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RESEARCH ARTICLE

Could thermal sensitivity of mitochondria determine species distribution in a changing climate?

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ABSTRACT

For many aquatic species, the upper thermal limit (T_{max}) and the heart failure temperature (T_{HF}) are only a few degrees away from the species' current environmental temperatures. While the mechanisms mediating temperature-induced heart failure (HF) remain unresolved, energy flow and/or oxygen supply disruptions to cardiac mitochondria may be impacted by heat stress. Recent work using a New Zealand wrasse (*Notolabrus celidotus*) found that ATP synthesis capacity of cardiac mitochondria collapses prior to T_{HF}. However, whether this effect is limited to one species from one thermal habitat remains unknown. The present study confirmed that cardiac mitochondrial dysfunction contributes to heat stress-induced HF in two additional wrasses that occupy cold temperate (*Notolabrus fucicola*) and tropical (*Thalassoma lunare*) habitats. With exposure to heat stress, *T. lunare* had the least scope to maintain heart function with increasing temperature. Heat-exposed fish of all species showed elevated plasma succinate, and the heart mitochondria from the cold temperate *N. fucicola* showed decreased phosphorylation efficiencies (depressed respiratory control ratio, RCR), cytochrome *c* oxidase (CCO) flux and electron transport system (ETS) flux. *In situ* assays conducted across a range of temperatures using naive tissues showed depressed complex II (CII) and CCO capacity, limited ETS reserve capacities and lowered efficiencies of pyruvate uptake in *T. lunare* and *N. celidotus*. Notably, alterations of mitochondrial function were detectable at saturating oxygen levels, indicating that cardiac mitochondrial insufficiency can occur prior to HF without oxygen limitation. Our data support the view that species distribution may be related to the thermal limits of mitochondrial stability and function, which will be important as oceans continue to warm.

KEY WORDS: Fish, Thermal limits, Cardiac mitochondria, Heart failure, Oxidative phosphorylation

INTRODUCTION

Critical environmental stressors can affect the dynamics and distribution of fish populations (Booth et al., 2011; Sunday et al., 2012). Currently, ocean temperatures are influenced by climate change and drive changes in the distribution of fish populations and species (Sunday et al., 2011). As ocean temperatures rise, the thermal limitation of fishes is indicated by a decreased capacity in aerobic performance (Pörtner and Knust, 2007). An understanding of the thermal tolerance windows for fishes has been called for so that the effects of ocean warming on temperature-related geographic distributions can be predicted (Pörtner, 2002). A comparative approach provides a powerful means to test the physiological mechanisms responsible for differences in thermal tolerances among related taxa with different thermal niches (Somero, 2010; Somero, 2011).

The thermal tolerances of fishes and other ectotherms appear to be reflected by the temperatures at which their hearts fail (T_{HF}), and therefore the heart is contended to be the most temperature-sensitive organ in fish (Pörtner and Farrell, 2008; Pörtner and Knust, 2007). The scope of thermal tolerance has also been proposed to result from limitations imposed by mitochondrial function and density (Pörtner, 2002). Alterations in mitochondrial function with temperature can contribute to trade-offs in energy budgets that in turn affect fish growth and fertility, and can ultimately influence population dynamics (Pörtner, 2002). However, some studies have indicated that the temperature at which liver and skeletal muscle mitochondrial respiration fail (T_{mt}) occurs above critical habitat temperatures (Pörtner et al., 2000; Pörtner, 2002; Somero et al., 1998; Weinstein and Somero, 1998). These studies, however, focused on maximal respiration capacities in terms of flux, with single electron inputs into respiratory chains, and did not use heart muscle or explore respirational efficiencies.

Recent work has demonstrated that T_{mt} can occur below the T_{HF} in heart muscle exposed to increasing environmental temperatures (Iftikar and Hickey, 2013). This work on the wrasse *Notolabrus celidotus* showed that, while increasing temperature elevated oxygen flux through respiring mitochondria, a greater fraction of the flux at high temperatures was to meet elevated inner mitochondrial membrane proton leak (proton leak represents non-phosphorylating respiration). This study was the first to directly measure ATP production simultaneously with respiration, and this declined at temperatures well below T_{HF}, indicating a loss of oxidative phosphorylation (OXP) system efficiency prior to T_{HF} (Iftikar and Hickey, 2013). The study also showed that mitochondrial coupling – traditionally measured as the respiratory control ratio (RCR), provides a reasonable measure of the coupling between the electron transport system (ETS) and OXP system. Mitochondria from fish acclimated to 17.5°C showed significant depressions of ATP synthesis at 25°C. Additionally, the non-OXP respiration fluxes with respiratory complex I substrates (Leak-I) had increased by 60% relative to Leak-I at 17.5°C, while the RCRs decreased to less than 4, such that Leak-I accounted for 25% of OXP (Iftikar and Hickey, 2013). Therefore, cardiac mitochondria had lost considerable efficiency prior to T_{HF}.

The aim of the present study was: (i) to determine whether heart T_{mt} occurs below T_{HF} in other wrasse species from habitats with different temperatures; and (ii) to explore differences in thermal responses among species. The family *Labridae* is a large group of wrasse species occupying reef habitats in both temperate and tropical waters (Cowman et al., 2009). Two species were investigated: the cold temperate *Notolabrus fucicola* (banded/purple

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List of symbols and abbreviations

ATP	adenosine-5′-triphosphate
CCO	cytochrome *c* oxidase
ETS	electron transport system
FCCP	carbonyl cyanide *p*-(trifluoro-methoxy)phenyl-hydrazone
HF	heart failure
NADH	nicotinamide adenine dinucleotide (reduced)
OXP	oxidative phosphorylation
RCR	respiratory control ratio
T_{HF}	temperature of heart arrhythmia/failure
T_{mt}	temperature of mitochondrial respiration failure
UCR	uncoupled control ratio

wrasse) (J. Richardson 1840), which is most abundant in southern coastal waters of the South Island of New Zealand (winter and summer temperatures of 9 and 13°C, respectively) (Denny and Schiel, 2001; Denny and Schiel, 2002), and the tropical *Thalassoma lunare* (lunar wrasse) (Linneaus 1758), which inhabits Indo-Pacific waters including North-Eastern Australian reefs (winter and summer temperatures of 25 and 28°C, respectively) (Ackerman, 2004; Randall et al., 1990). We also compared data from these two species with published data from *N. celidotus*, which inhabits temperate reefs of the North Island of New Zealand (winter and summer temperatures of 15 and 21°C, respectively) (Ayling and Cox, 1982).

We first tested the T_{HF} across all three wrasse species exposed to acute heat stress, and then tested the influence of heat stress on the mitochondria within permeabilised cardiac fibres from these fish (Fig. 1A,B). Mitochondrial function was then further tested at a range of temperatures across each species' thermal range. Lastly, the capacity of heart mitochondria to take up substrates with increasing temperature was investigated (Fig. 1A). These data were used to determine whether mitochondrial dysfunction contributes to HF in other related fish species, and therefore restrict species distribution.

RESULTS

Cardiac function with acute heat stress

The T_{HF} of cold-adapted *N. fucicola* was 21.7±0.3°C, while T_{HF} of the tropical wrasse *T. lunare* was much higher at 32.8±0.3°C (Fig. 2). The T_{HF} for *N. celidotus* falls between these values at 27.8±0.4°C (Iftikar and Hickey, 2013). The thermal scope, i.e. the temperature difference between T_{HF} and acclimation temperature ($T_{acclimation}$), for *N. fucicola* and *N. celidotus* was 8.7±0.3 and 9.1±0.4°C, respectively (Fig. 2, inset). In contrast, the thermal scope of heart function was limited to only 5.8±0.2°C in *T. lunare* (Fig. 2, inset).

