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RESEARCH ARTICLE

Air breathing in the Arctic: influence of temperature, hypoxia, activity and restricted air access on respiratory physiology of the Alaska blackfish *Dallia pectoralis*

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ABSTRACT

The Alaska blackfish (*Dallia pectoralis*) is an air-breathing fish native to Alaska and the Bering Sea islands, where it inhabits lakes that are ice-covered in the winter, but enters warm and hypoxic waters in the summer to forage and reproduce. To understand the respiratory physiology of this species under these conditions and the selective pressures that maintain the ability to breathe air, we acclimated fish to 5°C and 15°C and used respirometry to measure: standard oxygen uptake ($\dot{M}_{O_2}$) in normoxia (19.8 kPa P_{O_2}) and hypoxia (2.5 kPa), with and without access to air; partitioning of standard $\dot{M}_{O_2}$ in normoxia and hypoxia; maximum $\dot{M}_{O_2}$ and partitioning after exercise; and critical oxygen tension (P_{crit}). Additionally, the effects of temperature acclimation on haematocrit, haemoglobin oxygen affinity and gill morphology were assessed. Standard $\dot{M}_{O_2}$ was higher, but air breathing was not increased, at 15°C or after exercise at both temperatures. Fish acclimated to 5°C or 15°C increased air breathing to compensate and fully maintain standard $\dot{M}_{O_2}$ in hypoxia. Fish were able to maintain $\dot{M}_{O_2}$ through aquatic respiration when air was denied in normoxia, but when air was denied in hypoxia, standard $\dot{M}_{O_2}$ was reduced by ~30–50%. P_{crit} was relatively high (5 kPa) and there were no differences in P_{crit}, gill morphology, haematocrit or haemoglobin oxygen affinity at the two temperatures. Therefore, Alaska blackfish depends on air breathing in hypoxia and additional mechanisms must thus be utilised to survive hypoxic submergence during the winter, such as hypoxia-induced enhancement in the capacities for carrying and binding blood oxygen, behavioural avoidance of hypoxia and suppression of metabolic rate.

KEY WORDS: respiratory partitioning, bimodal respirometry, temperature acclimation, critical oxygen tension, haemoglobin oxygen affinity, gill remodelling

INTRODUCTION

Air-breathing fishes constitute ~450 of the 32,000 extant fish species. The majority occur in tropical regions (Johansen, 1970; Graham, 1997) where high temperatures and hypoxia are common (Diaz, 2001; Val et al., 2005; Diaz and Breitburg, 2009). Therefore, it has been hypothesised that air breathing in fish evolved to overcome the challenges of low oxygen availability (Barrell, 1916; Carter and Beadle, 1930; Carter, 1931; Carter, 1957; Packard, 1974; Randall et al., 1981a; Graham and Wegner, 2010; Sedmera and Wang, 2012; Lefevre et al., 2014a). Although hypoxia was probably a major drive during the evolution of air breathing in many species, it is also known that several extant fishes utilise it under conditions of increased oxygen demand, such as during swimming (reviewed by Lefevre et al., 2014b), digestion (e.g. Iftikar et al., 2008; Lefevre et al., 2012) and elevated temperature (e.g. Johansen et al., 1970).

For a few species, the benefit of breathing air appears less obvious because they inhabit cold regions where metabolism is lower and ice cover may prevent air breathing during the winter. Here, the Alaska blackfish (*Dallia pectoralis* Bean 1880) stands out as the only air-breathing fish inhabiting Arctic regions, specifically Alaska and the Bering Sea islands (Jordan and Evermann, 1897; Scott and Crossman, 1973; Armstrong, 1994; Campbell and Lopéz, 2014). It extracts oxygen from the air using a modified oesophagus (Crawford, 1971; Crawford, 1974), but because of its ancient lineage (Cavender, 1969; Nelson, 1972), it is difficult to relate the current habitat of the Alaska blackfish to the habitat in which the ability to breathe air once evolved. Presently, the Alaska blackfish inhabits lakes that freeze over in the winter (Ultsch, 1989; Gudkov, 1998), which prevents diffusion of atmospheric oxygen into the water and, particularly in combination with snow, limits light penetration and thereby photosynthesis, resulting in hypoxia. Indeed, field measurements show that the habitats of Alaska blackfish become severely hypoxic during the winter (S.L. and J.A.W.S., personal observation, see Materials and methods). Although the Alaska blackfish is reported to be active in the winter (Ostdiek and Nardone, 1959), it has its most active and reproductive period in the summer, when it is reported to migrate into shallow areas with dense vegetation, little mixing and resulting hypoxia (Blackett, 1962). The selective pressure that maintains the ability to breathe air could thus be the benefit of inhabiting hypoxic areas that are less accessible to other species (Armstrong, 1994). Air breathing may additionally support the elevated oxygen demand associated with higher temperature in the summer and the increased activity necessary for foraging and reproduction. These benefits would then have to outweigh the disadvantages, such as increased predation risk (Kramer et al., 1983) and costs in terms of time and energy (Kramer and McClure, 1981; Kramer, 1983; Kramer, 1987; Lefevre et al., 2013).

Overall, there is limited knowledge on the dependence on atmospheric oxygen (i.e. respiratory partitioning) in this species, because oxygen uptake ($\dot{M}_{O_2}$) has only been measured from water (Scholander et al., 1953; Crawford, 1971) or air at 20°C (Crawford, 1971), which is outside the normal temperature range for the Alaska blackfish (Ostdiek and Nardone, 1959). It is able to support its basic oxygen demand (standard $\dot{M}_{O_2}$) down to a critical oxygen tension (P_{crit}) of 4–5 kPa at 20°C, but theoretically P_{crit} can be expected to decrease with temperature as a result of a lower standard $\dot{M}_{O_2}$ (e.g. Fry and Hart, 1948; Clarke and Johnston, 1999). The objective of this study was therefore to investigate the dependence on air breathing in relation to temperature, oxygen level and exercise, and

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List of symbols and abbreviations

A	absorbance
A_0	absorbance at 0% oxygen
A_{100}	absorbance at 100% oxygen
AAS	absolute aerobic scope
AS	aerobic scope
FAS	factorial aerobic scope
ILCM	interlamellar cell mass
$\dot{M}_{O_2}$	total oxygen uptake from air and water
$\dot{M}_{O_2,max}$	maximum oxygen uptake
$\dot{M}_{O_2,w}$	oxygen uptake from water only (i.e. air access denied)
P_{50}	oxygen partial pressure of haemoglobin half saturation
P_{crit}	critical oxygen partial pressure
P_{O_2}	oxygen partial pressure
$P_{O_2,a}$	oxygen partial pressure in air
$P_{O_2,w}$	oxygen partial pressure in water
Q_{10}	temperature coefficient
RM	repeated measures
S	fractional saturation

the ability to extract oxygen from water when access to air is denied. We hypothesised that the Alaska blackfish: (1) increase air breathing to support the larger standard $\dot{M}_{O_2}$ associated with elevated temperature and exhaustive exercise ($\dot{M}_{O_2,max}$), (2) can maintain standard $\dot{M}_{O_2}$ in hypoxia without air breathing at cold temperature, but not at high temperature, and (3) has a lower P_{crit} at cold temperature, as a result of the lower standard $\dot{M}_{O_2}$. To investigate these hypotheses, we acclimated fish to 5°C and 15°C and measured the following: (1) standard $\dot{M}_{O_2}$ in normoxia (19 kPa) and hypoxia (2.5 kPa), with and without access to air; (2) respiratory partitioning of standard $\dot{M}_{O_2}$ in normoxia and hypoxia; (3) $\dot{M}_{O_2,max}$ and respiratory partitioning after exhaustive exercise; and (4) P_{crit} and the commonly associated physiological variables, namely haematocrit, haemoglobin oxygen affinity, secondary lamellar length and inter-lamellar cell mass (ILCM).

RESULTS

Standard oxygen uptake and respiratory partitioning

When moved to the respirometer, total $\dot{M}_{O_2}$ was initially high and required ~10 h (Fig. 1A,C) to 15 h (Fig. 1B) to stabilize, except in 15°C-acclimated fish in hypoxia (Fig. 1D). For both 5°C and 15°C fish in normoxia, $\dot{M}_{O_2}$ was initially three-times higher than the apparent resting $\dot{M}_{O_2}$ (Fig. 1A,C), whereas for 5°C hypoxic fish, the initial elevation was ~twofold (Fig. 1B).

