% (marked as 'Pejus') and 10% (marked as 'Critical') of the maximum aerobic scope for each group and the intersection of these lines with the Fry aerobic scope curves represents the pejus temperature (T_{pej}) and critical temperature (T_{crit}). Owing to the similarity of aerobic scope values at T_{crit}, the 'Critical' lines overlap with each other. Dashed lines represent fitting with the polynomial quadratic function as an alternative way to extrapolate the rate transition temperatures.

breakpoint temperature (T_{AB}) values (Table 2, Fig. 4C). The T_{AB} values ranged from 20.3±0.3 to 20.7±0.4°C among groups (Table 2).

Ventricle mass, haemoglobin (Hb) and haematocrit (Hct)

The fish from the $f_{H,max}$ measurements had a mean (±s.e.m.) body mass of 36.9±1.2 g (Table 3) and a mean (±s.e.m.) wet ventricle

The Journal of Experimental Biology (2015) 218, 803-812 doi:10.1242/jeb.113993

Table 1. Estimates of optimum temperature for aerobic scope (T_{opt}), pejus temperature (T_{pej}), critical temperature (T_{crit}) and their corresponding aerobic scope as derived from the Fry aerobic scope curves for three family groups of H-strain rainbow trout (*Oncorhynchus mykiss*)

	Family group 1		Family group 2		Family group 5		Family mean	
	Temperature (°C)	Aerobic scope (mg O_2 h^{-1} kg^{-1})	Temperature (°C)	Aerobic scope (mg O_2 h^{-1} kg^{-1})	Temperature (°C)	Aerobic scope (mg O_2 h^{-1} kg^{-1})	Temperature (°C)	Aerobic scope (mg O_2 h^{-1} kg^{-1})
Lower T_{crit}	−2.4±0.7	55.0±2.6	−1.0±1.4	56.0±2.2	−4.3±2.5	54.1±2.4	−2.6±1.0	55.0±1.3
Lower T_{pej}	12.4±0.4	495.0±23.1	11.6±0.2	503.7±19.8	11.5±1.0	487.0±21.9	11.8±0.4	495.2±11.8
T_{opt}	16.5±0.6	550.0±25.6	15.3±0.2	559.7±22.0	15.8±0.7	541.2±24.3	15.8±0.3	550.3±13.2
Upper T_{pej}	20.5±0.8	495.0±23.1	19.0±0.4	503.7±19.8	20.2±0.5	487.0±21.9	19.9±0.3	495.2±11.8
Upper T_{crit}	35.3±1.6	55.0±2.6	31.6±1.7	56.0±2.2	36.0±1.6	54.1±2.4	34.3±1.0	55.0±1.3

Estimates were generated from individual fish data using a Gaussian three-parameter function to generate the Fry aerobic scope curve (similar to the fitting for the mean value in Fig. 3B). Using these equations, T_{opt} was defined as the temperature with maximum aerobic scope, upper and lower T_{pej} were defined as the maximum and minimum temperatures at which aerobic scope remained above 90% of the maximum aerobic scope, and upper and lower T_{crit} were defined as the maximum and minimum temperatures at which aerobic scope was at least 10% of the maximum aerobic scope at T_{opt}. All values are means±s.e.m.

mass of 30.6±1.2 mg (Table 4). There were no statistically significant differences among family groups. The relative wet ventricle mass (RVM) was between 0.081% and 0.084% (Table 4). Hct level and Hb content also were not significantly different among family groups. Hct level ranged from 32.9±1.1% to 36.9±2.6%; Hb content was around 10.1 g dl^{-1} (Table 4).

DISCUSSION

For the first time, the present study has comprehensively examined the upper thermal tolerance of an introduced fish species (PFRC H-strain of rainbow trout) that has undergone significant thermal selection over 19 generations of hatchery culture. We have provided experimental support for the two hypotheses that we set out to test: (1) PFRC H-strain rainbow trout are able to maintain aerobic scope and $f_{H,max}$ at a high temperature; and (2) phenotypic variation has been reduced as a result of the intensive selection. $f_{H,max}$ was sustained without showing arrhythmia until 25°C, at which temperature aerobic scope was still at 40% greater than RMR. Limited variation was particularly evident for the rate transition temperatures as indicated by the subtle differences among independently tested groups (Table 1, 2). For CT_{max}, the family group differences that did emerge as significant were quantitatively small (Fig. 2).