Enzyme and metabolite profiles from heart and plasma

Citrate synthase (CS) activity was similar for heart tissues of all three wrasse species (Table 1), indicating similar mitochondrial volumes, assuming that CS is constant across species. The anaerobic glycolytic enzyme marker lactate dehydrogenase (LDH) increased activity with acute heat stress in *N. fucicola* and *N. celidotus*, but not

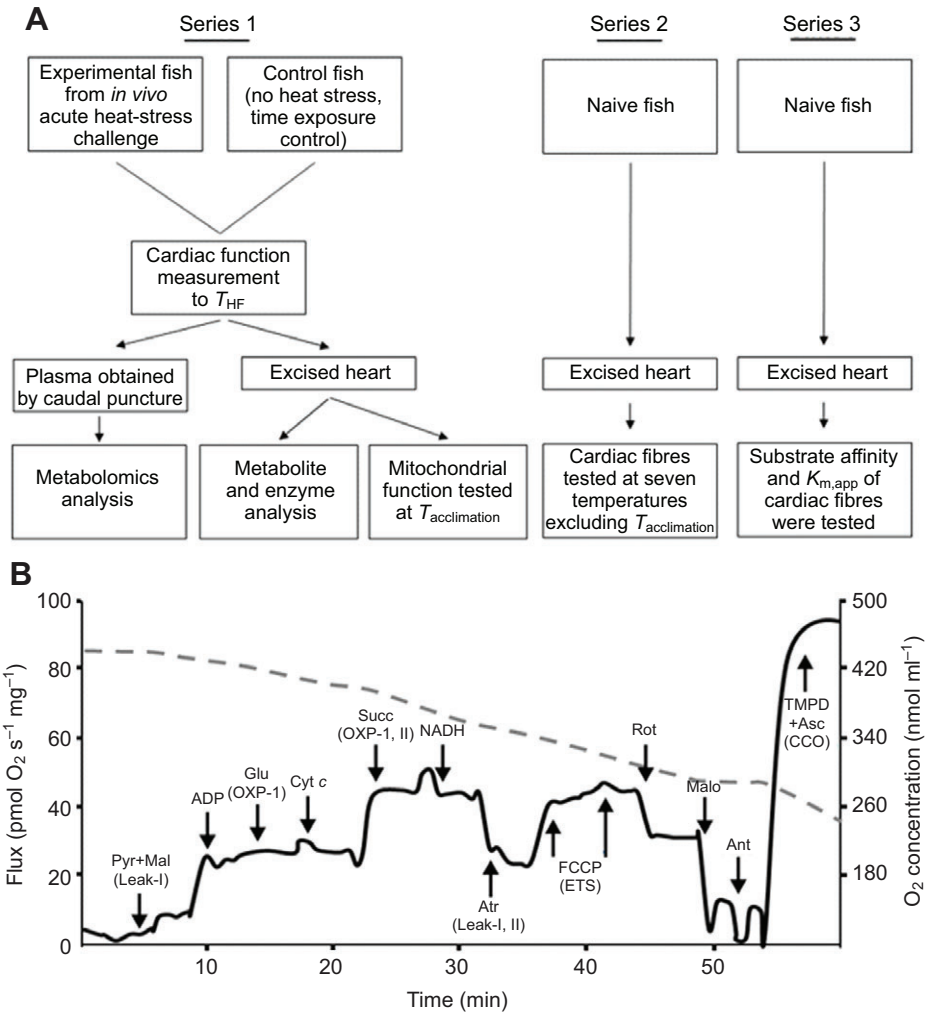

Fig. 1. Diagram of the experimental protocol for this study. (A) The experimental design illustrated as a flow chart depicting the three experimental series. (B) The SUIT assay (see Iftikar and Hickey, 2013), measuring mitochondrial flux (black line, left *y*-axis) and oxygen concentration (dashed grey line, right *y*-axis) over time. Titrations of mitochondrial substrates, poisons and inhibitors and their time of addition are shown with arrows, and the resulting respiratory state is given in parentheses. Pyr, pyruvate; Mal, malate; Glu, glutamate; Cyt *c*, cytochrome *c*; Succ, succinate; Atr, atractyloside; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenyl-hydrazone; Rot, rotenone; Malo, malonate; Ant, antimycin-a; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; Asc, ascorbate; Leak-I, state 2 respiration through complex I (CI) in the absence of ADP; OXP-I, state 3 respiration; OXP-I, II, parallel electron transport from CI and complex II (CII); Leak-I, II, leak respiration flux rate through CI and CII; ETS, maximal flux of the electron transport system; CCO, activity of complex IV (CIV), cytochrome *c* oxidase.

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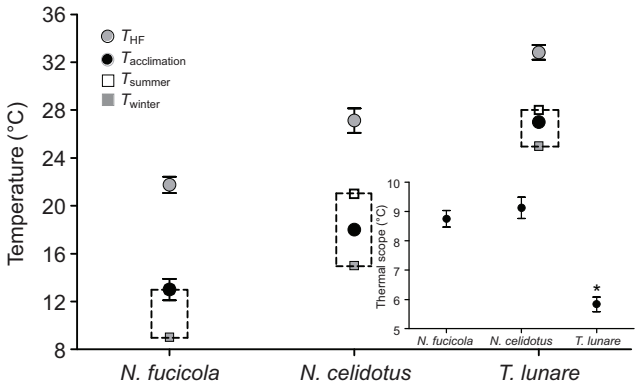

Fig. 2. Thermal tolerance profile for three fish species. T_{HF} is the mean (±s.d.) temperature of heart failure (HF) for each species (N=6). $T_{acclimation}$ is the mean (±s.d.) holding temperature of each species prior to experiments (N=6). T_{summer} and T_{winter} are the average temperatures experienced by each species in the summer and winter, respectively. The temperature range between T_{summer} and T_{winter} is demarcated with a dashed box. Inset, thermal scope is the difference between T_{HF} and $T_{acclimation}$ (means ± s.e.m., N=6). The asterisks indicate a significant difference (P≤0.05) in thermal scope from other species.

in *T. lunare* (Table 1). Acute temperature exposure significantly elevated lactate levels in heart tissue of both *N. fucicola* and *T. lunare*, but not in *N. celidotus* (P=0.07) (Iftikar and Hickey, 2013) (Table 1).

Two-dimensional principal component analysis (PCA) showed the largest separation between metabolic profiles of control and acutely heat-stressed *T. lunare* (Fig. 3) implying that heat stress impacted the intermediary metabolism of this species the most. In contrast, less separation was apparent in *N. fucicola* relative to *N. celidotus* and *T. lunare* (Fig. 3). When metabolite changes were compared across species, lactate accumulation increased in heat-stressed *N. celidotus* and *T. lunare* plasma but not in *N. fucicola* (Table 2). Exposure to acute heat stress also induced significant changes in some TCA cycle intermediates in the blood. Succinate was elevated in all three species, while citrate and cis-aconitate accumulation was elevated only in *T. lunare* (Table 2).

Mitochondrial function

Series 1: *in vivo* acute heat-stress exposure

Acute heat stress lowered RCR-I values in heat-exposed fish (Fig. 4A). OXP-I, II respiration (state III) was not impacted by acute heat stress in any of the three species (Fig. 4B) but acutely heat-stressed *N. fucicola* had lower uncoupled (ETS) and cytochrome *c* oxidase (CCO) fluxes relative to control fish (Fig. 4C,D).

