

RESEARCH ARTICLE

Aerobic scope fails to explain the detrimental effects on growth resulting from warming and elevated CO₂ in Atlantic halibut

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ABSTRACT

As a consequence of increasing atmospheric CO₂, the world's oceans are becoming warmer and more acidic. Whilst the ecological effects of these changes are poorly understood, it has been suggested that fish performance including growth will be reduced mainly as a result of limitations in oxygen transport capacity. Contrary to the predictions given by the oxygen- and capacity-limited thermal tolerance hypothesis, we show that aerobic scope and cardiac performance of Atlantic halibut (*Hippoglossus hippoglossus*) increase following 14–16 weeks exposure to elevated temperatures and even more so in combination with CO₂-acidified seawater. However, the increase does not translate into improved growth, demonstrating that oxygen uptake is not the limiting factor for growth performance at high temperatures. Instead, long-term exposure to CO₂-acidified seawater reduces growth at temperatures that are frequently encountered by this species in nature, indicating that elevated atmospheric CO₂ levels may have serious implications on fish populations in the future.

KEY WORDS: Oxygen consumption rate, Optimal temperature, Climate change, Ocean acidification, Respirometry, Oxygen and capacity limited thermal tolerance, OCLTT, Carbon dioxide

INTRODUCTION

Fishes have adapted to almost all aquatic habitats on Earth, and they can be found living at temperatures from close to –2°C in the polar regions to +44°C in some African lakes (Nelson, 2006). However, no single species can tolerate the entire temperature range and individual species have evolved the capacity to function within species-specific thermal windows that can be quite narrow (Nelson, 2006). Consequently, there is great concern over the ability of fish to acclimate and adapt to the current ocean warming resulting from rising levels of atmospheric greenhouse gases (Caldeira and Wickett, 2003; Haugan and Drange, 1996; Levitus et al., 2000; Turley et al., 2010). Indeed, biological effects attributed to rising ocean temperatures, such as reduced growth rate and shifts in distribution and abundance, have already been observed for various marine fish species (Perry et al., 2005; Pörtner and Knust, 2007; Southward et al., 1995).

The Atlantic halibut [*Hippoglossus hippoglossus* (Linnaeus 1758)] is an ecologically and economically important species in the

North Atlantic Ocean. They are demersal top predators with specimens weighing over 300 kg, ranking them among the largest teleost species in the world (Haug, 1990). Consequently, fluctuations in their abundance can significantly influence the ecosystem they inhabit (Haug, 1990). The Atlantic halibut is a highly appreciated fish for human consumption, but wild catches have declined drastically over the last decade, concomitant with an increased interest in Atlantic halibut aquaculture (Haug, 1990; Imsland and Jonassen, 2001; Jonassen et al., 1999). The adult Atlantic halibut is a strong swimmer capable of long-distance migrations between summer feeding on the continental shelf and winter spawning at great depths off the continental shelf (Haug, 1990). As juveniles, Atlantic halibut are localized and found in well-defined nursery grounds (Haug, 1990). This more sedentary lifestyle in combination with the fact that young specimens are less resilient to temperature fluctuations makes juvenile Atlantic halibut an interesting model organism when trying to understand how current changes in climate may affect marine fish populations (Imsland and Jonassen, 2001; Jonassen et al., 1999).

It has been hypothesized that temperature limitations of aquatic ectotherms are mainly set by reductions in aerobic scope, which is the difference between standard and maximum metabolic rates (Clark et al., 2013; Farrell et al., 2009; Pörtner and Farrell, 2008; Pörtner and Knust, 2007; Wang and Overgaard, 2007). This hypothesis, often called the oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis, has been the subject of a number of recent reviews (Farrell et al., 2009; Pörtner, 2010; Pörtner and Farrell, 2008; Wang and Overgaard, 2007). Reductions in aerobic scope are suggested to occur when increasing environmental temperature raises the standard metabolism in ectothermic animals, while maximum oxygen supply fails to increase correspondingly (Melzner et al., 2009b; Pörtner and Farrell, 2008). This hypothesis further states that aerobic scope and fitness-related performance traits such as growth rate, locomotion and reproductive success are closely and causally linked. Aerobic scope is therefore often used as a unifying physiological principle to explain reduced fitness and abundance of animals living outside their optimal thermal window (Brett, 1971; Farrell et al., 2009; Fry, 1947; Melzner et al., 2009b; Pörtner and Farrell, 2008; Pörtner and Knust, 2007; Wang and Overgaard, 2007). However, experimental evidence following long-term exposure to elevated temperatures is scarce (Franklin and Seebacher, 2009; Wang and Overgaard, 2007; Clark et al., 2013a; Clark et al., 2013b).