The occurrence and intensity of local adaptation relies on many factors: the scale and magnitude of selection; the level of standing genetic variation in the population; the extent of gene flow; and the heritability of adaptive traits. Because these factors differ widely between species and populations, so too does the magnitude of local adaptation (Leimu and Fischer, 2008). Artificially translocating fish to a new environment could potentially accelerate and intensify adaptation. Indeed, precedents exist to indicate a genetic basis for adaptation of PFRC rainbow trout to the hot climate. Ward et al. (2003) examined the genetic structure of the Western Australia rainbow trout, comparing Serpentine Dam wild-type S-strain and PFRC hatchery H-strain with North American rainbow trout. The Australian hatchery fish were genetically more homozygous than their North American counterparts. Furthermore, a study of gene expression in the rainbow trout introduced to Japan (Ojima et al., 2012; Tan et al., 2012) revealed a higher expression of heat shock genes after the strain was raised at high temperatures (20–24°C, occasionally 30°C) in summer for more than 14 successive generations. Similarly, transplanted sticklebacks (*Gasterosteus aculeatus*) became more cold tolerant in just three generations (Barrett et al., 2011). However, phenotypic plasticity can cause exceptional variability in thermal performance (Currie et al., 1998) and must be investigated as a complement to evolutionary adaptation for thermal tolerance (Narum et al., 2013). In the present study, we had maintained the fish at 15°C in a common garden environment since hatching stage to minimize any acclimation effect.

CT_{max}

CT_{max} is a widely accepted measure of acute thermal tolerance and is defined as 'the thermal point at which locomotion becomes disorganized and the animal loses its ability to escape from conditions that will promptly lead to its death' (Lutterschmidt and Hutchison, 1997). Thus, CT_{max} can be used to infer the ability of animals to tolerate a rapid regional or a diurnal temperature increase in the wild. For example, CT_{max} has been found to be coincident with the extreme temperatures of the habitat in a number of fish species (Somero and DeVries, 1967; Beitinger et al., 2000). The present study found that CT_{max} of H-strain rainbow trout is between 28 and 30°C, which is very close to the annual highest water temperature of PFRC based on historic data (Molony et al., 2004). In the summer of 2014, a relatively cool year in Western Australia, the highest water temperature was recorded as 26.1°C for 3 h (Fig. 1) and this extreme did not produce any thermal-related fish mortality. Fish survival was possible in previous summers when ambient water temperatures (>30°C) clearly exceed the measured CT_{max} only because cooling towers were used at the hatchery to chill the hatchery water for each pond as well as possible.

When compared with CT_{max} values for rainbow trout populations from other locations and habitats, we found a large variance among studies (26.9–31.8°C, Table 5). However, acclimation temperature played a significant role in this variability (Fig. 5). CT_{max} has a smaller range between 27.5 and 30°C for indigenous strains in British Columbia, Canada, when acclimated at 10°C (Scott et al., 2014) and in California, USA (Myrick and Cech, 2000, 2005), and in recently established strains in Pennsylvania, USA (Carline and Machung, 2001). For related redband trout that have adapted to a desert environment (Rodnick et al., 2004), where summer water temperature can oscillate from 18 to 30°C daily, CT_{max} (29.7±0.3°C) was close to the upper daily water temperature recorded for those streams. Even a Japanese strain, originally from California, which has been intensely selected for upper temperature tolerance over multiple generations, had a CT_{max} of 30.4°C that was no different from that of the non-selected strains (Ineno et al., 2005). These results suggest that variability of CT_{max} may be lower at a population level (arising both naturally and through artificial selection) compared with the larger differences that are known to exist among species, which range from <10°C in Antarctic 'icefish' (Somero and DeVries, 1967) to ~40°C in mosquito fish (Beitinger et al., 2000). Significant differences do exist among families in the