Series 2: impact of *in situ* increase in assay temperature

Although all species increased leak-I flux with increasing temperature, *T. lunare* showed the greatest elevation in flux

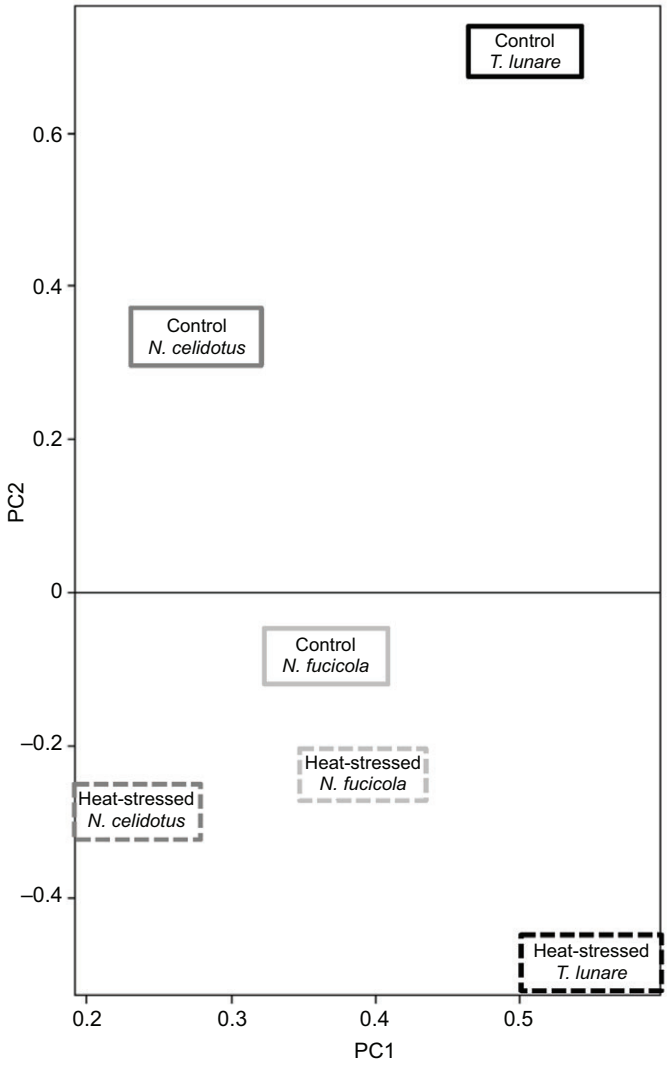

Fig. 3. Principal component analysis (PCA) projection of metabolite profiles for control and heat-stressed fish. Data for *N. fucicola*, *N. celidotus* (Iftikar and Hickey, 2013) and *T. lunare* were projected onto a 2D space and metabolic profiles were generated from mean metabolites that had statistically significant (P≤0.05) accumulation (N=6 per treatment per species).

(Fig. 5A). *Notolabrus fucicola* had the least increase in OXP-I, while *N. celidotus* and *T. lunare* showed similar increases (Fig. 5B). *Notolabrus celidotus* had the largest elevation in OXP-I, II flux as assay temperatures increased (Fig. 5C) (Iftikar and Hickey, 2013). Similar to OXP-I, *N. fucicola* had the highest OXP-I, II rates for a given assay temperature compared with *N. celidotus* and *T. lunare*. Arrhenius plots for RCRs indicated different thermal relationships

Table 1. Metabolite concentration and enzyme activity in heart tissue of *Notolabrus fucicola*, *Notolabrus celidotus* and *Thalassoma lunare*

	N. fucicola		*N. celidotus*		*T. lunare*	
	Control	Experimental	Control	Experimental	Control	Experimental
CS (μmol min^{-1} mg^{-1})	80.26±8.14		80.38±20.88		126.27±43.76	
Lactate (μmol g^{-1})	96.64±14.61	142.29±16.28*	106.61±18.46	142.39±10.44‡	97.19±18.05	142.29±16.28*
LDH (μmol min^{-1} mg^{-1})	1086.08±98.42	1382.46±64.10*	1050.11±92.75	1682.83±168.00*	1043.17±57.46	1382.46±64.10*

CS, citrate synthase; LDH, lactate dehydrogenase.
Data are means ± s.e.m. (N=6). *Notolabrus celidotus* data are from a previous study (Iftikar and Hickey, 2013).
The asterisks indicate significant differences between control and acutely heat-stressed fishes (P≤0.05). ‡P=0.07.

Table 2. Accumulation of glycolytic and TCA cycle intermediates in control and acutely heat-stressed fish plasma for *N. fucicola*, *N. celidotus* and *T. lunare*

	N. fucicola		*N. celidotus*		*T. lunare*	
	Increase/decrease	Fold-change	Increase/decrease	Fold-change	Increase/decrease	Fold-change
Glycolytic intermediates						
Lactate	↑	1.22	↑	1.55*	↑	2.47*
TCA cycle intermediates						
Succinate	↑	1.76*	↑	1.35*	↑	1.93*
Citrate	↓	0.98	↑	1.55	↑	2.62*
Cis-aconitate	↓	0.86	↑	1.58	↑	2.98*

N=6. *Notolabrus celidotus* data are from a previous study (Iftikar and Hickey, 2013).
Arrows indicate the increase/decrease in intermediate accumulation in the plasma from heat-stressed fish compared with control fish, and the fold-change is given in the adjacent column. The asterisks denote that accumulation is significant in plasma from heat-stressed fish ($P \leq 0.05$).

for RCR-II versus RCR-I because absolute break temperatures (ABTs) for RCR-II were found in two species, *N. celidotus* (21.8±1.7°C) and *T. lunare* (28.5±1.3°C) (Fig. 5D). These ABTs were close to $T_{acclimation}$ and below their respective T_{HF}. Only *N. celidotus* showed an ABT for RCR-I at 24.6±2.8°C below the T_{HF} (Fig. 5D).

Arrhenius plots for complex I (CI) respiration showed a greater response to increasing temperature compared with complex II (CII) respiration in *N. celidotus* and *T. lunare* (Fig. 6B,C), as reflected by steeper slopes, significant in *T. lunare* (−7.7±1.7 CI versus −3.1±1.3 CII) (Fig. 6C). In comparison, CI respiration of *N. fucicola* (−4.0±0.7) responded less to temperature compared with CII respiration (−5.1±1.1) (Fig. 6A). However, CCO respiration had the greatest temperature-driven response in *N. fucicola* (−7.9±0.5, Fig. 6A) relative to *N. celidotus* (−6.0±0.8, Fig. 6B) and *T. lunare* (−5.3±0.9, Fig. 6C).

Notolabrus fucicola had the greatest fractional capacity to chemically uncouple with the uncoupling agent FCCP, and increased flux by ~57% at 25°C relative to rates at $T_{acclimation}$ (Fig. 7A). However, this required a higher FCCP concentration (1.58±0.15 μmol l^{-1}; Fig. 7B, inset). Additionally, *N. fucicola* maintained uncoupled control ratios (UCRs) above a value of 1 at all assay temperatures (Fig. 7B), indicating a greater ETS reserve capacity or stability compared with OXP. *Notolabrus celidotus* had also maximised the ETS rate by 32.5°C (Fig. 7A), although ETS rate was more sensitive to FCCP, requiring only half (0.81±0.07 μmol l^{-1}) the concentration used by *N. fucicola* (Fig. 7B, inset). *Thalassoma lunare* had the lowest uncoupling capacity, requiring on average 0.5 μmol l^{-1} FCCP (Fig. 7B, inset) to achieve a maximal ~45% uncoupling compared with $T_{acclimation}$ rates (Fig. 7A). Moreover, UCR values of *N. celidotus* and *T. lunare* were significantly depressed below their T_{HF} at 25 and 27.5°C, respectively (Fig. 7B). In *T. lunare*, from 27.5°C upwards, UCRs were equal to or below a value of 1, indicating that the total ETS reserve capacity was required to support maximal OXP.