Overall, temperature significantly influenced standard $\dot{M}_{O_2}$ (Fig. 2; three-way ANOVA, $F_{1,36}$=104.5, P<0.001) with a temperature coefficient (Q_{10}) of 2.2. Standard $\dot{M}_{O_2}$ was thus significantly higher at 15°C compared with 5°C in normoxia with air (two-way ANOVA, P<0.001), normoxia without air (P=0.005), hypoxia with air (P<0.001) and hypoxia without air (P=0.002). Furthermore, $\dot{M}_{O_2}$ was significantly affected by an interaction between the level of oxygen and access to air (three-way ANOVA, $F_{1,36}$=33.0, P<0.001).

Fish were able to maintain standard $\dot{M}_{O_2}$ without access to air in normoxia [two-way ANOVA with repeated measures (RM), $F_{1,10}$=0.629, P=0.446], compared with normoxia with air, at both 5°C (P=0.135) and 15°C (P=0.626). Similarly, when fish were exposed to hypoxia and allowed to breathe air, standard $\dot{M}_{O_2}$ was maintained compared with normoxia with air (two-way ANOVA, $F_{1,18}$=3.6, P=0.074), and there was a tendency for standard $\dot{M}_{O_2}$ to be higher in hypoxia at 5°C (P=0.091), but not at 15°C (P=0.380). When access to air was denied in hypoxia, standard $\dot{M}_{O_2}$ was reduced by 28% at 5°C (Student's t-test, P=0.004) and 39% at 15°C (P=0.01), compared with normoxia with air. There was also a significant reduction in standard $\dot{M}_{O_2}$ when compared with hypoxia with air (two-way RM ANOVA, $F_{1,8}$=30.0, P<0.001). Specifically, it was reduced by 53% at 5°C (P=0.012) and 45% at 15°C (P=0.002). The first fish exposed to hypoxia without air access at 5°C lost equilibrium after 13 h, requiring us to shorten the exposure period to 4–6 h, and none of the remaining 5°C fish lost equilibrium over this period. By contrast, all the 15°C fish lost equilibrium after 2.9±0.8 h (mean ± s.d.).

Hypoxia significantly influenced respiratory partitioning of standard $\dot{M}_{O_2}$ (Fig. 3; two-way ANOVA, $F_{1,18}$=34.5, P<0.001), and the percentage of standard $\dot{M}_{O_2}$ obtained from air was 3.5-times higher in hypoxia at both 5°C (P=0.002) and 15°C (P<0.001). The effect of temperature on partitioning was lower (Q_{10}=1.2) than the general effect on standard $\dot{M}_{O_2}$, and was not significant (two-way ANOVA, $F_{1,18}$=1.5, P=0.244).

Critical oxygen tension

There was a difference in the behavioural response to acute gradual hypoxia between 5 and 15°C fish (Fig. 4). Five of the six

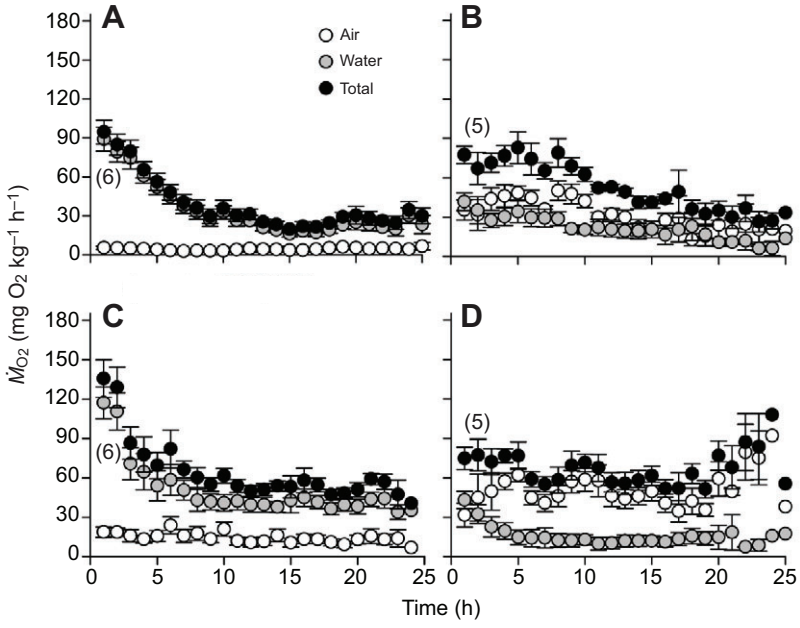

Fig. 1. Bimodal oxygen uptake over 24 h in the Alaska blackfish *Dallia pectoralis*. Oxygen uptake ($\dot{M}_{O_2}$) from air, water and in total recorded over 24 h for 5°C- (A,B) and 15°C-acclimated (C,D) fish in normoxia (A,C) and hypoxia (B,D). Values are means ± s.e.m. Different individuals were used in the four experimental treatment groups (see Fig. 7), for which sample sizes are indicated in parentheses.

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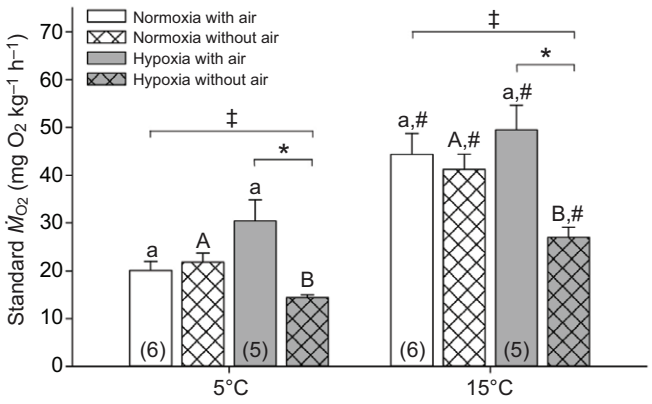

Fig. 2. Effects of temperature, hypoxia and restricted air access on standard oxygen uptake in the Alaska blackfish. Standard $\dot{M}_{O_2}$ of 5°C- and 15°C-acclimated fish exposed to normoxia or hypoxia, with or without access to air. Within each of the four experimental treatments, standard $\dot{M}_{O_2}$ was measured on the same individual; first with access to air using bimodal intermittent-closed respirometry, then without access to air using single-phase respirometry (see Fig. 7 for details). For all fish with access to air, standard $\dot{M}_{O_2}$ was calculated as the lowest 10th percentile during the 24 h measurement period (Fig. 1). For the normoxic fish without access to air, standard $\dot{M}_{O_2,w}$ was calculated as the lowest 10th percentile of the $\dot{M}_{O_2,w}$ values taken prior to measurement of P_{crit}. For the hypoxic fish without access to air, standard $\dot{M}_{O_2,w}$ was calculated as the lowest 10th percentile during the 3–6 h measurement period. Values are means ± s.e.m. and sample size is indicated in parentheses for the four different treatment groups. Hash signs indicate significant difference between 5 and 15°C within each of the four exposure groups (two-way ANOVA). Asterisks indicate significant effect of restricted air access within normoxia or hypoxia, at 5°C or 15°C (RM two-way ANOVA). Daggers indicate significant difference between normoxia with air and hypoxia without air (Student's *t*-test). Dissimilar lowercase letters indicate a significant effect of hypoxia within temperature, when air breathing was allowed (two-way ANOVA). Dissimilar uppercase letters indicate a significant effect of hypoxia within temperature, when air breathing was restricted (two-way ANOVA).

5°C fish were largely quiescent throughout the exposure (Fig. 4A–D,F), whereas four of the five 15°C fish became agitated immediately when P_{O_2} started to decrease, as evidenced by the higher $\dot{M}_{O_2}$ from water ($\dot{M}_{O_2,w}$) (Fig. 4H–K) than the standard $\dot{M}_{O_2}$ previously measured in normoxia with air (Fig. 2, white bars). The individually measured P_{crit} did not differ significantly between 5°C- and 15°C-acclimated fish (Table 1; Mann–Whitney rank sum test, P=0.662). The higher $\dot{M}_{O_2,w}$ of 15°C- compared with 5°C-acclimated fish appeared to be maintained even at P_{O_2} levels below P_{crit}, and the slope of the line through oxygen-dependent $\dot{M}_{O_2,w}$ values was thus significantly steeper at 15°C (Table 1; Student's *t*-test, P=0.017).