The Journal of Experimental Biology (2015) 218, 803-812 doi:10.1242/jeb.113993

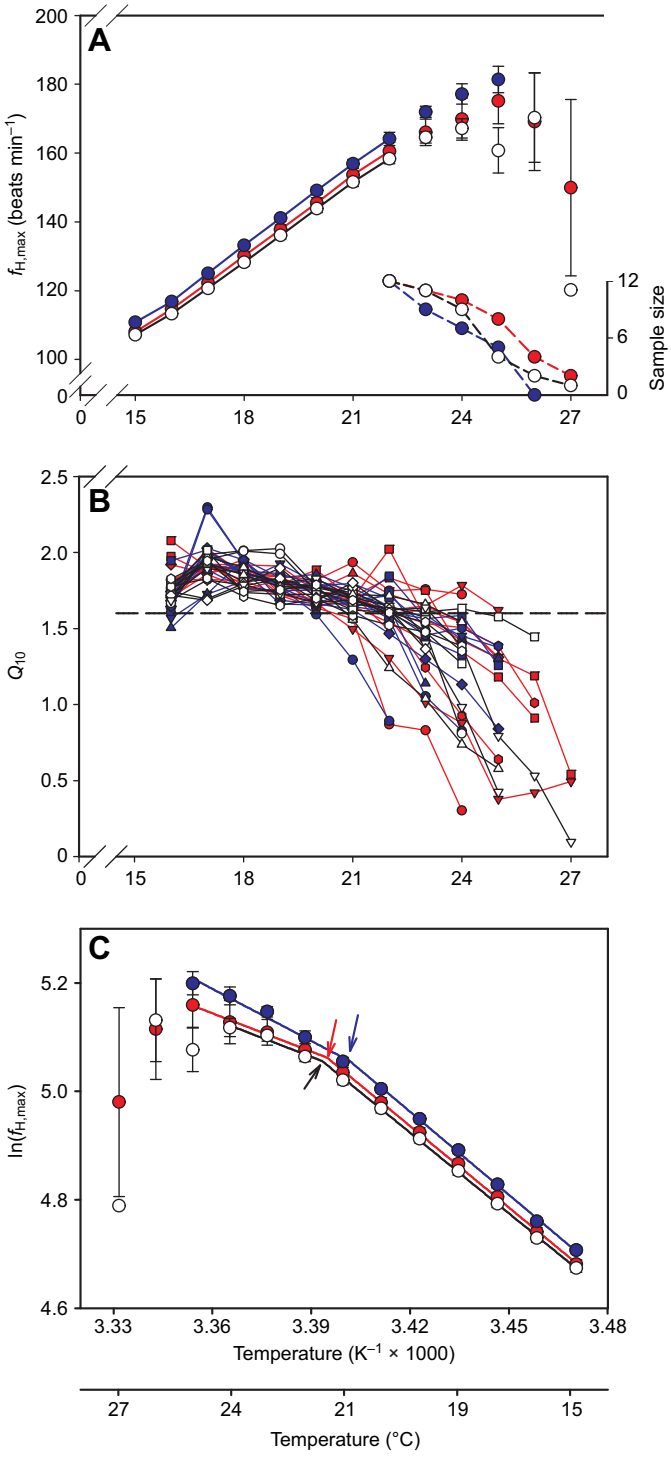

Fig. 4. Maximum heart rate ($f_{H,max}$) in response to temperature increase in three family groups of rainbow trout with the Q_{10} plot and Arrhenius plot. In all panels, red indicates family group 1 of PFRC H-line rainbow trout, blue indicates family group 2, white indicates family group 5. (A) Pharmacologically stimulated $f_{H,max}$ during incremental warming of three family groups. Mean (±s.e.m.) $f_{H,max}$ data for each group are connected by a solid line to indicate that all fish had a regular heart rhythm (N=12 for each group). After some fish developed cardiac arrhythmia, the number of remaining fish without cardiac arrhythmia (symbols with dashed lines) is also given and their mean $f_{H,max}$ data are shown by the unconnected symbols. (B) Incremental Q_{10} values for $f_{H,max}$ during incremental warming of three family groups. Each line represents an individual fish's responses. The rate transition temperature for Q_{10} (T_{QB}) was arbitrarily set as the temperature beyond which the incremental Q_{10} for $f_{H,max}$ fell below 1.6 (the horizontal dashed line). Specifically, T_{QB} is the last temperature where Q_{10} is above the dashed line. (C) Arrhenius plot of mean $f_{H,max}$ during incremental warming for three family groups. The mean $f_{H,max}$ was derived from the individual fish responses summarized in A. It was possible to fit two segmental linear regressions to the data to identify the Arrhenius breakpoint temperature (T_{AB}), which is indicated by the arrows.

present and many previous studies, even though the absolute values of CT_{max} are generally within 2°C in common garden environments (Carline and Machung, 2001; Myrick and Cech, 2005). The underlying reason for the intraspecific variance of CT_{max} in rainbow trout is unclear, but may arise from modification of key biochemical pathways (Hochachka and Somero, 2002) during adaptation. With the rapid development of high-throughput next-generation sequencing, it may be possible to address this question in the near future. All the same, artificially selecting a particular strain of rainbow trout (and perhaps other species) to perform at significantly higher temperatures may prove difficult using CT_{max} as the only metric to make such a distinction, because CT_{max} is a measure of tolerance rather than performance.

Metabolic rates and aerobic scope

It is proposed that the thermal performance of an aquatic ectotherm is limited by the capacity to deliver oxygen to its tissues (Pörtner and Knust, 2007; Pörtner and Farrell, 2008). Thus, the curve for aerobic scope as a function of temperature, as originally conceived for fishes by Fry (1947), graphically describes the thermal performance of a fish by illustrating the capacity to increase its tissue oxygen delivery above and beyond routine needs (Farrell, 2009). According to the Fry aerobic scope curve, an elevated RMR without a corresponding increase in MMR will reduce aerobic scope, which consequently will cause a decrease of upper critical temperature. In an earlier research on Italian rainbow trout (3–10 g), aerobic scope at 20°C averaged 355.2 mg O_2 kg^{-1} h^{-1} (Wieser et al., 1985), which is lower than in the present findings (474.3 mg O_2 kg^{-1} h^{-1}), suggesting the PFRC H-line trout have a greater capacity for performance at these warm temperatures. However, naturally warm-adapted populations of redband trout may have evolved an even greater aerobic scope compared with the artificially selected stock in the present study. For example, RMR of redband trout at 24°C (200±13 mg O_2 kg$^{-0.83}$ h^{-1}; Rodnick et al., 2004) was 49% lower than the values generated here, yet MMR was the same in the two studies. As a result, the aerobic scope of redband trout at 24°C (533±22 mg O_2 kg$^{-0.882}$ h^{-1}) is 1.6 times higher than that of the rainbow trout from Western Australia, which suggests that redband trout may be better adapted to warm temperatures even though they have a similar CT_{max} (Table 5).