Series 3: CI substrate affinity with increasing assay temperature

OXP respiration of *N. celidotus* and *T. lunare* cardiac mitochondria preferred pyruvate as its main CI substrate, compared with cardiac mitochondria of *N. fucicola*, which preferred malate as the main CI substrate. The apparent K_m ($K_{m,app}$) for malate in *N. fucicola* did not change with increasing temperature (Fig. 8A). In *N. celidotus*, the $K_{m,app}$ for pyruvate remained unchanged up to 30°C and then increased at 32.5°C by 19-fold (Iftikar and Hickey, 2013). Similarly, in *T. lunare*, $K_{m,app}$ pyruvate was 3-fold higher at 37.5°C (Fig. 8A). V_{max} rates for *N. fucicola* were unaffected by temperature, while they increased with temperature for *N. celidotus* (Fig. 8B). *Thalassoma lunare* increased V_{max} up to 32.5°C, and then decreased at 37.5°C to

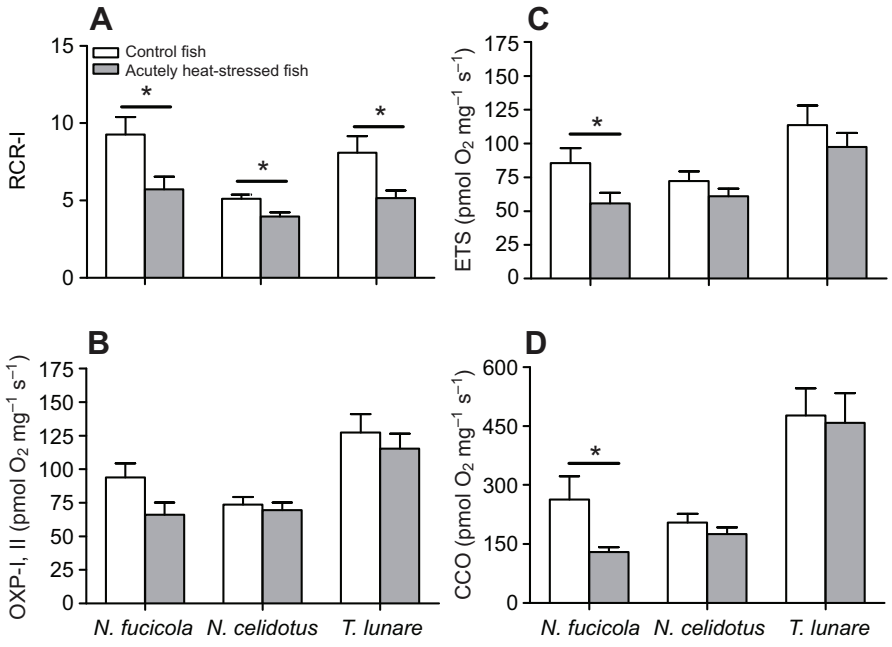

Fig. 4. Cardiac mitochondrial function of three fish species exposed to *in vivo* acute heat stress. (A) Respiratory control ratio (RCR-I) determined as OXP-I/Leak-I. (B) OXP-I, II rate. (C) Uncoupled (ETS) rate. (D) Cytochrome *c* oxidase (CCO) rate. Data are means ± s.e.m. for control (*N*=6) and heat-stressed fish (*N*=6). The asterisks indicate a significant difference ($P \leq 0.05$) between control and heat-stressed fish for each species.

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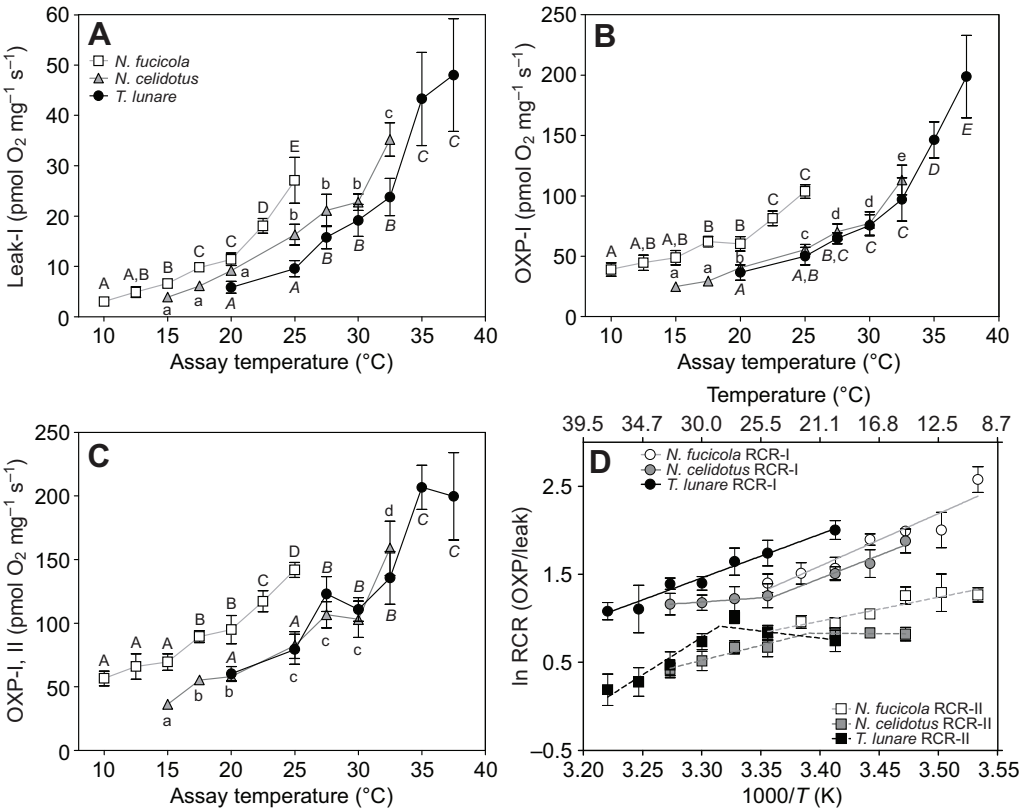

Fig. 5. Cardiac mitochondrial function of three fish species exposed to increasing *in situ* assay temperatures. (A) Leak-I rate. (B) OXP-I rate. (C) OXP-I, II rate. (D) Arrhenius plot depicting respiratory control ratios; RCR-I determined as OXP-I/leak-I and RCR-II determined as OXP-I, II/leak-I, II. Solid lines and dashed lines represent linear/non-linear regression fitted for RCR-I and RCR-II, respectively. For all mean (±s.e.m.) rates and percentages (N=6) within a species, letters of the same case and style are not significantly different (P≥0.05) from one another.

initial rates. Apparent substrate oxidation efficiencies, as determined by the $V_{max}/K_{m,app}$ ratio (as a proxy for the traditional measure of enzyme efficiency of k_{cat}/K_m), were depressed for *N. celidotus* and *T. lunare* at their maximal assay temperatures (Fig. 8C), but predicted optimal temperatures for substrate oxidation were below their T_{HF} at 26.6±1.0 and 30.6±0.7°C, respectively. $V_{max}/K_{m,app}$ ratios remained unaffected by heat stress in *N. fucicola* (Fig. 8C) but it should be noted that fibres of this species were oxidising malate.