Maximum oxygen uptake and aerobic scope

Fish acclimated to 5°C and 15°C showed a considerably elevated $\dot{M}_{O_2}$ immediately following exhaustive exercise (Fig. 5A), and there was a strong tendency for $\dot{M}_{O_2,max}$ to be higher at 15°C (Student's *t*-test, P=0.090). The absolute aerobic scope (AAS) also tended to be higher at 15°C (Fig. 5B), although the difference was not significant (Student's *t*-test, P=0.283). Factorial aerobic scope (FAS) (Fig. 5C) was significantly lower at 15°C (Student's *t*-test, P=0.045). The use of air breathing during recovery from exhaustive exercise (Fig. 5D) was even lower than that measured under resting conditions (Fig. 3), and there was no difference between the two temperatures (Student's *t*-test, P=0.395).

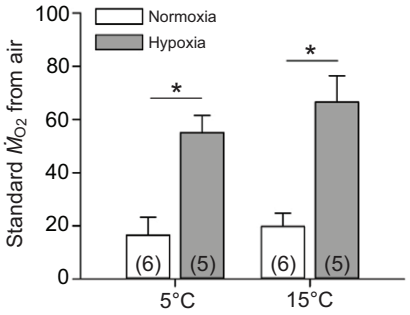

Fig. 3. The effect of temperature and hypoxia on respiratory partitioning in the Alaska blackfish. Respiratory partitioning as a percentage of standard $\dot{M}_{O_2}$ derived from air of 5°C- and 15°C-acclimated fish exposed to normoxia or hypoxia for 24 h in a bimodal intermittent-closed respirometer. Values are means ± s.e.m. Asterisks indicate significant effect of hypoxia within an acclimation temperature (two-way ANOVA).

Haemoglobin oxygen affinity and haematocrit

Haemoglobin O_2 affinity was measured as the partial pressure of oxygen at haemoglobin half saturation (P_{50}) in haemolysates (i.e. still containing endogenous co-factors). Under these conditions, there was a strong effect of temperature on haemoglobin P_{50} (RM two-way ANOVA, $F_{1,27}$=55.4, P<0.001, Table 1) as a result of the exothermic nature of haemoglobin O_2 binding. Acclimation temperature, however, did not affect haemoglobin P_{50} ($F_{1,27}$=0.01, P=0.916,), indicating no regulation of haemoglobin O_2 affinity during acclimation to 5°C or 15°C. Likewise, there was no significant difference in haematocrit between acclimation groups (Student's *t*-test, P=0.650, Table 1).

Gill morphology

Acclimation to 5°C or 15°C did not result in alterations of gill morphology (Fig. 6; Table 2). Quantitative measurements did not reveal any significant effects of temperature acclimation on lamellar length (Student's *t*-test, P=0.691), ILCM area (P=0.699) or the ratio of ILCM height to lamellar height (P=0.775).

DISCUSSION

Standard metabolism and air breathing

The standard $\dot{M}_{O_2}$ values of Alaska blackfish measured in the present study are comparable to previous measurements on Alaska blackfish (Scholander et al., 1953; Crawford, 1971), as well as to values obtained for other air-breathing fishes (Lefevre et al., 2014c). It is interesting that up to 10 h was required for the Alaska blackfish to enter a resting state in the bimodal respirometer, but a similar pattern has been observed in the striped catfish *Pangasianodon hypopthalmus* (Lefevre et al., 2011). Unfortunately, prolonged measurements comparable to these two studies have seldom been carried out, or are not published, making it difficult to ascertain whether it is a general pattern of air-breathing fish. In any case, because $\dot{M}_{O_2}$ was measured for 24 h and stabilized within this time, we feel assured that the values reported here are representative of standard $\dot{M}_{O_2}$. $\dot{M}_{O_2}$ has also been measured in the central mudminnow *Umbra limi* (Currie et al., 2010), which is the closest air-breathing relative to the Alaska blackfish (Peckham and Dineen, 1957). At 15°C, the standard $\dot{M}_{O_2}$ measured in Alaska blackfish (44 mg O_2 kg^{-1} h^{-1}) was lower than that of the central mudminnow [60 mg O_2 kg^{-1} h^{-1}, calculated from Currie et al. (Currie et al., 2010) by scaling to a body mass of 50 g using an exponent of –20 (Clark and Johnston, 1999)]. As expected, the standard $\dot{M}_{O_2}$ of Alaska blackfish increased from 5 to 15°C, with a Q_{10} of 2.2. This Q_{10} value

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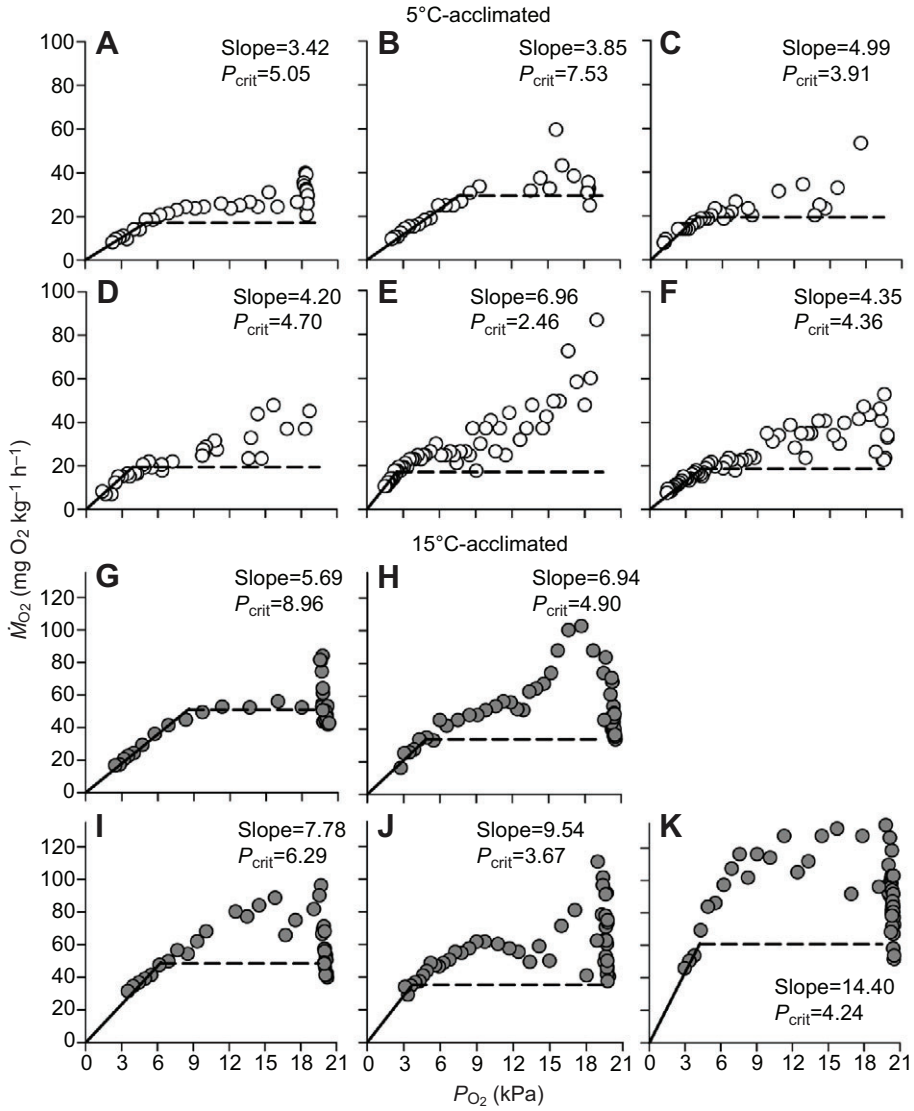

Fig. 4. Oxygen uptake by the Alaska blackfish from water during submergence in a closed respirometer as a function of water P_{O_2}. Data are shown for individual fish acclimated to 5°C (A–F) and 15°C (G–K). These fish had also been used to measure standard $\dot{M}_{O_2}$ with air access in normoxia (Fig. 1A,C, white bars in Fig. 2). Oxygen uptake from water ($\dot{M}_{O_2,w}$) was measured during submergence in a closed respirometer. Because of increased spontaneous activity of most fish in the respirometer as P_{O_2} declined, standard $\dot{M}_{O_2}$ values calculated as the lowest 10th percentile for the same individuals during 24 h bimodal intermittent-closed respirometry in normoxia with air (white bars in Fig. 2) were utilised as oxygen-independent $\dot{M}_{O_2}$ (presented as a horizontal dashed line for each fish). Oxygen-dependent $\dot{M}_{O_2,w}$ was determined from the slope of a line fitted to the $\dot{M}_{O_2,w}$ values that fell below standard $\dot{M}_{O_2}$ (solid line). For each individual, P_{crit} was determined as the intersection of the two lines. The slope and P_{crit} values are provided for each fish, and the mean values are presented in Table 1.

is lower than the 2.4 reported for an increase in temperature from 15 to 31°C (Currie et al., 2010) and the 2.7 found when temperature increased from 5 to 15°C (Klinger et al., 1982) in the central mudminnow, indicating that the $\dot{M}_{O_2}$ of Alaska blackfish has a slightly lower temperature sensitivity.