While Gaussian curves fitted the MMR and aerobic scope data well, extrapolation of these lines beyond the actual data points must be undertaken with great caution. For example, here, the extrapolated values for the upper T_{crit} exceeded the CT_{max} values, which is a highly unlikely situation. Instead, this anomaly reflects the difficulty in measuring metabolic rates at a test temperature >25°C, beyond which aerobic scope falls precipitously with increasing temperature. Thus, we included a quadratic fit (dashed lines) to the data in Fig. 3B as an alternative extrapolation for T_{crit}. Gaussian functions (solid lines in Fig. 3B), however, provided a better prediction of T_{opt} for aerobic scope (from 15.3 to 16.5°C). Furthermore, 90% of maximum aerobic scope could be maintained up to 19.0–20.5°C depending on the family, and factorial aerobic scope was an impressive 2.7–3.1 at these temperatures. By comparison with other salmonids, the upper T_{pej} for the PFRC H-strain was at the upper end of the upper T_{pej} range

The Journal of Experimental Biology (2015) 218, 803-812 doi:10.1242/jeb.113993

Table 2. Estimates of Arrhenius breakpoint temperature (T_{AB}), Q_{10} breakpoint temperature (T_{QB}), temperature of the highest $f_{H,max}$ (T_{max}) and cardiac arrhythmia temperature (T_{AR}) and their corresponding maximum heart rate ($f_{H,max}$) for rainbow trout family groups

	Family group 1		Family group 2		Family group 5		Family mean	
	Temperature (°C)	$f_{H,max}$ (beats min^{-1})	Temperature (°C)	$f_{H,max}$ (beats min^{-1})	Temperature (°C)	$f_{H,max}$ (beats min^{-1})	Temperature (°C)	$f_{H,max}$ (beats min^{-1})
T_{AB}	20.7±0.4^{b}	152.6±4.9^{a}	20.3±0.3^{b}	151.0±3.4^{a}	20.6±0.1^{b}	148.6±1.5^{a}	20.5±0.2	150.8±2.1
T_{QB}	22.0±0.4^{b}	162.8±4.5^{b}	21.6±0.2^{b}	162.9±2.5^{b}	22.0±0.3^{b}	159.2±3.1^{b}	21.9±0.2	161.7±2.1
T_{max}	24.0±0.4^{a}	173.7±4.6^{b}	23.5±0.4^{a}	173.1±3.5^{b}	23.7±0.3^{a}	168.0±2.7^{b}	23.7±0.2	171.6±2.2
T_{AR}	25.7±0.4^{a}		24.7±0.4^{a}		24.9±0.4^{a}		25.1±0.3	

T_{AB} represents the transition temperature beyond which the exponential change of $f_{H,max}$ slows. T_{QB} represents the transition temperature where the incremental Q_{10} for $f_{H,max}$ decreases and remains below 1.6. T_{max} is the highest absolute value achieved for $f_{H,max}$. T_{AR} is the temperature where cardiac arrhythmia first develops. Different superscript letters indicate significant differences within each group. All values are means±s.e.m.

for *O. nerka* populations (16.4–20.7°C; Eliason et al., 2011). Where the PFRC H-strain truly stands out is in its ability to maintain a large aerobic scope very close to CT_{max}. At 25°C, RMR could still be increased by 1.4- to 1.8-fold depending on the family groups. Given that salmonids that consume large meals can double their metabolic rate post-prandially (Farrell et al., 2001; Fu et al., 2005; Eliason and Farrell, 2014), we can conclude that the PFRC H-strain at 25°C would have some (but not a full) capacity for both digestion and activity. This conclusion is consistent with empirical observation of fish swimming and eating in the holding aquaria even at the hottest summer temperature (26.1°C) during the present study. Even so, the temperature margin between these activities and death is small. Previous research found that all fish were dead within 20 h when held at 27°C, but with a variable mortality rate across time (Molony et al., 2004). The group variability in aerobic scope and RMR observed here might help explain the variable mortality observed earlier. Further studies will be needed to explain how aerobic scope can be maintained at such high temperatures in this strain.

$f_{H,max}$

The heart plays a critical role in delivering oxygen to tissues. The Fick equation states that oxygen uptake is the product of cardiac output and tissue oxygen extraction from the blood. Moreover, cardiac performance becomes even more critical during acute warming because it is predominately heart rate, much more so than either cardiac stroke volume or tissue oxygen extraction, that increases as metabolic rate increases (Farrell, 2009). In salmonids, data suggest that the inability of $f_{H,max}$ to continue to increase exponentially with temperature may be the initial trigger for the levelling of aerobic scope as T_{opt} is approached (Farrell, 2009; Casselman et al., 2012; Eliason et al., 2013). Here, as in earlier studies (Casselman et al., 2012; Chen et al., 2013; Sidhu et al., 2014), we used pharmacological approaches to generate $f_{H,max}$ and to examine the effect of acute warming. The proximity of T_{AB} (Table 2) and upper T_{pej} (Table 1) contrasts with the previous findings that T_{AB} was closer to either T_{opt} (Casselman et al., 2012) or lower T_{pej} (Ferreira et al., 2014). Another important association is that cardiac arrhythmias began when T_{pej} was exceeded and aerobic scope was in decline. The reason is that if the $f_{H,max}$ cannot increase further, then the only means to increase maximum aerobic scope (or maintain it if $f_{H,max}$ is in decline) would be to increase cardiac stroke volume and/or tissue oxygen extraction, which are less efficient. Indeed, the present study found that cardiac arrhythmias developed well beyond the upper T_{pej} (Table 1), but always at temperatures prior to reaching CT_{max}.