DISCUSSION

The present study indicates that while mechanisms differ, some form of cardiac mitochondrial dysfunction occurs below T_{HF}, and that species from narrow-ranging thermal habitats were more susceptible to heat stress. The tropical *T. lunare* was most greatly impacted by acute heat stress, with a limited ability to increase cardiac scope (Fig. 2) and showed the greatest shifts in plasma metabolite profiles (Fig. 3). The TCA cycle intermediate succinate was most elevated in heat-stressed *T. lunare*, indicating a more severe mitochondrial insufficiency in this species (Table 2). RCR-II also altered substantially just above $T_{acclimation}$ for *T. lunare* and *N. celidotus*, indicating that OXP was compromised below T_{HF} (Fig. 5D). Furthermore, the tropical *T. lunare* showed limited CII activity (Fig. 6C) and a decreasing ETS reserve capacity with rising temperature, depicted by the inability of their cardiac mitochondria to uncouple with increasing assay temperatures (Fig. 7A,B). Cardiac mitochondria from acutely *in vivo* heat-stressed *N. fucicola* had

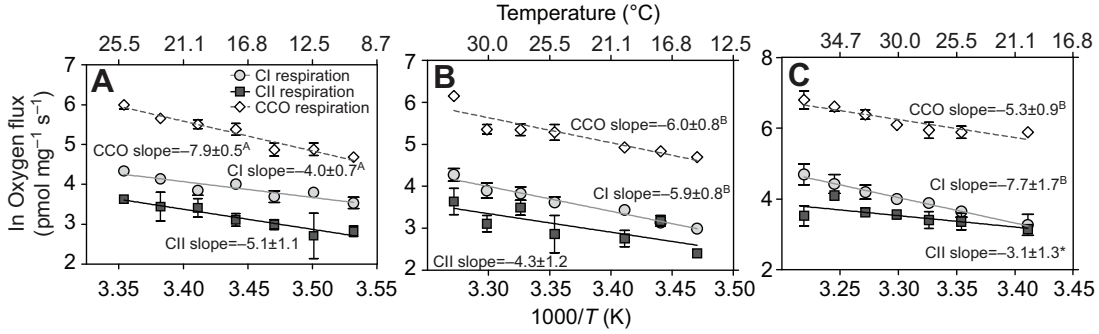

Fig. 6. Arrhenius plots depicting respiration of mitochondrial complexes. CI respiration (calculated as OXP-I–Leak-I), CII respiration (calculated as OXP-I, II–OXP-I) and CCO respiration (cytochrome *c* oxidase, CIV) of permeabilised cardiac fibres from (A) *N. fucicola*, (B) *N. celidotus* (Iftikar and Hickey, 2013) and (C) *T. lunare*. Linear regression was fitted to data and respective slopes (±s.e.m.) are given within the graph. Statistical analysis comparing mean slopes for CI and CCO respiration across fish species show significant differences (P≤0.05) with uppercase letters. Mean slopes for CII respiration across fish species showed no significant difference. The asterisk indicates a significant difference (P≤0.05) between the mean slopes for CI and CII respiration within *T. lunare*. The activation energy (E_a) for CCO respiration was calculated as 18.9±1.1 kJ mol^{-1} for *N. fucicola*, 14.5±2.0 kJ mol^{-1} for *N. celidotus* and 13.2±2.2 kJ mol^{-1} for *T. lunare*.

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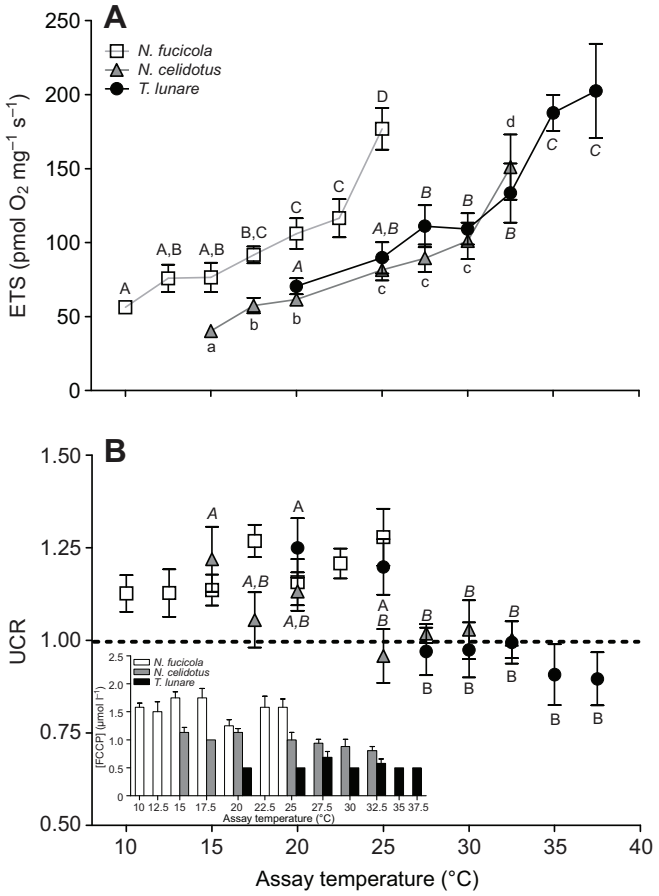

Fig. 7. Mitochondrial respiration of uncoupled permeabilised cardiac fibres from three fish species exposed to increasing *in situ* assay temperature. (A) ETS rate. (B) Uncoupled control ratio (UCR) determined as ETS/OXP-I, II. The dotted line at 1 represents when ETS reserve capacity=OXP. Inset, FCCP concentration required to uncouple cardiac mitochondria. For all mean (±s.e.m.) rates and ratios (N=6) within a species, letters of the same case and style are not significantly different (P≥0.05) from one another.

depressed RCR-I and ETS and CCO fluxes, relative to control fish (Fig. 4A,C,D). In addition, when cardiac fibres from *N. fucicola* were assayed across temperatures (*in situ*), CI respiration showed a diminished response (Fig. 6A). Overall, this study confirmed that T_{mt} occurs below T_{HF} for *N. fucicola* and *T. lunare* that inhabit relatively stable thermal environments.

The impact of acute heat stress on the wrasse heart

The tropical wrasse *T. lunare* had the lowest scope to increase heart rate relative to $T_{acclimation}$ (Fig. 2). Such a narrow thermal scope for cardiac function has been established previously in warm-adapted ectotherms (Stillman and Somero, 1996; Stillman and Somero, 2000; Vernberg and Tashian, 1959) where the ABT for heart rate in warm-adapted intertidal porcelain crabs was close to the maximal microhabitat temperatures (Stillman and Somero, 1996). Therefore, despite higher absolute thermal tolerances, under heat stress warm-adapted species have lower thermal scopes compared with temperate relatives (Stillman and Somero, 2000). PCA analysis also confirmed that *in vivo* heat stress caused the greatest change in the plasma metabolite profile of *T. lunare* relative to the colder wrasse species (Fig. 3). Specifically, the increase in all TCA cycle intermediates, in particular succinate in acutely heated *T. lunare* plasma indicates that this species must increase its anaerobic dependence with heat stress (Table 2). The plasma membrane is considered to be impermeable to succinate (Stadlmann et al., 2006). The accumulation of succinate in fish blood probably indicated mitochondrial disruption at CII, also known as succinate dehydrogenase (Grieshaber et al., 1994). Data presented here reveal that the tropical *T. lunare* is closer to HF and is more metabolically limited than its temperate equivalents *N. fucicola* and *N. celidotus*, and as such would be the most susceptible of the three species to rising temperatures given predictions of global warming.