Unexpectedly, respiratory partitioning remained below 20% from air, even at 15°C. Alaska blackfish thus depended mainly on $\dot{M}_{O_2,w}$ despite increased temperature. The central mudminnow appears to show a similar response (Gee, 1980), but in other air-breathing species, increased air breathing with temperature has been reported (Johansen et al., 1970; Horn and Riggs, 1973; Vivekanandan and Pandian, 1977; Smatresk and Cameron, 1982; Patra et al., 1983; Yu and Woo, 1985; McMahon and Burggren, 1987; Fernandes and Perna, 1995; Geiger et al., 2000). The present measurements also show that the Alaska blackfish can extract sufficient oxygen from the water alone to maintain standard $\dot{M}_{O_2}$ in normoxia at both 5°C and 15°C, and also to increase $\dot{M}_{O_2}$ during activity. When exposed to hypoxia, the Alaska blackfish compensated fully through increased air breathing, which was expected at 15°C but not at 5°C because of the lower standard $\dot{M}_{O_2}$ at this temperature. There was even a slight tendency for standard $\dot{M}_{O_2}$ to be elevated in hypoxia, which could result from a possible energetic cost of surfacing (Kramer and McClure, 1981; Kramer, 1983; Kramer, 1987; Lefevre et al., 2013). A larger response to hypoxia than temperature has also

Table 1. Respiratory variables in Alaska blackfish *Dallia pectoralis* acclimated to 5°C and 15°C

Acclimation temperature	P_{crit} (kPa)	Slope (mg O_2 kg^{-1} h^{-1} kPa^{-1})	Haematocrit (%)	P_{50} at 5°C (kPa)	P_{50} at 15°C (kPa)
5°C	4.7±0.6	4.5±0.5^{b}	29.6±2.3	0.69±0.07^{A}	1.44±0.14^{B}
15°C	5.6±0.9	8.9±1.5^{a}	28.5±1.4	0.69±0.07^{A}	1.40±0.18^{B}

Critical oxygen tension (P_{crit}), slope ($\dot{M}_{O_2,w}$ below standard $\dot{M}_{O_2}$), haematocrit (Hct) and haemoglobin oxygen affinity (P_{50}). Values are means ± s.e.m. Different lowercase letters indicate significant difference between acclimation temperatures in the slope of the line through oxygen-dependent $\dot{M}_{O_2,w}$ values for each fish (Student's *t*-test). Different uppercase letters indicate significant differences between the P_{50} means (RM two-way ANOVA) between both acclimation temperature and measure temperature. N=6 for 5°C and N=5 for 15°C P_{crit}, respectively. N=7 for P_{50} at each temperature. Of these fish, six in each group had also been used in the respirometry experiment (Fig. 7).

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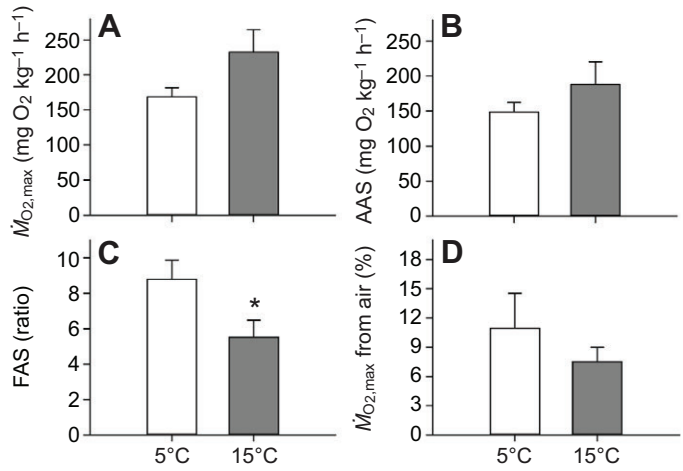

Fig. 5. Oxygen uptake and respiratory partitioning after exhaustive exercise in the Alaska blackfish. Maximum oxygen uptake ($\dot{M}_{O_2,max}$) (A), absolute aerobic scope (AAS) (B), factorial aerobic scope (FAS) (C), and respiratory partitioning following exercise (percentage of $\dot{M}_{O_2,max}$ from air) (D) for fish acclimated to 5°C (white) and 15°C (grey). Values are means ± s.e.m. *N*=6 in each group. Asterisks indicate significant differences (Student's *t*-test).

been reported for the central mudminnow (Gee, 1980). The fact that hypoxia induced air breathing at both 5°C and 15°C, whereas increased temperature in itself did not, might indicate that the Alaska blackfish (and the central mudminnow) relies more on stimulation of external branchial O_2 receptors rather than internal receptors, to induce and control air breathing (e.g. Smatresk, 1986).

When air breathing was denied in hypoxia, however, $\dot{M}_{O_2}$ was significantly reduced, indicating that the fish were not able to extract enough oxygen from the water to maintain standard $\dot{M}_{O_2}$. This was not surprising considering that the oxygen level (2.5 kPa) was well below P_{crit} (5 kPa) at both temperatures, although it was hypothesized that 5°C fish would have a lower P_{crit} (discussed below) and therefore do better. The data suggest that to survive, the Alaska blackfish would have to significantly suppress overall metabolic rate enough to match the amount of oxygen it can extract, or partly suppress metabolic rate and compensate by anaerobic metabolism (e.g. Richards, 2009; Richards, 2010), in which case survival time would be limited. Involvement of anaerobic metabolism has been demonstrated for other air-breathing fish (MacCormack et al., 2003; da Cruz et al., 2013). In the present study, 15°C fish lost equilibrium after about 3 h, whereas none of the 5°C fish had lost equilibrium after 4–6 h of hypoxic exposure without air access, although the first fish tested did lose equilibrium after 13 h. That the Alaska blackfish lost equilibrium indicates that they were not able to suppress metabolic rate enough to match oxygen supply, and the shorter coping time at 15°C could reflect a higher metabolic rate and thereby faster build-up of anaerobic endproducts and depletion of glycogen stores. Assessment of the extent to which anaerobic metabolism and suppression of metabolic rate is used by the Alaska blackfish at different temperatures and oxygen levels requires detailed measurements of both aerobic and anaerobic metabolism, but it would be interesting and worthwhile to include these in future investigations.

Critical oxygen tension

We initially expected that P_{crit} would be lower at 5°C because of the lower standard $\dot{M}_{O_2}$ at this temperature, but surprisingly, a relatively high P_{crit} of 5 kPa was measured for both 5°C- and 15°C-acclimated fish. A similar P_{crit} has even been reported for Alaska blackfish at 20°C (Crawford, 1971). The P_{crit} is well above the 1–4 kPa typical of hypoxia-tolerant teleosts (Nilsson and Randall, 2010), but falls within the range of P_{crit} values reported for other air-breathing species (Lefevre et al., 2014c). The effect of temperature acclimation on the P_{crit} of air-breathing fish has, to our knowledge, only been