Ventricle mass, Hb and Hct

Hb concentration was similar to previous findings in redband trout (Rodnick et al., 2004), while Hct level was higher than average in rainbow trout (Gallaugher and Farrell, 1998), suggesting the blood O_2 carrying capacity of the PFRC H-line is comparable with that of warm-adapted redband trout. However, the RVM of PFRC rainbow trout was smaller (7.7–33.1%) than RVM in redband trout (Rodnick et al., 2004), which may be a hatchery effect. If the difference in cardiac mass translates to improved cardiac output and arterial blood pressure, it may help explain why the redband trout appears to be better adapted to warm temperatures.

Conclusions

On the whole, despite the fact that Western Australia is a marginal habitat for rainbow trout (high temperatures, low water oxygen content and loss of aquatic habitat as creeks and dams dry up), the stocking programme carried out annually by PFRC is able to maintain a viable recreational fishery based on the PFRC H-strain of rainbow trout in stocked habitats in Western Australia. The present study provides physiological evidence to support the previous discovery that the PFRC H-strain has undergone certain levels of selection and demonstrated the ability to survive the hot climate. Further comparative physiological and genomic research could help to elucidate the mechanism of broader thermal adaptation at both the inter- and intra-specific level. Because of the adequate performance in both growth (Molony et al., 2004) and temperature tolerance, rainbow trout in the current study represent a promising strain suited to aquaculture in a warming climate, and thus merit further investigation.

Table 3. Body size of the H-strain rainbow trout used for the various tests performed in this study

	CT_{max} measurements			Aerobic scope			$f_{H,max}$ measurements	
Family group	*N*	Mass (g)	Length (cm)	*N*	Mass (g)	*N*	Mass (g)	Length (cm)
1	75	27.7±0.6^{c}	12.6±0.08^{c}	6	33.6±0.7	12	35.8±2.4	14.0±0.3
2	75	31.0±0.6^{a,b}	13.1±0.08^{a,b}	6	33.0±0.5	12	39.6±1.9	14.3±0.2
3	75	29.5±0.6^{b,c}	12.8±0.09^{b,c}					
4	75	32.8±0.5^{a}	13.4±0.06^{a}					
5	75	30.1±0.5^{b}	13.0±0.08^{b}	6	34.2±0.8	12	35.4±1.9	13.9±0.3
Mean	375	30.2±0.3	13.0±0.04	18	33.6±0.4	36	36.9±1.2	14.1±0.2

Different superscript letters indicate significant differences among groups (P<0.05). All values are means±s.e.m.

The Journal of Experimental Biology (2015) 218, 803-812 doi:10.1242/jeb.113993

Table 4. Wet ventricle mass, haematocrit (Hct) and haemoglobin concentration (Hb) for H-strain rainbow trout

Family group	Ventricle size			Blood analysis		
	N	Mass (mg)	RVM (%)	*N*	Hct (%)	Hb (g dl^{-1})
1	12	29.1±2.3	0.081±0.003	5	33.8±3.7	11.1±0.1
2	12	32.1±1.6	0.081±0.002	3	35.3±2.1	10.0±0.2
3				5	36.9±2.6	9.8±0.5
5	12	30.6±2.4	0.084±0.003	4	32.9±1.1	9.9±0.3
Mean	36	30.6±1.2	0.082±0.002	17	34.7±1.3	10.1±0.2

RMV, relative wet ventricular mass to body mass. All values are means±s.e.m.