The impact of heat stress on mitochondrial bioenergetics

The RCR provides an indication of mitochondrial efficiency, as the constituents used to calculate this ratio are dependent on the components of OXP being intact (Brand and Nicholls, 2011). It is known that mild heat stress *in vitro* will depress OXP coupling, and that extreme heat stress will cause irreversible changes in mitochondrial inner membrane integrity in the mammalian heart (Žūkienė et al., 2007). This acts through increased inner mitochondrial membrane proton leakage, and therefore decreased OXP efficiency. Here, it resulted in a lowering of RCR-I in all three heat-stressed species when assayed *in vitro* (Fig. 4A).

When tested at various temperatures, RCR-I was most affected in *N. celidotus* (Fig. 5D). Arrhenius plot analysis revealed a breakpoint in the RCR-I for *N. celidotus* at temperatures below T_{HF}, suggesting a lowered OXP efficiency in this species (Hilton et al., 2010). High temperatures are known to increase the inner membrane proton leak while decreasing membrane potential, often coinciding with escalating oxygen flux that does not contribute to ATP production (Žūkienė et al., 2007). This depresses OXP capacity in rat heart mitochondria (Žūkienė et al., 2007) and substantially depresses ATP

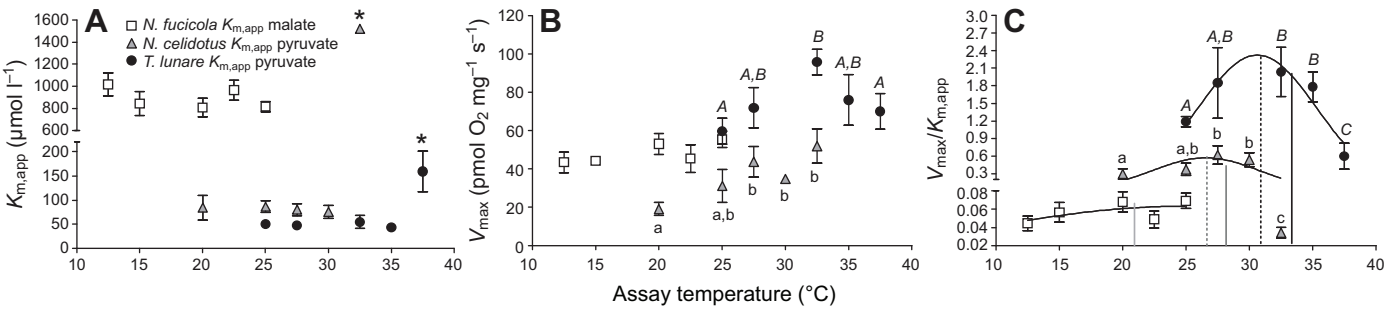

Fig. 8. Substrate kinetics for three fish species with increasing assay temperature. (A) The apparent affinity ($K_{m,app}$) to primary CI substrate specified in the key. The asterisks indicate that within a species the $K_{m,app}$ is significantly different (P≤0.05). (B) V_{max}. (C) $K_{m,app}/V_{max}$ ratio indicative of enzyme efficiency. Solid and dashed lines represent T_{HF} and optimal enzyme efficiency temperature determined by non-linear regression (parabola), respectively. For all rates and ratios (means ± s.e.m., N=6) within a species, letters of the same case and style are not significantly (P≥0.05) different from one another.

production by 25°C below T_{HF} in *N. celidotus* (Iftikar and Hickey, 2013).

Increasing assay temperature impacted the RCR-II of *N. celidotus* and *T. lunare* the most (Fig. 5D). Arrhenius breakpoints occurred at 21.8±1.7°C for *N. celidotus* and 28.5±1.3°C for *T. lunare*, which was just above their respective $T_{acclimation}$. As the RCR-II is determined from OXP-I, II, it putatively more closely reflects mitochondrial OXP and its efficiencies *in vivo*. Part of CII is succinate dehydrogenase, which is reported to be sensitive to heat stress in ectotherm mitochondria (O'Brien et al., 1991). This perhaps explains the more prominent inflexion in the RCR-II and decreased responsiveness in CII flux with increasing temperature in *N. celidotus* and significantly so in *T. lunare* relative to CI fluxes (Fig. 6B,C). This may also explain the accumulation of plasma succinate, as inadequate succinate dehydrogenase capacity will promote succinate release into the blood (Table 2), indicating a relative insufficiency of CII in *T. lunare* at temperatures prior to HF.

CCO had a greater overall flux, or excess capacity, compared with CI and CII respiration in all three species (Fig. 6A–C), and this is common for ectotherms (Blier and Lemieux, 2001; Dahlhoff and Somero, 1993). CCO is an indicator of maximal aerobic capacity and the activity of CCO in ectotherms is typically highest in the heart because of its high metabolic demands (Ludwig et al., 2001). While warm acclimation has been shown to increase CCO activity in fish hearts (Cai and Adelman, 1990; Foster et al., 1993), the thermal sensitivity of CCO was greatest in the colder *N. fucicola* (Fig. 6A) compared with the tropical *T. lunare* (Fig. 6C). The decreased thermal response of *T. lunare* CCO relative to *N. fucicola* and *N. celidotus* CCO may limit ETS flux as temperatures increase. This may also explain the greater overall CCO capacities in the tropical species.

Both *N. fucicola* and *N. celidotus* showed a greater capacity to uncouple mitochondria with increasing assay temperature (Fig. 7A). The cold-adapted *N. fucicola* also needed higher FCCP concentrations for similar assay temperatures to *N. celidotus* (Fig. 7B, inset), indicating a tightly coupled ETS in *N. fucicola*, or differences in FCCP mobility and inner membrane composition (Gnaiger et al., 2000). This was also reflected in the UCR observed for *N. fucicola*, which remained unaffected as assay temperatures increased (Fig. 7B). In comparison, both *N. celidotus* and *T. lunare* had significantly depressed UCRs at temperatures well below their T_{HF}, indicating either less coupled OXP systems or less ETS reserve capacity. *Thalassoma lunare* in particular had UCRs at or below the value of 1 (Fig. 7B), signifying a loss in ETS reserve capacity. This also indicates a loss of capacity to generate mitochondrial membrane potentials with increasing temperature. We note that uncouplers such as FCCP can depress ETS respiration in fish heart mitochondria (Hilton et al., 2010). This may result from effects on the electrogenic importation of substrates (e.g. pyruvate, which requires a membrane potential).