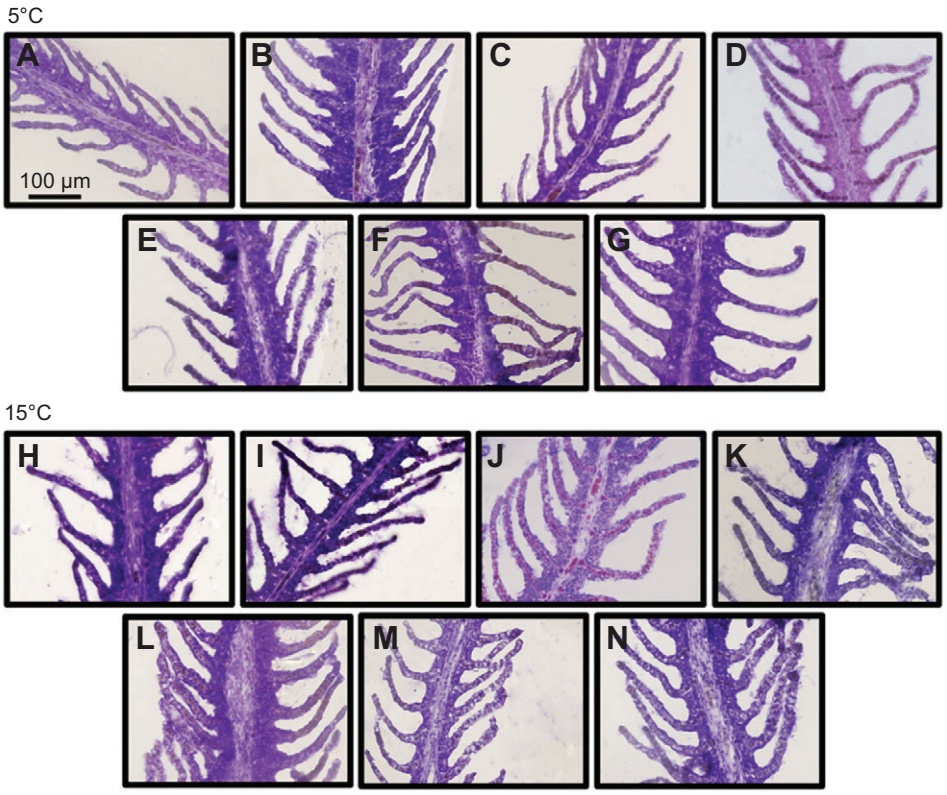

Fig. 6. Gill morphology in the Alaska blackfish after acclimation to 5°C and 15°C. Haematoxylin and eosin stained images of the second left gill arch filaments from Alaska blackfish that had been acclimated to 5°C (A–G) or 15°C (H–N). The scale bar in A applies to all panels and each panel is representative of an individual fish.

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Table 2. Gill morphology parameters of Alaska blackfish acclimated to 5°C and 15°C

Acclimation temperature	Lamellar length (μm)	ILCM area (μm^2)	ILCM height/ lamellar height
5°C	196.0±6.5	1473.1±106.2	0.13±0.01
15°C	202.1±4.4	1594.5±80.8	0.13±0.01

Values are means ± s.e.m. *N*=7 at each temperature. Of these fish, six in each group had also been used in the respirometry experiment and measurement of haemoglobin P_{50} (Fig. 7). No statistically significant differences existed for any parameter between acclimation temperatures (Student's *t*-tests).

investigated in the tropical fish *Hypostimus regani*, which appear to maintain P_{crit} from 20 to 25°C, and actually reduce it from 25 to 30°C (Fernandes et al., 1999). The temperature independence of the Alaska blackfish P_{crit} means that 5°C-acclimated fish did not have an improved ability to take up oxygen from the water in hypoxia, even though this would seem highly adaptable, given their likelihood of being submerged for the whole winter. In other words, the 15°C-acclimated fish could take up more oxygen at any given hypoxic oxygen level than the 5°C-acclimated fish. Greater oxygen uptake at a given P_{O_2} can be achieved by increasing haematocrit, haemoglobin concentration or erythrocyte number (Weber et al., 1976; Fernandes et al., 1999), blood oxygen affinity (i.e. decreased blood P_{50}) (Grigg, 1969; Weber et al., 1976; Albers et al., 1983; Andersen et al., 2009) or respiratory surface area (Sollid et al., 2005; Tzaneva et al., 2011; Nilsson et al., 2012). The Alaska blackfish does not appear to adopt any of these mechanisms. Alternatively, the ability of warmer fish to sustain increased $\dot{M}_{O_2}$ at a given hypoxic P_{O_2} could also be explained by the temperature dependence of heart function leading to increased cardiac output at higher temperatures, allowing more oxygen to be transported from the gill to the tissues (unless gill oxygen uptake is limited by diffusion). Numerous studies show that fish cardiac performance is increased at high temperature, with or without acclimation (Butler and Taylor, 1975; Barron et al., 1987; Bailey and Driedzic, 1990; Matikainen and Vornanen, 1992, Anttila et al., 2014). In addition, increasing the oxygen-diffusive capacity of the tissues at higher temperature (e.g. through increased capillary density), would allow for an increased unloading of oxygen at the tissues and a better utilization of the venous oxygen reserve. To examine such mechanisms, the Alaska blackfish cardiovascular system and its regulation during temperature acclimation need to be characterized, and procedures have to be developed for this species for measuring aortic blood flow and preferentially also monitoring arterial and venous P_{O_2}. This may be a challenging, but not impossible, task in this relatively small fish.

The haemoglobin P_{50} was substantially lower than P_{crit}, being 0.7 kPa at 5°C and 1.4 kPa at 15°C (no difference between acclimation groups), which is within the range of other air-breathing fish, although only tropical species have been investigated (e.g. Johansen et al., 1978a; Johansen et al., 1978b; Damsgaard et al., 2014). A high haemoglobin oxygen affinity not only favours oxygen extraction at the gills under hypoxic conditions, but also reduces the risk of diffusive loss of the oxygen taken up from the oesophagus, when the oxygenated blood flows through the gills (Johansen, 1970; Olson, 1994). The difference in P_{50} and P_{crit} may seem paradoxical because the blood could theoretically be fully saturated with oxygen when $\dot{M}_{O_2}$ starts to decrease at P_{crit}. A difference between P_{50} and P_{crit} has been reported for some species (e.g. Sollid et al., 2005; Porteus et al., 2014) but not for others (e.g. Mandic et al., 2009). It might indicate that Alaska blackfish are suppressing metabolic rate, and thereby $\dot{M}_{O_2}$, at a certain P_{O_2} when they are exposed to hypoxia without access to air, but it would require detailed measurement of anaerobic metabolism to confirm a suppression of metabolic rate. The result could also simply reflect the fact that P_{crit} is influenced more by other factors, such as diffusion distance, respiratory surface area and overall oxygen-carrying and cardio-respiratory capacity (Gamperl and Driedzic, 2009; Richards, 2009). In relation to this, it is important to bear in mind that prolonged exposure to hypoxia without access to air, as occurs during the winter, would probably initially make the fish hypoxemic (Randall et al., 1981b; Hedrick et al., 1994), and this hypoxemia could induce compensatory changes in, for example, oxygen-carrying capacity (Graham, 1983; Petersen and Gamperl, 2011), oxygen affinity (Weber et al., 1979; Graham, 1983) and cardio-respiratory parameters (Graham, 1983; Burleson et al., 2002; Petersen and Gamperl, 2011; Porteus et al., 2014), which would ultimately improve P_{crit} (e.g. Fu et al., 2011). Inclusion of acclimation to hypoxia without access to air in future studies on the Alaska blackfish is of obvious interest, though it may be challenging and demand careful considerations regarding how to best replicate field conditions in the laboratory.

Exhaustive exercise and aerobic scope

There was a tendency for both $\dot{M}_{O_2,max}$ and AAS to be increased at 15°C, which would be beneficial as this corresponds to the summer temperature where the most energy demanding activities are carried out (Blackett, 1962; Morrow, 1980). It may then seem counterintuitive that FAS decreased, but this is explained by the larger effect that temperature has on standard $\dot{M}_{O_2}$ compared with $\dot{M}_{O_2,max}$. The functional significance of FAS versus AAS is regularly being debated, and the conclusions reached in different experiments may differ substantially depending on which variable is used (Clark et al., 2013), as is also evident in the present data. Arguably, AAS says quantitatively more about the functional aerobic capacity, because the value relates more directly to available energy for processes such as protein synthesis and physical activity for which the cost is essentially independent of temperature (Brett, 1979; Brett and Groves, 1979). Generally, there are few air-breathing fishes for which AAS has been measured (Lefevre et al., 2014b) and no previous study has measured AAS of an air-breathing fish at different temperatures. It is, nonetheless, well established that AAS generally increases with temperature in water-breathing fishes (Fry, 1947; Johnston and Dunn, 1987; Claireaux et al., 2000; Clark et al., 2013). It is important to note that it is becoming increasingly clear that the optimal temperature for AS may not correspond to the overall optimal temperature or preferred temperature (Clark et al., 2013).

Despite a pronounced elevation in $\dot{M}_{O_2}$ following exhaustive exercise, and contrary to expectations, the Alaska blackfish did not resort to air breathing to any significant degree during recovery, even at 15°C. A similar phenomenon has been observed in other air-breathing fishes (Wells et al., 2007; Lefevre et al., 2013). That exercise, like increased temperature, did not induce air breathing might indicate that hypoxic stimulation of external rather than internal O_2 receptors is needed to initiate air breathing. It could also be an adaptive strategy that lowers the risk of aerial predation at a time when the fish has little capacity left for anaerobic burst swimming and thus escape responses, while also facilitating excretion of CO_2 (Shartau and Brauner, 2014). It would be interesting to investigate whether hypoxia restricts sustained swimming performance in Alaska blackfish, as observed in the banded knifefish (*Gymnotus carapo*) (McKenzie et al., 2012).