MATERIALS AND METHODS

Study strain and location

All procedures were approved by the University of British Columbia Committee on Animal Care in accordance with the Canadian Council on Animal Care (A10-0335). Rainbow trout at PFRC, Pemberton, Western Australia, originated from the San Francisco Bay area – Sonoma Creek and/or Russian Creek (Department of Fisheries, Western Australia, 2002). After the new introductions to Western Australia ceased in the 1970s, rainbow trout at PFRC have been self-sustaining for nearly half a century. For the present experiments, the brood stock had been reared in shallow and circular concrete ponds (0.7 m deep, 7.5 m diameter). These ponds were aerated and received fresh creek water from Big Brook Dam. The temperature of the ponds reflected water temperature in the dam, which increases in summer as a result of insolation and a greatly reduced water level. All fish in this study were bred on 10 and 11 June 2013 using 25 males and 25 females that were dry stripped. Milt from five males was pooled and used to fertilize eggs from five females to generate a group with five half-sibling families. In total, we produced five such family groups (Table 3), each of which contained five half-sibling families. Fertilized eggs were incubated in boxes made from rigid fly wire (length×width×height: 120×50×35 mm). Prior to hatching, eggs were removed from the incubator and placed into 25 hatching/larval rearing boxes (340×270×200 mm) made from a perforated (1.0 mm diameter holes) stainless steel sheet. These boxes were suspended (half submerged) in separate 200 l cones, with a common water supply in a recirculating system. Animals were raised in thermally controlled water (15.0±0.4°C) with 12 h:12 h light:dark cycles. The system received an input of fresh creek water at the rate of 6 l min^{-1}. Two biological filters (1 mm) filtered excess food and waste and were cleaned daily. Water nitrite and ammonia levels were checked twice a week using an aquarium water quality test kit (Tetratest, Tetra, Germany). After hatching, 200 fish were released from hatching boxes into the cones. Fish were fed twice a day with commercial freshwater trout diet (Skretting, Cambridge, Tasmania, Australia), but not on the day before an experiment, which resulted in a 48 h fast for the experimental fish. The following experiments were conducted between November 2013 and January 2014. Each fish was subjected to only one of the following tests.

CT_{max}

Each CT_{max} measurement used a batch of 15 fish from each half-sibling family (75 fish from each family group, Table 3). Fish were quickly dip netted into a 40 l plastic container with aerated and temperature-controlled circulating water (F32 – MD, Julabo Labortechnik GmbH, Seelbach, BW, Germany). Fish were given 1 h to recover from handling stress at 15°C. Water temperature was then increased at 0.3°C min^{-1} to 22°C and at 0.1°C min^{-1} thereafter (Beitinger et al., 2000) until CT_{max} was reached, defined as the temperature when an individual fish lost equilibrium continuously for 10 s. Subsequently, fish were immediately removed from the test chamber and put into a separate bucket at 15°C to recover. After the experiment, fish were anaesthetized in 80 mg l^{-1} MS222 buffered with 160 mg l^{-1} $NaHCO_3$ and body size was measured. Water temperature was measured using a digital thermometer (precision: 0.1°C; Total-Range Thermometer, Fisher Scientific, Nepean, ON, Canada). Post-testing fish mortality was 3.2%. All the thermometers were calibrated in an ice water bath (0°C) and 50°C water bath. Two mercury thermometers were used to validate the water bath temperatures.

Aerobic scope

RMR and MMR were measured to calculate aerobic scope (MMR–RMR). Given the limited amount of individual variation observed with the CT_{max} measurements, aerobic scope was only measured in the three family groups

Table 5. Comparison of critical thermal maximum (CT_{max}) for various populations of rainbow trout under different acclimation temperatures (T_{acclim})

T_{acclim} (°C)	CT_{max} (°C)	Heating rate (°C min^{-1})	Mass (g)	Length (cm)	Strain source	References
8	26.9±0.12	0.1		11.0–18.0	Washington	Becker and Wolford, 1980
9.8	27.9±0.05	0.3		15.3±0.3	Pennsylvania	Carline and Machung, 2001
10	28.5±0.28	0.02		15.0–20.0	Arizona	Lee and Rinne, 1980
10	28.0±0.12	0.3	~15.0	~10.0	Missouri	Currie et al., 1998
10	27.7±0.08	0.3	12.9±0.6		California	Myrick and Cech, 2000
11	~27.5	0.3	8.0±1.6		California	Myrick and Cech, 2005
11*	29.0±0.05	0.3	2.4±0.5		British Columbia	Scott et al., 2014
13	27.9±0.14	0.33	21.8±0.4		Ontario	Leblanc et al., 2011
14	28.5±0.11	0.3	13.8±0.8		California	Myrick and Cech, 2000
14	29.4±0.1	0.033‡	41.0–140.0		Oregon	Rodnick et al., 2004
15	29.4±0.08	0.3				Strange et al., 1993
15	29.1±0.09	0.3	~15.0	~10.0	Missouri	Currie et al., 1998
15	27.7±0.03	0.0014§	89.9±5.4	19.9±3.6	North Carolina	Galbreath et al., 2006
15	~28.4	0.3	9.3±2.0		California	Myrick and Cech, 2005
15	~29.7	0.083¶			Miyazaki, Japan	Ineno et al., 2005
15	29.0±0.02	0.3/0.1	30.2±0.3	13.0±0.4	Western Australia	Present study
18	~31.2	0.3		4.1–20	Arizona	Recsetar et al., 2012
19	~29.6	0.3	14.3±2.9		California	Myrick and Cech, 2005
19	29.9±0.17	0.3	11.8±0.7		California	Myrick and Cech, 2000
20	29.4±0.19	0.02		15–20	Arizona	Lee and Rinne, 1980
20	29.8±0.12	0.3	~2.0	~4.1	Missouri	Currie et al., 1998
20	~30.4	0.083¶			Miyazaki, Japan	Ineno et al., 2005
22	30.9±0.13	0.3	9.3±1.0		California	Myrick and Cech, 2000
25	31.8±0.1	0.3	6.1±0.6		California	Myrick and Cech, 2000

*Fish held at 10–12°C; ‡temperature was increased at 2°C h^{-1}; §temperature was increased at 2°C day^{-1}; ¶temperature was increased at 5°C h^{-1}.
'~' indicates an estimated or calculated mean by the original author(s). Values separated by '–' represent the range of the traits. Other values were given as means ±s.e.m. In all studies, loss of equilibrium was used as the indication of CT_{max}. The association between acclimation temperature and CT_{max} is analysed in Fig. 5.