High mitochondrial respirational flux in most animals is fuelled by pyruvate followed by glutamate (Johnston et al., 1994; Lemieux et al., 2008; Moyes et al., 1990). The preferential use of malate for cardiac mitochondrial respiration in *N. fucicola* can perhaps be attributed to an abundance of malic enzyme activity (Fig. 8A). Malate supports cardiac mitochondrial respiration in cold-adapted teleosts (Skorkowski et al., 1984; Skorkowski et al., 1985) and flux could be attributed to mitochondrial malic enzyme (Skorkowski, 1988), which converts malate to pyruvate. Therefore, pyruvate oxidation is still potentially driving OXP-I flux, as it can be derived from malic enzyme. However, heat stress did not impact malate uptake by heart mitochondria from *N. fucicola* for all assay temperatures measured (Fig. 8A), indicating that the malate–aspartate shuttle and subsequent downstream enzymes are thermostable in this temperature range. In contrast, high temperatures substantially decreased the affinity for pyruvate uptake in *N. celidotus* and *T. lunare* at their respective maximal assay temperatures of 32.5 and 37.5°C (Fig. 8A) (Iftikar and Hickey, 2013). Although these temperature maxima are above the T_{HF} for both species, the peak temperature for enzyme efficiency derived from the ratio of $V_{max}/K_{m,app}$ for pyruvate was below T_{HF} at 26.6±1.0 and 30.6±0.7°C, respectively (Fig. 8C). Subsequently, substrate transport or turnover efficiency is lost prior to HF in *N. celidotus* and *T. lunare*.

Is habitat a limiting factor in a changing climate?

The upper thermal limits on aerobic scope were postulated to govern fish species distribution, as the aerobic scope of fishes declines with increasing temperature (Pörtner and Farrell, 2008; Pörtner and Knust, 2007). It has been contended that this loss of aerobic scope coincides with apparent decreases in tissue oxygen supplies and T_{HF} (Farrell et al., 2009; Pörtner and Farrell, 2008; Pörtner and Peck, 2010; Wang and Overgaard, 2007). However, recent reappraisals indicate that thermal limitations on aerobic scope occur at temperatures above those constraining other crucial parameters, such as fish growth, reproduction and locomotion (Clark et al., 2013; Gräns et al., 2014; Healy and Schulte, 2012). Moreover, oxygen supply may still be adequate where these parameters fail, and potentially at and above temperatures where aerobic scope fails (Gräns et al., 2014; Iftikar and Hickey, 2013).

Our work shows that while mechanisms vary among species, cardiac mitochondrial function is likely to also be disrupted at temperatures below T_{HF} for these wrasse species, and may therefore contribute significantly to each species' thermal habitat range. The cardiac mitochondria of the cold temperate *N. fucicola* appears to be less stable under *in vivo* thermal stress, while cardiac mitochondria of temperate *N. celidotus* and tropical *T. lunare* showed rapid losses in OXP efficiencies (RCR-I, II) above $T_{acclimation}$. *Thalassoma lunare* also has the narrowest thermal window for heart function, with decreased ETS reserve capacity, and a lowered mitochondrial substrate affinity that coincides with T_{HF}. Additionally, CII and CCO appear to be most limited in terms of thermal plasticity in *T. lunare*. Importantly, these conditions manifest at saturating oxygen concentrations, indicating that cardiac mitochondria can become impaired without invoking oxygen limitation. Our data conclude that heat stress can mediate cardiac mitochondrial insufficiency in *N. fucicola*, *N. celidotus* and *T. lunare*, and therefore cardiac mitochondria can provide insight into species' thermal limits. Understanding mitochondrial function, or dysfunction in ectotherms such as fish, still requires study across a greater range of species to better understand the potential ramifications of climate change.

MATERIALS AND METHODS

Experimental animals

Notolabrus fucicola were collected by hook and line and held at the Portobello Marine Laboratory (University of Otago) on the Otago Peninsula of the South Island of New Zealand. Fish were housed at ambient temperatures (13.0±1.0°C) for 4 weeks. *Notolabrus celidotus* were collected and held as described previously (Iftikar and Hickey, 2013) at 18.0±0.5°C for 4 weeks. *Thalassoma lunare* were netted around Flinders reef, QLD, Australia, and held at Moreton Bay Research Station (University of Queensland) on North Stradbroke Island. Fish were maintained at 27.0±0.5°C for 4 weeks. All three species were fed daily but fasted for 24 h before experimentation. All experiments were conducted according to the guidelines of the Universities of Otago, Auckland and Griffith animal ethics committees.

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Cardiac function protocol

The protocol of Iftikar and Hickey (Iftikar and Hickey, 2013) was followed for both *N. fucicola* and *T. lunare*. Water pumped into the buccal cavity induced atonic immobility (Wells et al., 2005) and fish were then held for 3 h prior to experiments to dissipate any associated handling stress. Heart rates were measured non-invasively by fetal Doppler probes (Sonotrax B, Contec Medical Systems, Qinhuangdao, China) to avoid anaesthetics. Probes were placed immediately above the heart of fish held supine in a submerged sponge within a 4 l plastic tank. This was further immersed in a larger 20 l water reservoir with a recirculating seawater system at each species' $T_{acclimation}$. The reservoir water was gradually increased by 1°C every 10 min for heat-stressed fish and the temperature was determined by placing a thermocouple (Digitech QM-1600, Jaycar Electronics, Auckland, New Zealand) inside the fish's mouth. Sonograms were measured after each temperature was reached, for 1 min (N=6). The acute T_{HF} was determined as the heart rate became arrhythmic or failed. Control fish were held in parallel yet with no heat stress and for the same duration (N=6). Sonograms from control and heat-stressed fish were recorded using Audacity 1.2.6 (http://audacity.sourceforge.net/) and analysed to determine heart rates. Heart rate sonogram data were normally distributed and analysed using a repeated measures analysis of variance (ANOVA) followed by a *post hoc* test (Tukey's). Fish were killed by cephalic concussion and a blood sample was taken from the caudal vein and rapidly frozen at −80°C for subsequent metabolomics analysis. The hearts were excised for mitochondrial respirometry (Fig. 1A).

Metabolite and enzymes analysis from heart tissue or plasma

Metabolites from plasma of control and experimental fish were extracted using −30°C methanol (Villas-Bôas et al., 2003). Initially, 20 µl of internal standard (10 mmol l^{-1} solution of DL-alanine-2,3,3,3-d4) was added to 100 µl of plasma, vortexed and frozen at −80°C. Samples were then freeze dried (Virtis freeze dryer) and metabolites extracted by adding 500 µl cold methanol:water (1:1 v/v) at −30°C. The solution was mixed vigorously for 1 min and centrifuged at 4°C for 5 min at 16,000 ***g***. The supernatant was collected in a separate tube and the pellet was re-suspended and extracted a second time in 500 µl cold methanol:water (4:1 v/v). This second extracted supernatant was then pooled with the first extract. Then, 5 ml of cold bi-distilled water (4°C) was added to extracted plasma, frozen to −80°C and freeze dried. Metabolites were chemically derivatised using methyl chloroformate and the samples were analysed by GC-MS with no modifications from the protocol of Villas-Bôas et al. (Villas-Bôas et al., 2003). The relative level of the metabolites in plasma was based on the base peak height as detected by gas chromatography. Values were normalised by the base peak height of the internal standard (alanine-d4). Metabolites in the plasma samples were identified using an in-house methyl chloroformatate (MCF) MS library of derivatised metabolites. These contained MS spectra obtained from ultra-pure standards with the mass spectra saved and analysed in AMDIS 2.65 software (www.amdis.net) (Fig. 1A).

Lactate, CS (an aerobic marker of mitochondrial content) (Srere, 1969) and LDH (an anaerobic marker enzyme) (Hochachka et al., 1983) in cardiac tissue from control and heat-stressed fish were measured similar to Iftikar et al. (Iftikar et al., 2010) with modifications from previous investigators (Hickey and Clements, 2003; Newsholme and Crabtree, 1986).