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Rectifying the paradox of needing to breathe, but not being able to, in the winter

It is evident from the present study that the Alaska blackfish is quite dependent on air breathing to survive severe hypoxia, both at 5°C and 15°C. The fact that these fish are commonly found in lakes known to be covered by ice in the winter is an apparent paradox in light of these results, and other physiological strategies must obviously be utilized by the Alaska blackfish to survive the winter. In addition to the possible hypoxemia-induced changes in the ability to extract oxygen from hypoxic water discussed above, mechanisms could include utilising oxygen from gas pockets under the ice, holes in the ice, entering a state of deep metabolic rate depression or avoidance of behavioural hypoxia.

It has been proposed that the Alaska blackfish and the related central mudminnow breathe air from gas pockets under the ice (Klinger et al., 1982; Magnuson et al., 1983), and it is also common Alaskan folklore that a symbiotic relationship exists between the muskrat and the Alaska blackfish. It is told that the muskrat digs breathing and feeding holes in the ice, and the ensuing aggregation of Alaska blackfish churns the water, preventing the hole from freezing over (Sisinyak, 2006). Although data indicate an importance of gas pockets and possible muskrat symbiosis for survival of the central mudminnow (Klinger et al., 1982), identical mechanisms do not appear to apply to the winter survival of Alaska blackfish, because this cannot explain how the Alaska blackfish survives in lakes where muskrats are absent or where the ice is at least 1 m thick (S.L. and J.A.W.S., personal observations). Nevertheless, to fully exclude the importance of gas pockets and/or muskrats for winter survival of the Alaska blackfish, more dedicated surveys of occurrence and distribution during summer and winter are necessary, in addition to experiments investigating the behaviour of the Alaska blackfish in its natural winter environment.

During collection we observed that fish appeared to be completely absent from an artificial pond (Duck Hunters Training Pond, Rabbit Slough, Palmer, Alaska) from which Alaska blackfish were previously trapped during the spring, summer and autumn. The pond lacked inflow and oxygen was measured to be 0.0 kPa. At the same time, we found Alaska blackfish to be abundant in another lake (Little Campbell Lake, Anchorage, AK, USA), which was natural, had an inlet from a neighbouring creek and an outlet, and oxygen levels that were relatively high in the water column (3.6 kPa at 2 m, 2.5°C), but decreased with depth (0.8 kPa at 3.25 m, 3.3°C). It is a theoretical possibility that Alaska blackfish were present in the anoxic pond, but in a dormant state, which we failed to induce in the laboratory. Even so, we find this unlikely because very few animals are tolerant of long-term anoxia, and these are either ethanol-producing fish or turtles that tolerate extreme lactate levels by buffering and storing it in their shell (Hochachka and Lutz, 2001; Lutz et al., 2003). Because the Alaska blackfish does neither, it would have to enter complete metabolic arrest during this dormant state, which would then have to last the whole winter. To our knowledge, anoxic survival through complete metabolic arrest has not been observed in any adult fish.

That being said, it cannot be ruled out that Alaska blackfish strongly suppress metabolic rate and activity during the winter. It would aid the fish in surviving in severely hypoxic (but not anoxic) areas of a lake, without becoming acidotic. The cue for entering this dormant state could be a combination of low temperature, short daylight, severe hypoxia and ice coverage. A winter dormant state has been observed in several water-breathing fish (Walsh et al., 1983; Corkum and Gamperl, 2009; Costa et al., 2013) and estivation during drought is also well-known from some amphibious air-breathing fishes (Smith et al., 1930; Janssens, 1964; Delaney et al., 1974; Eduardo et al., 1979) but has not been reported for any aquatic air-breathing fish. The fact that fish were easily caught at certain depths (and thereby certain oxygen levels, as described above) reveals that at least a proportion of them were not dormant. Rather, it could point to a hypoxia-avoidance strategy, which is common in fish (Hill et al., 1973; Burleson et al., 2001; Bell and Eggleston, 2005; Herbert et al., 2011).

In summary, this study shows that the Alaska blackfish is as much an air-breathing fish as other species, despite inhabiting the Arctic. It has a high capacity for air breathing, and uses it to support its basic oxygen demands when faced with hypoxia, which is thus the likely selective pressure that maintains the ability to breathe air in this species. There are still unanswered questions regarding the physiological acclimation and adaptation of Alaska blackfish to hypoxia and cold, particularly the possible mechanisms involved in hypoxic survival during prolonged winter submergence. Future studies should attempt to incorporate investigations of behavioural and ecological aspects of Alaska blackfish biology.

MATERIALS AND METHODS

Animal collection

All procedures were approved by the University of Alaska Anchorage (UAA) Institutional Animal Care and Use Committee, and animals were collected under appropriate Alaska Department of Fish and Game permits. Alaska blackfish were collected from Rabbit Slough and Duck Hunters Training Pond (Palmer, AK, USA) in summer and autumn months (batch 1), or Little Campbell Lake (Anchorage, AK, USA) in March 2013 (batch 2), using minnow traps. At the collection time of batch 1, there was no ice and water temperature was high (~12–15°C). At the collection time of batch 2, Little Campbell Lake was covered with a ~1-m-thick ice layer and the water temperature was 3–4°C. The average P_{O_2} was 3–4 kPa, but approached 0 kPa near the bottom. During initial collection attempts in Little Campbell Lake it became clear that the depth at which the traps were positioned under the ice was crucial for success. Fish were easily captured from shallower depths, but no fish were captured when the traps were placed on the severely hypoxic bottom. Similarly, no fish could be collected from Duck Hunters Training Pond in March, where P_{O_2} was 0.0 kPa at all depths, despite numerous attempts.

Temperature acclimation

Fish (N=28, 48±4 g; mean ± s.e.m.) were maintained in the UAA vivarium under a 12 h:12 h light:dark photoperiod. Individuals from batch 1 had been kept in the facilities at 12–15°C for more than 12 months prior to acclimation to 5°C or experimentation at 15°C, and were distributed evenly between the two acclimation temperatures and oxygen levels (four experimental treatments) to avoid bias. Individuals from batch 2 were kept in the facilities at 5°C for a minimum of 1 week prior to acclimation to 15°C or experimentation at 5°C and were likewise distributed evenly between the four experimental treatments. Individuals from both batch 1 and 2 were thus kept at either their original temperature (15°C for batch 1 and 5°C for batch 2) or acclimated to the other temperature (5°C for batch 1 and 15°C for batch 2). Individuals were randomly assigned to two tanks per temperature. Final acclimation temperature was obtained by decreasing the temperature gradually ~1°C per day or raising the temperature gradually ~2°C per day. This ultimately resulted in individuals that had been kept at the target temperature for 8 to 40 days, except four of the 15°C fish from batch 1 that had been kept at 15°C for 12 months (see supplementary material Fig. S1B,D) and seven of the 5°C fish from batch 2 that were captured at ~4°C and thus had been acclimatized to low temperature for several months in nature (see supplementary material Fig. S1A,C). Despite the differences in acclimation history, there was no significant correlation between standard $\dot{M}_{O_2}$ and duration of acclimation (supplementary material Fig. S1), indicating that the acclimation period to both 5°C and 15°C was sufficient. Four cooler/heater systems (Teco-TR20, Senkor Group, Inc., Terrell, TX, USA) were used to regulate temperature. During acclimation, fish were fed to

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satiation every 3 or 4 days with bloodworms, but food was withheld at least 24 h prior to respirometry. Two-thirds of the water in each aquarium was changed once a week and levels of nitrite, nitrate and ammonia were below recommended levels (Tetra EasyStrips, Tetra, Blacksburg, VA, USA).

Respirometry setup

The experimental setup consisted of a large oval tank (190 l) that housed both a bimodal respirometer [2.3 l, poly(methyl methacrylate)] (for details, see Lefevre et al., 2011; Lefevre et al., in press) and a traditional respirometer [1.9 L, poly(methyl methacrylate), tube-shaped, Ø=11 cm, L=20 cm]. Temperature of the oval tank was controlled by a Teco-TR20 cooler/heater system. Temperature and P_{O_2} in air and water in the bimodal respirometer were measured continuously 12 times per minute using two fibre optic oxygen sensors with integrated temperature sensors (Visiferm DO, Hamilton Company, Bonaduz, GR, Switzerland) and data collected with hardware and software designed at Aarhus University, Denmark. The system also controlled the pumps that flushed the two phases. Water P_{O_2} and temperature in the traditional respirometer was recorded 12 times per minute with an optical oxygen meter and accompanying temperature sensor (FireStingO_2, PyroScience GmbH, Aachen, Germany) using the Firesting Logger software. An on–off timer with 15 min intervals was used to control the pump flushing the chamber with aerated or hypoxic water. To obtain hypoxia, the tank water was bubbled with nitrogen gas and the flow rate manually adjusted to achieve the appropriate level of hypoxia. The hypoxic level (P_{O_2} ~2.5 kPa) was chosen in relation to the average P_{O_2} measured at the time and place where the fish (batch 2) were collected in Little Campbell Lake in March 2013.