The Journal of Experimental Biology (2015) 218, 803-812 doi:10.1242/jeb.113993

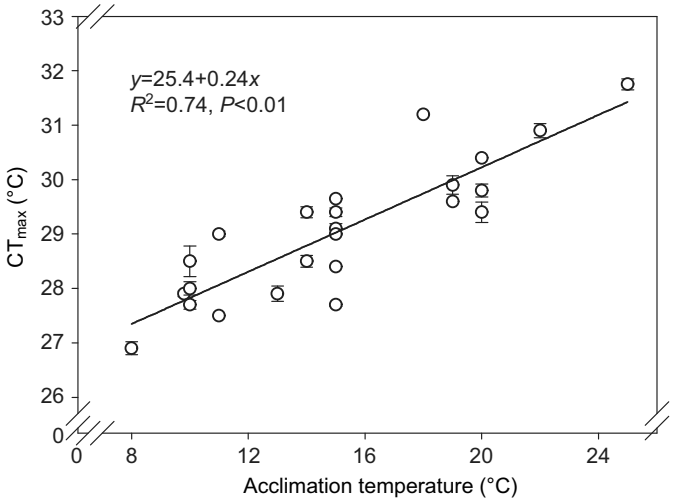

Fig. 5. Simple linear regression analysis of CT_{max} and acclimation temperature using data from various rainbow trout populations.

(1, 2 and 5) that showed the greatest collective variability. Aerobic scope measurements were completed in 18 days, with daily measurements of six fish (two from each of the three family groups) (Table 3). As a result, fish growth during the course of the measurements was not a significant factor (see below). RMR and MMR were measured on individual fish (N=6 per group) at each of six test temperatures (8, 12, 15, 18, 21 and 25°C) as follows. Two weeks before the experiment, six fish were randomly selected and placed into separate floating boxes made from rigid plastic mesh (diameter 15 cm, height 20 cm). After a 48 h fasting period, fish were individually transferred from the holding tank to one of six custom-made, intermittent-flow respirometers (~500 ml). Water temperature (15°C) in the respirometers was controlled with in-line, recirculating chillers (F32 – MD, Julabo Labortechnik GmbH). Fish were left overnight for a minimum of 12 h to adjust to the respirometer. Water temperature was increased at a rate of 2°C h^{-1} to the desired test temperature. Then, the fish were held at the test temperature for 1 h before RMR was measured by closing the respirometer and recording the depletion of oxygen from the water with a fibre optic oxygen meter (Firesting O_2, PyroScience GmbH, Aachen, Germany). Water oxygen content was allowed to decrease by no more than 25% (i.e. >75% saturation), which generally took less than 10 min. After the RMR measurement, one fish at a time was removed from the respirometer and placed in a 50 cm diameter circular tank containing aerated water at the test temperature, where it was exercised to exhaustion over a roughly 5 min period by combining hand chasing and gentle tail pinches until the fish became unresponsive to touch. The fish was immediately returned to the respirometer (<1 min) and oxygen consumption was measured for 3 min. MMR was determined by taking the maximum rate of oxygen removal for a minimum of 1.5 min during this recording period, which typically occurred immediately after the fish was returned to the respirometer. After the MMR measurement, fish were weighed and returned to the holding tank. Each fish was given a minimum of 3 days to recover between temperature tests. At the time of the experiments, the fish weighed a mean (±s.e.m) of 31.8±0.9 g (N=18 in total). Body mass did not differ significantly among family groups. Body mass increased by an average of 7.9% during the 18 days of testing despite the food restriction, handling and tests.

RMR and MMR were calculated as:

$$\dot{V}_{O_2} = ([O_2]t_0 - [O_2]t_1) \times V/(T \times M_b), \quad (1)$$

where $\dot{V}_{O_2}$ is oxygen consumption rate (mg O_2 kg^{-1} h^{-1}); $[O_2]t_0$ is oxygen concentration at time t_0 (mg O_2 l^{-1}); $[O_2]t_1$ is oxygen concentration at time t_1 (mg O_2 l^{-1});and V is the respirometer volume minus the volume of the experimental animal (l), where the value of animal volume (l) was assumed to be the same as the value of animal mass (kg); T=t_1−t_0 (h); and M_b is the body mass of the experimental animal (kg).