Mitochondrial respirometry

Three series of experiments were conducted for all fish species. A substrate-uncoupler-inhibitor titration (SUIT) protocol that investigates different components of the phosphorylation system was applied in experimental series 1 and 2, which differed only in assay temperatures. In series 1, permeabilised fibres from hearts of fish exposed *in vivo* to acute heat stress were tested. Permeabilised cardiac fibres were assayed close to the fish's $T_{acclimation}$, which for *N. fucicola*, *N. celidotus* and *T. lunare* was 12.5, 20 and 27.5°C, respectively (Fig. 1A). In series 2, we determined the temperature at which cardiac mitochondrial function appeared to alter significantly (T_{mt}). For *N. fucicola*, mitochondrial function within cardiac fibres *in situ* was measured at 10, 12.5, 15, 17.5, 20, 22.5 and 25°C; for *T. lunare*, it was measured at 20, 25, 27.5, 30, 32.5, 35 and 37.5°C; previously collected data was used for *N. celidotus* measured at 15, 17.5, 20, 25, 27.5, 30 and 32.5°C (Fig. 1A) (Iftikar and Hickey, 2013).

Permeabilised fibre preparation

Fish hearts were rapidly dissected and immersed in 2 ml modified ice-cold relaxing buffer (BIOPS, 2.77 mmol l^{-1} CaK_2EGTA, 7.23 mmol l^{-1} K_2EGTA, 5.77 mmol l^{-1} Na_2ATP, 6.56 mmol l^{-1} $MgCl_2 \cdot 6H_2O$, 20 mmol l^{-1} taurine, 20 mmol l^{-1} imidazole, 0.5 mmol l^{-1} dithiothreitol, 50 mmol l^{-1} K-MES, 15 mmol l^{-1} sodium phosphocreatine and 50 mmol l^{-1} sucrose, pH 7.1) (Gnaiger et al., 2000; Iftikar and Hickey, 2013). The hearts were then teased into fibre bundles and placed in 1 ml ice-cold BIOPS in a plastic culture plate; 50 µg ml^{-1} saponin was then added while the fibres were shaken on ice for 30 min. Fibres were rinsed three times for 10 min in 2 ml of modified mitochondrial respiratory medium (Fish-MiRO5, 0.5 mmol l^{-1} EGTA, 3 mmol l^{-1} $MgCl_2 \cdot 6H_2O$, 60 mmol l^{-1} potassium lactobionate, 20 mmol l^{-1} taurine, 10 mmol l^{-1} KH_2PO_4, 20 mmol l^{-1} Hepes, 160 mmol l^{-1} sucrose and 1 g l^{-1} BSA, essentially free fatty acid, pH 7.24 at 20°C) (Gnaiger et al., 2000; Iftikar and Hickey, 2013). The fibres were then blotted dry and weighed into 2–3 mg bundles for respiration assays. All chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA).

The SUIT protocol

Oxygen was added into the gas phase above media prior to closing chambers to supersaturate Fish-MiRO5. Oxygen concentrations were maintained above 280 nmol ml^{-1} to ensure saturation and to maximise flux. CI substrates (2 mmol l^{-1} malate and 10 mmol l^{-1} pyruvate) were added to measure state II respiration through CI in the absence of ADP (denoted 'Leak I') (Fig. 1B). Excess ADP (2.5 mmol l^{-1}) stimulated oxidative phosphorylation (OXP-I, state III respiration), and glutamate (10 mmol l^{-1}) was added to saturate CI. Cytochrome *c* (10 µmol l^{-1}) was added to test outer membrane integrity. Phosphorylating respiration with complex I and II substrates (OXP-I, II) was measured by the addition of succinate (10 mmol l^{-1}). NADH (0.5 mmol l^{-1}) was then added to assess inner mitochondrial membrane damage (Fig. 1B). Leak respiration rates were also measured on combined CI and CII substrates by addition of atractyloside (750 µmol l^{-1}, Leak-I, II), followed with repeated titrations of carbonyl cyanide *p*-(trifluoromethoxy)phenyl-hydrazone (FCCP, 0.5 µmol l^{-1}) to uncouple mitochondria (denoted 'ETS'). CI, II and III activities were inhibited by the addition of rotenone (0.5 µmol l^{-1}), malonate (15 mmol l^{-1}) and antimycin a (1 µmol l^{-1}), respectively. Finally, the activity of cytochrome *c* oxidase (CCO, complex IV) was measured by the addition of the electron donor couple *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD, 0.5 mmol l^{-1}) and ascorbate (2 mmol l^{-1}) (Fig. 1B). All chemicals were obtained from Sigma-Aldrich.

Substrate affinity and apparent K_m calculation

In series 3, the affinity of primary substrates in cardiac mitochondria with increasing assay temperature was tested (Fig. 1A). After preliminary tests, we found that OXP respiration of *N. fucicola* cardiac mitochondria was greater with malate than with pyruvate. The apparent K_m for malate was determined at 12.5, 15, 20, 22.5 and 25°C for *N. fucicola*. For *N. celidotus* and *T. lunare*, the main oxidative substrate was pyruvate. The apparent K_m for pyruvate was measured at 25, 27.5, 32.5, 35 and 37.5°C for *T. lunare* and 20, 25, 27.5, 30 and 32.5°C for *N. celidotus*.

The capacity ($K_{m,app}$) of mitochondria within permeabilised fibres to take up the primary CI oxidative fuel was measured in the presence of ADP (2.5 mmol l^{-1}). The respective substrate was titrated in at minute volumes by step-wise substrate additions using microinjection pumps (Oroboros Tip O2-K) until flux was maximal (saturated). Michaelis–Menten curves were generated and substrate-saturation curve kinetics were applied to determine $K_{m,app}$ and V_{max} values using non-linear regression.

Calculations and statistical analyses

A comparative metabolite profile was generated between control and acutely heat-stressed fish and data were analysed using R-software (Aggio et al., 2011). Differences in metabolite profiles between control and heat-stressed fish were compared using PCA and data were projected on a 2D plane (PC1 versus PC2). All mitochondrial respiration rates were expressed per mg wet mass of cardiac fibres. In mitochondrial respiration assays, differences across temperatures, and between control and experimental fish, were evaluated with a one- or two-factor ANOVA, followed by a *post hoc* test

(Tukey's). RCR-I was calculated as OXP-I/Leak-I, and RCR-II was calculated as OXP-I, II/Leak-I, II. RCR-I and RCR-II were graphed as Arrhenius plots and segmented regression analysis was applied to determine Arrhenius break temperature (ABT). While the Arrhenius plot is intended for kinetic data, the purpose of the natural logarithm/reciprocal plot was to linearize exponential data and resolve changes in states such as those associated with thermodynamic transitions. Therefore, in this study, this analysis was applied to RCR-I and RCR-II. UCR was calculated as ETS/OXP-I, II. The point at which $V_{max}/K_{m,app}$ peaked was determined using non-linear regression peak analysis. The level of significance for all statistical tests was set at $P<0.05$. All statistical tests were run using SigmaPlot version 12 (Systat Software Inc., San Jose, CA, USA) unless stated otherwise.

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Competing interests
The authors declare no competing financial interests.

Author contributions
F.I.I. and A.J.R.H. designed the research; F.I.I. conducted the research, carried out statistical analyses of the data and wrote the first draft of the article; A.J.R.H. had significant input on data analysis; J.R.M. and D.W.B. performed mitochondrial assays and surgical procedures; G.M.C.R. organised animal husbandry and participated in field experiments at Moreton Bay Research Station, and contributed to drafting of the manuscript; all authors revised and approved the article.

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