In addition to the oceans becoming warmer, uptake of anthropogenic carbon dioxide (CO₂) from the atmosphere decreases ocean pH (Turley et al., 2010). Ocean acidification has been suggested to reduce aerobic scope further, but even in this case empirical studies are rare and available information is conflicting (Pörtner, 2010; Pörtner and Farrell, 2008). For example, aerobic

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scope is reduced, unaffected or even increased in different species of tropical reef fish following exposure to CO₂-acidified water (Couturier et al., 2013; Munday et al., 2009; Rummer et al., 2013), whereas no effect is observed in Atlantic cod (*Gadus morhua*) living in temperate areas (Melzner et al., 2009a). However, the differences may not be biological but instead may be explained by methodological artifacts associated with the different experimental setups. The reef fish were assessed using unusual respirometry techniques and they experienced short-term exposure to elevated partial pressure of CO₂ (P_{CO_2}) at near-lethal temperatures, while the cod were measured in a swim tunnel respirometer and exposed to elevated P_{CO_2} for 4–12 months at 5°C.

The aim of this study was to test the core assumption of the OCLTT hypothesis, that thermal tolerance is limited by oxygen supply to the tissues, and further to test how the interaction between temperature and reduced pH affects the performance of a marine fish species. Specifically, the effects on growth rate and multiple steps in the oxygen transport cascade were assessed in juvenile Atlantic halibut acclimated for 14–16 weeks to a range of temperatures in either water with the current ocean pH or water where pH was reduced by 0.4 units by CO₂ injection to simulate predicted ocean acidification for the year 2100 (Caldeira and Wickett, 2003; Turley et al., 2010).

RESULTS

The statistical analysis revealed that there were no tank effects for any of the measured variables. Mortality was low, with ~6% of the fish dying during the experimental period, leaving us with a total of 463 fish. Mortalities appeared random, with no correlation to temperature or pH.

Oxygen consumption rate and aerobic scope

There was a significant general increase in aerobic scope with increased temperature ($P < 0.0001$; Fig. 1A). There was also a significant general positive effect of temperature on both standard ($P < 0.0001$) and maximum ($P < 0.0001$) metabolic rates, but the increase in aerobic scope was mainly due to a greater temperature effect on maximum metabolic rate (Fig. 1B). The fish at the two highest temperatures had significantly higher aerobic scope than fish at lower temperatures (Fig. 1A). Fish kept at low pH showed a significant general increase of $14 \text{ mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$ ($\Delta 7\%$) in aerobic scope compared with fish kept at current ocean pH ($P = 0.021$; Fig. 1A).

Oxygen transport variables

Both maximum cardiac flow-generating capacity ($P < 0.0001$) and haematocrit ($P < 0.0001$) showed a significant general increase with temperature (Fig. 2A,B), while increasing temperature significantly decreased the relative ventricular mass ($P < 0.0001$; Fig. 2C). Consistent with the general positive effect of low pH on aerobic scope, there was also a significant general positive effect of low pH on maximum cardiac flow-generating capacity (10% increase; $P = 0.024$; Fig. 2A). The pH effect on maximum flow-generating capacity significantly interacted with temperature, making the pH effect larger at the two higher temperatures ($\Delta 58\%$ and $\Delta 38\%$ at 12 and 18°C, respectively) than the general effect of 10% ($P = 0.0012$; Fig. 2A). No effects of low pH were seen in either haematocrit or relative ventricular mass.

Specific growth rate

The specific growth rate was highest in the three intermediate temperature groups (10–14°C) and was significantly depressed in fish exposed to the two highest and the lowest temperatures