Measurement of standard $\dot{M}_{O_2}$ and respiratory partitioning in normoxia and hypoxia

A carefully planned sequence of measurements was performed on each individual, as illustrated in Fig. 7. Temperature-acclimated individuals were randomly assigned to one of two treatments (normoxia, 19.8 kPa; hypoxia, 2.5 kPa), for a total of four measurement groups: 5°C normoxia, 5°C hypoxia, 15°C normoxia and 15°C hypoxia. Average oxygen and temperature values for the different groups during respirometry are provided in Table 3. Each fish was first placed in the bimodal intermittent-closed respirometer and $\dot{M}_{O_2}$ measured for 24 h. This first step of the protocol was identical for all four groups, and was chosen over a randomized design (e.g. measuring half the fish without access to air first) because it was not known to what degree restricted air access in hypoxia would cause hypoxemia and potentially affect subsequent measures of respiratory partitioning. At 5°C (both normoxia and hypoxia), the water in the respirometer was renewed for 15 min every hour, and the air space was flushed for 2 min every hour, giving one $\dot{M}_{O_2}$ point per hour. At 15°C the water and air phases were flushed every 30 min (5 min in water, 2 min in air) because of the more rapid decline of P_{O_2}. To make the 5°C and 15°C measurements comparable, one point per hour is presented. Background measurements were performed in both normoxia and hypoxia, and all reported $\dot{M}_{O_2}$ values have been corrected accordingly. Water and air $\dot{M}_{O_2}$ were calculated as described in detail by Lefevre et al. (Lefevre et al., 2011). Total $\dot{M}_{O_2}$ was calculated for each point as the sum. Standard $\dot{M}_{O_2}$ with access to air was then calculated as the 10th percentile (Van den Thillart et al., 1994; Davoodi and Claireaux, 2007; Dupont-Prinet et al., 2010; Nelson and Chabot, 2011) of all the $\dot{M}_{O_2}$ points during the 24 h measurement (for each fish). Fish that did not appear to enter a resting state during this period were omitted from further calculations (Fig. 7).

Measurement of $\dot{M}_{O_2}$ without access to air in normoxia and under declining P_{O_2} (P_{crit})

Fish that had been exposed to normoxia were moved directly from the bimodal respirometer to a traditional respirometer (without access to air), thereby allowing for a within individual assessment of the effect of restricted air access in normoxia (Fig. 7). This sequence was chosen deliberately over a random design (e.g. exposing half of the individuals to restricted air access

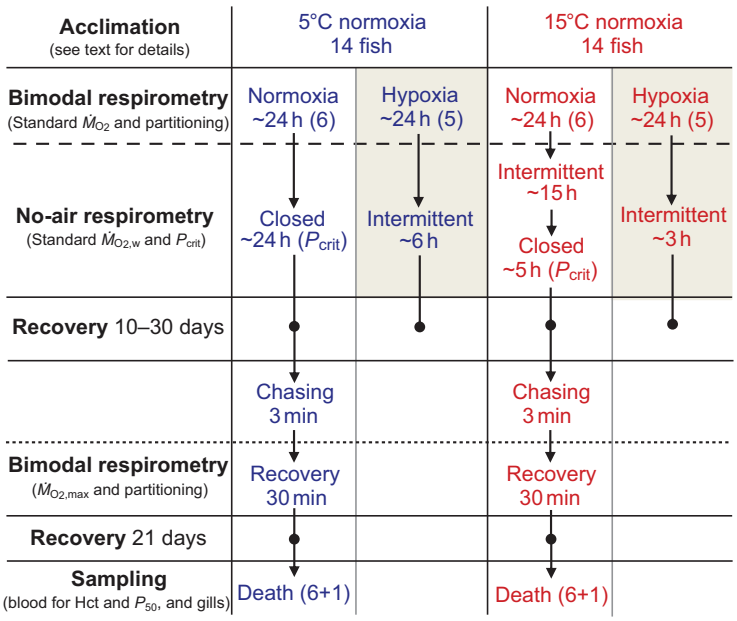

Fig. 7. Illustration of the experimental protocol. Arrows indicate sequential measurements on the same individual. Numbers in parentheses indicate the number of fish included in data analysis in each treatment. Acclimation time varied because only one fish could be measured at a time, overall from 8–40 days up to 12 months (four 15°C fish had been kept at 15°C for 12 months and ten 5°C fish were captured at ~5°C and had thus been acclimatised to ~5°C for several months). A total of 28 fish were acclimated (14 at each temperature), but some fish (1 at each temperature in normoxia and 2 at each temperature in hypoxia) were omitted because a resting state was not obtained during the 24 h measurement period (bimodal intermittent-closed respirometry). The two omitted fish in normoxia were, however, included in the sampling of blood and gills at the end. The experimental design allowed for repeated-measures analysis of the effect of restricted air access at both acclimation temperatures in normoxia or hypoxia (Fig. 2). The dashed horizontal line indicates that the fish was moved directly from the bimodal to the no-air respirometer. Fish that had been exposed to prolonged hypoxia (grey boxes) were not used for other measurements. Normoxic fish that had been exposed to a P_{crit} measurement (gradual short-term hypoxia) were also used for measurement of $\dot{M}_{O_2}$ after chasing (i.e. $\dot{M}_{O_2,max}$) and the partitioning of oxygen uptake after chasing. A minimum of 10 days recovery in normoxia was allowed after measurement of P_{crit}. The dotted horizontal line indicates that the fish was immediately placed into the bimodal respirometer after chasing. After a further 3 weeks of recovery from $\dot{M}_{O_2,max}$ measurements, blood and gills were sampled from these fish for analyses of haematocrit, haemoglobin P_{50}, and parameters of gill morphology.

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Table 3. Oxygen and temperature levels during respirometry

	Acclimation at 5°C		Acclimation at 15°C	
	Normoxia	Hypoxia	Normoxia	Hypoxia
Measurement temperature (°C)	5.4±0.1	6.0±0.3	16.2±0.2	16.0±0.2
$P_{O_2,w}$ (with air, kPa)	20.8±0.2	2.4±0.5	19.5±0.4	2.8±0.8
$P_{O_2,a}$ (kPa)	20.9±0.1	19.1±0.4	20.5±0.2	18.4±1.1
$P_{O_2,w}$ (no air, kPa)	18.9±0.2	2.1±0.3	19.9±0.5	2.5±0.3

Values are means ± s.d. Measurement temperature is the average temperature measured in the water phase in the bimodal respirometer. $P_{O_2,w}$ is the mean P_{O_2} in the water phase. $P_{O_2,a}$ is the mean P_{O_2} in the air phase.

first) because the effect of restricted air access on subsequent measurements was unknown. At both 5°C and 15°C, the same fish was used to measure standard $\dot{M}_{O_2}$ without air access (standard $\dot{M}_{O_2,w}$) in normoxia, and subsequently characterize the response in $\dot{M}_{O_2,w}$ to gradually decreasing P_{O_2} (using closed respirometry). The latter also allowed for determination of P_{crit}. For the 5°C fish, P_{O_2} decreased very slowly, and water flow was therefore ceased immediately and measurements continued until P_{O_2} reached 1.3 kPa (~24 h), allowing calculation of both standard $\dot{M}_{O_2,w}$ and P_{crit}. At 15°C, P_{O_2} decreased more rapidly as a result of the generally higher $\dot{M}_{O_2}$, and this allowed for ~15 h of intermittent-closed respirometry (15 min closed, 15 min open) before the respirometer was sealed and P_{O_2} allowed to decrease to ~2.6 kPa (~5 h). For all fish, the standard $\dot{M}_{O_2,w}$ was calculated as the lowest 10th percentile of the oxygen-independent data points (i.e. taken prior to P_{crit}). For each individual fish at both 5°C and 15°C, P_{crit} was determined as the intersection between lines representing oxygen-independent and oxygen-dependent $\dot{M}_{O_2,w}$ (Ultsch et al., 1980; Berschick et al., 1987; Pörtner and Grieshaber, 1993; Nilsson et al., 2010). Because a number of the fish were spontaneously active in the closed respirometer (see Fig. 4), and because it was not known whether the $\dot{M}_{O_2,w}$ measured without access to air would reflect the 'true' standard $\dot{M}_{O_2}$, standard $\dot{M}_{O_2}$ measured in normoxia with access to air (white empty bars in Fig. 2) was utilized as oxygen-independent standard $\dot{M}_{O_2}$. Oxygen-dependent $\dot{M}_{O_2,w}$ was determined as the slope of a line fitted to all $\dot{M}_{O_2,w}$ values that fell below the standard $\dot{M}_{O_2}$.