$f_{H,max}$

$f_{H,max}$ was measured in individual anaesthetized fish, as originally developed by Casselman et al. (2012) and since then widely used in a variety of fish species (see Introduction). Briefly, each fish was anaesthetized (80 mg l^{-1} MS222 buffered with 160 mg l^{-1} $NaHCO_3$; Sigma-Aldrich, St Louis, MO, USA) and placed in the electrocardiogram (ECG) measuring system, where the gills were continuously supplied with aerated, temperature-controlled water containing a maintenance anaesthetic concentration (65 mg l^{-1} of MS222 and 130 mg l^{-1} $NaHCO_3$). Two silver electrodes (30 gauge, 5 cm in length) were placed to touch the ventral side of the body (the positive one in the middle of the pectoral fins, and the negative one 4 cm away from the positive electrode towards the pelvic fins) to capture the ECG signal. The electrodes were connected to an Animal Bio Amp (ADInstruments Inc., Bella Vista, NSW, Australia) that amplified (1000×) and filtered (60 Hz line filter; low-pass 30–50 Hz; high-pass 0.1–0.3 kHz) the ECG signal. The conditioned ECG signal was digitalized using a Powerlab 8/35 data acquisition system and analysed using Labchart software version 7 (ADInstruments Inc., Bella Vista, NSW, Australia). Anaesthetized fish were stabilized at the initial test temperature (15°C) for 1 h. An intraperitoneal injection of atropine sulphate (1.8 mg kg^{-1}, Sigma-Aldrich) blocked inhibitory vagal tonus to the heart and an isoproterenol injection (6 µg kg^{-1}, Sigma-Aldrich) stimulated cardiac adrenergic β-receptors. These injections resulted in a stable $f_{H,max}$. Then the water was heated in 1°C increments every 6 min, which was a sufficient equilibration time to reach a new stable level for heart rate. Acute warming continued until the heart developed an arrhythmia, at which point the experiment was terminated. The ventricle was immediately separated from the fish and weighed (Table 4).

Hb and Hct analysis

Subsequent to the completion of the above tests, a subsample of fish was removed from the remaining stock of group 1, 2, 3 and 5. Following acclimation at 15°C, five individuals from each group were randomly selected for determination of individual Hct and Hb levels. Fish were anaesthetized (80 mg l^{-1} MS222 buffered with 160 mg l^{-1} $NaHCO_3$; Sigma-Aldrich) and weighed, and blood samples were taken immediately using heparinized vacutainers. Hb was measured with a handheld haemoglobin analyzer (Hemacue 201+, Ängelholm, Sweden) using 0.1 ml of blood and Hct was measured using microhematocrit capillary tubes centrifuged at 10,000 ***g*** for 5 min.

Statistical analysis

All data analysis was performed using SigmaPlot version 11.0 (Systat Software Inc., San Jose, CA, USA) except where specified. Values are presented as means±s.e.m. Statistical significance for all analyses was set at α=0.05. Analysis of covariance (SPSS version 21.0, IBM Corp., Armonk, NY, USA) was used to test for differences in CT_{max} among family groups and account for the effect of body size. RMR, MMR and aerobic scope were determined for each individual fish at each of the six test temperatures. Differences in RMR, MMR and aerobic scope between family groups were tested using a two-way ANOVA with Holm–Sidak *post hoc* test. In addition, regression analysis was used to generate a best-fit curve for each of the three groups (polynomial quadratic function for RMR, and Gaussian three-parameter function for AS and MMR). The equations of these curves were used to generate the rate transition temperatures for aerobic scope: (1) T_{opt} was the temperature for the maximum aerobic scope; (2) T_{pej} was the temperature when aerobic scope was reduced to 90% of maximum; (3) T_{crit} was the temperature when aerobic scope was 10% of maximum. These rate transition temperatures were compared among family groups using a one-way ANOVA with a Holm–Sidak *post hoc* test (Table 1). In $f_{H,max}$ analysis, the rate transition temperatures (T_{AB}, the first Arrhenius breakpoint temperature; T_{QB}, the breakpoint temperature for Q_{10} of $f_{H,max}$; T_{max}, the temperature at which $f_{H,max}$ reached its maximum absolute value; T_{AR}, the arrhythmic temperature) were determined from the responses of individual fish and are reported as mean values in Table 2. T_{AB} analysis was performed according to Yeager and Ultsch (1989) and represents the intersection of two regression lines in an Arrhenius plot of $f_{H,max}$ versus test temperature. T_{QB} was arbitrarily set

as the temperature beyond which the incremental Q_{10} for $f_{H,max}$ fell below 1.6. For each individual, incremental Q_{10} values for $f_{H,max}$ during incremental warming were plotted against temperature (Fig. 4B). The last temperature where the Q_{10} was above 1.6 is defined as T_{QB}.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
A.P.F. conceived the experiment; Z.C., S.R.N., R.H.D. and A.P.F. designed the research; Z.C. and M.S. performed experiments; Z.C. and A.P.F. analysed the data and drafted the manuscript; Z.C., M.S., S.R.N., R.H.D. and A.P.F. revised and edited the manuscript; M.S., C.S.L. and A.R.C. bred the fish; A.R.C. cared for the fish.

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