Measurement of $\dot{M}_{O_2}$ without access to air in hypoxia

Fish that had been exposed to 24 h hypoxia (with access to air) were moved directly from the bimodal respirometer to a traditional respirometer (without access to air), and used to measure $\dot{M}_{O_2,w}$ without air in hypoxia, using intermittent-closed respirometry (Fig. 7). The first of the 5°C fish lost equilibrium after 13 h, and the following fish were therefore taken out after 4–6 h, to avoid loss of equilibrium during the measurement. The corresponding measurements on 15°C fish were terminated after ~3 h because of loss of equilibrium. Standard $\dot{M}_{O_2,w}$ was then calculated as the 10th percentile.

Measurement of $\dot{M}_{O_2,max}$

Following 10–30 days after measurement of standard $\dot{M}_{O_2}$ and P_{crit}, $\dot{M}_{O_2,max}$ was measured in the individuals from the 5°C and 15°C normoxic experiments, using body mass, length and pictures to identify individual fish (Fig. 7). $\dot{M}_{O_2,max}$ was measured by placing a fish in an oval tank (190 l; at the acclimation temperature of the fish), chasing the fish to exhaustion for 3 min and then immediately transferring the fish to the bimodal respirometer. The chase-protocol was considered to be the most appropriate way to induce $\dot{M}_{O_2,max}$ in this species and is generally considered a robust method for inducing $\dot{M}_{O_2,max}$ (Reidy et al., 1995; Clark et al., 2013). $\dot{M}_{O_2}$ was measured for 10–20 min after the fish was placed in the respirometer, to determine respiratory partitioning after exercise, but only the initial 1–3 min, where $\dot{M}_{O_2}$ was highest, were used to calculate $\dot{M}_{O_2,max}$. AAS was calculated as $\dot{M}_{O_2,max}$ minus the standard $\dot{M}_{O_2}$ previously determined for each fish using bimodal intermittent-closed respirometry. FAS was calculated as $\dot{M}_{O_2,max}$/standard $\dot{M}_{O_2}$.

Blood and tissue measurements

Fish utilized in the respirometry experiments were maintained in the UAA vivarium at their respective acclimation temperatures for an additional 3 weeks, giving a total temperature acclimation time of at least 2 months. Thereafter, they were anaesthetized with buffered tricaine methanesulphonate (MS-222) (0.2 g l^{-1} MS-222 + 0.2 g l^{-1} $NaHCO_3$) until opercular movements ceased and sampled for measurements of haemoglobin O_2 affinity, haematocrit and gill morphology. Fish were subsequently killed by decapitation followed by pithing. Fish from the hypoxic experiments, at both temperatures, were not used for these measurements (Fig. 7).

Haemoglobin oxygen affinity

Haemoglobin O_2 affinity was measured in haemolysate to evaluate whether temperature acclimation was associated with changes in blood O_2 affinity. Blood was sampled by cardiac puncture immediately following anaesthetisation. Red blood cells were separated from the plasma by centrifugation (4000 r.p.m., 5 min at 4°C) and washed three times in physiological saline. The red blood cells were stored at −80°C before being shipped on dry ice from UAA to Aarhus University (Denmark), where the analyses were performed. Aliquots of frozen red blood cells obtained from individual fish were added to 2× volume 0.2 M HEPES buffer (pH=7.4), and samples were centrifuged (4000 ***g***, 5 min) to remove cellular debris. Haemolysates (unstripped haemoglobin containing endogenous allosteric cofactors) were diluted in 0.1 M HEPES buffer (pH=7.4 at 20°C) to a final haem concentration of 0.6 mmol l^{-1}. Equilibrium between haemoglobin and O_2 was monitored using a modified gas diffusion chamber. Pure N_2 gas (>99.998%) was mixed with atmospheric air using two serial connected Wösthoff gas mixing pumps (Bochum, Germany), to create humidified gas mixtures at varying P_{O_2} that were allowed to equilibrate over an ultrathin 4 µl haemoglobin sample. The full saturation range was found by monitoring the absorbance at 426 nm using a photometer (Model 1100 M, Eppendorf AG, Hamburg, Germany) and a linearizer (Type L1853, Eppendorf AG, Hamburg, Germany) during equilibration with pure N_2 and pure O_2, and fractional saturations (S) were then found by stepwise increases in gas mixture P_{O_2}:

$$S = \frac{A - A_0}{A_{100} - A_0}, \quad (1)$$

where A is the absorbance at a given P_{O_2}, and A_0 and A_{100} are the absorbances recorded during equilibration with pure N_2 and pure O_2, respectively. O_2 equilibrium curves were generated from S as a function of P_{O_2} and P_{50} was found from the zero intercept of the Hill plot {log[S/(1−S)] vs logP_{O_2}} using ≥4 equilibrium steps in the 20–80% saturation range (R^2>0.99). Haemoglobin O_2 equilibrium curves were determined at constant temperatures of 5 and 15°C (±0.2°C).

Haematocrit

Haematocrit was determined as the fractional erythrocyte volume in a capillary tube following centrifugation at 10,000 ***g*** for 3 min.

Gill morphology

The second left gill arch from individual fish was dissected, fixed overnight in 4% paraformaldehyde in 0.1 mol l^{-1} phosphate-buffered saline (PBS; pH 7.4), cryo-protected by overnight treatment in 20% and then 30% sucrose solution, embedded in Tissue-Tek (optimal cutting temperature medium, Sakura Finetechnical Co. Ltd, Tokyo, Japan) and frozen at −80°C. Frozen gill arches were serially sectioned in 14 µm increments with a HM 505 N microtome cryostat (Microm International GmbH, Waldorf, Germany) and mounted on Superfrost slides (VWR, Radnor, PA, USA). The sections were dried at room temperature before storage at −80°C. Frozen sections were

thawed, washed in PBS (3×5 min), stained with haematoxylin and eosin and mounted with an aqueous mounting medium (Vecta Mount AQ, Vector Laboratories, Inc., Burlington, CA, USA) prior to being viewed and digitally captured with an Olympus FSX100 light microscope (Tokyo, Japan) at ×20 magnification. Lamellar height, ILCM height, and ILCM area was measured using ImageJ (Schneider et al., 2012), and the ratio of the ILCM height to lamellar height (Ong et al., 2007; Nilsson et al., 2012) calculated for 6–7 lamellae per 6–7 filaments for a total of 44 measurements per individual fish.

Statistics

Data were analysed in SigmaPlot 12.5 (Systat Software, Inc., Chicago, IL, USA). A Shapiro–Wilk's test was used to confirm normal distribution of the data and the assumption of equal variance was tested using *F*-tests. Initially, a three-way ANOVA was used to analyse the overall effects of temperature, oxygen level and restricted air access, and to identify possible interactions. Because there was a significant interaction between oxygen level and air access, a two-way ANOVA was used to analyse temperature and air access effect on standard $\dot{M}_{O_2}$ in normoxia or hypoxia, whereas a RM two-way ANOVA was used to analyse the effect of temperature and hypoxia on standard $\dot{M}_{O_2}$ with or without restricted air access. A Student's *t*-test was used specifically to compare standard $\dot{M}_{O_2}$ in normoxia with air with standard $\dot{M}_{O_2}$ in hypoxia without air, because this comparison was not included in the other analyses. A two-way ANOVA was also used to analyse effects of temperature and hypoxia on respiratory partitioning (an arcsine transformation was performed to normalize percentage data), whereas a RM two-way ANOVA was used to analyse P_{50}. ANOVA analyses were followed by Holm–Šídák's multiple comparison tests, when overall effects were significant ($P<0.05$). A Student's *t*-test was used to analyse the temperature acclimation effect on $\dot{M}_{O_2,max}$ partitioning during recovery (after arcsine transformation), AAS, FAS, haematocrit, lamellar height, ILCM area and ratio of ILCM height to lamellar height, whereas a Mann–Whitney rank sum test was used for P_{crit}.

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Competing interests

The authors declare no competing financial interests.

Author contributions

S.L. and J.A.W.S. conceptualized the study. S.L., J.A.W.S., C.D. and D.R.P. performed the experiments and analysed the data. S.L., J.A.W.S. and G.E.N. wrote the manuscript.

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Supplementary material

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