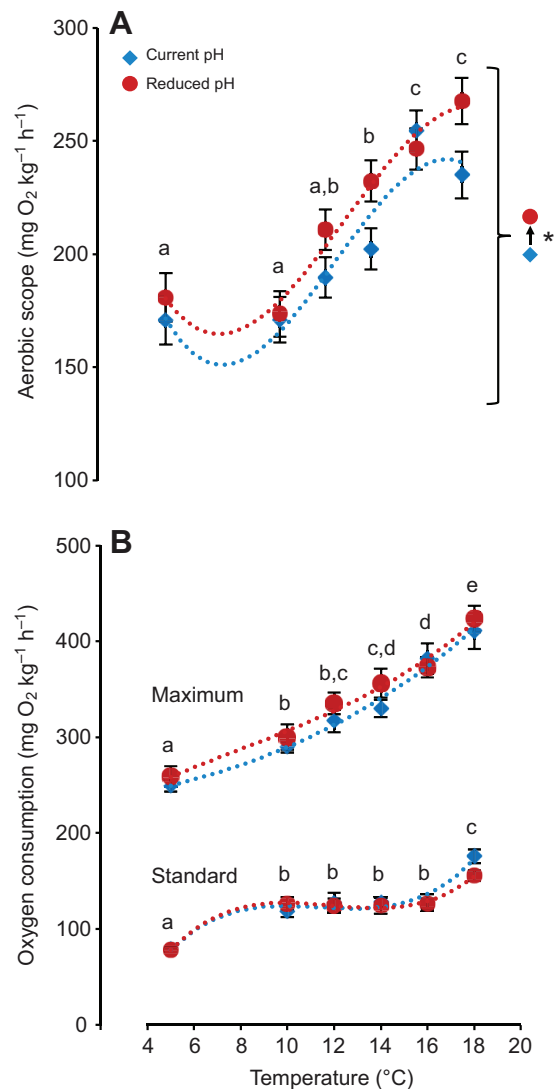


Fig. 1. Effects of temperature and CO₂ on aerobic scope and metabolic rates. Oxygen consumption in Atlantic halibut (*Hippoglossus hippoglossus*) after acclimation for 14–16 weeks to temperatures between 5 and 18°C in seawater with current ocean pH and in seawater with pH reduced by 0.4 units using CO₂ ($n = 8$). Data for (A) aerobic scope and (B) standard metabolic rate and maximum metabolic rate are presented as means \pm s.e.m. with a third-order polynomial trend-line fitted for illustrative purposes only. Different letters indicate significant differences between temperatures ($P < 0.05$) and the asterisk indicates a general significant increased aerobic scope in the groups acclimated to reduced ocean pH ($P = 0.021$).

($P < 0.0001$; Fig. 3). There was no general effect on growth by exposure to low or normal pH. In fish at 5°C, however, growth rate was significantly suppressed (-24%) at the low pH compared with fish at normal pH ($P = 0.008$; Fig. 3).

DISCUSSION

We show that aerobic scope and cardiac performance of Atlantic halibut, a marine teleost species, increase following long-term acclimation to elevated temperatures alone and in interaction with reduced seawater pH. However, this increase in aerobic scope does not translate into improved growth. Instead, growth decreases at the two highest temperatures. Low pH also reduces growth at the lowest temperature, which is a temperature regularly encountered by this species in nature (Langston et al., 2002).

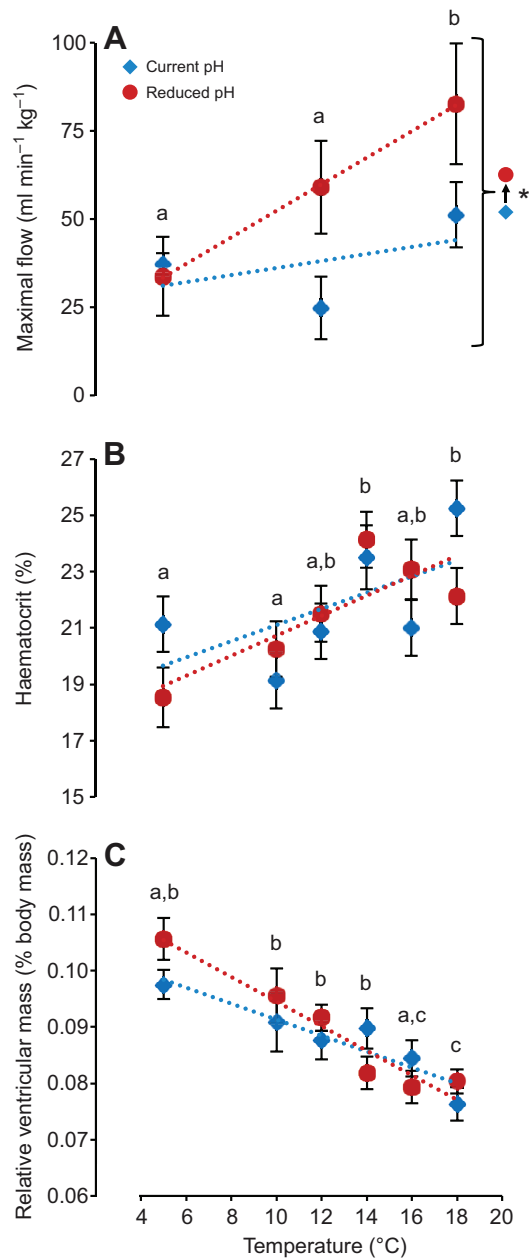


Fig. 2. Effects of temperature and CO₂ on cardiac performance, haematocrit and ventricle size. Oxygen transport variables in Atlantic halibut (*Hippoglossus hippoglossus*) after acclimation for 14–16 weeks to temperatures between 5 and 18°C in seawater with current ocean pH and in seawater with pH reduced by 0.4 units using CO₂. Data for (A) maximum cardiac flow-generating capacity ($N=6$), (B) haematocrit ($N=13$ – 23) and (C) relative ventricular mass ($N=21$ – 31) are presented as means \pm s.e.m. with a linear trend-line fitted for illustrative purposes only. Different letters indicate significant differences between temperatures ($P<0.05$) and the asterisk indicates a general significant increased maximum cardiac flow-generating capacity in the groups acclimated to reduced ocean pH ($P=0.024$).

The present study probably represents the most rigorous and comprehensive long-term experimental test of the hypothesis of OCLTT across a broad temperature range in any ectothermic animal. Thus, the temperature range encompasses: temperatures that juvenile Atlantic halibut typically encounter in nature (2–8°C) (Bigelow and Schroeder, 1953; Cargnelli et al., 1999; Haug, 1990); the optimal temperature range for growth (12–15°C) (Imsland and Jonassen,

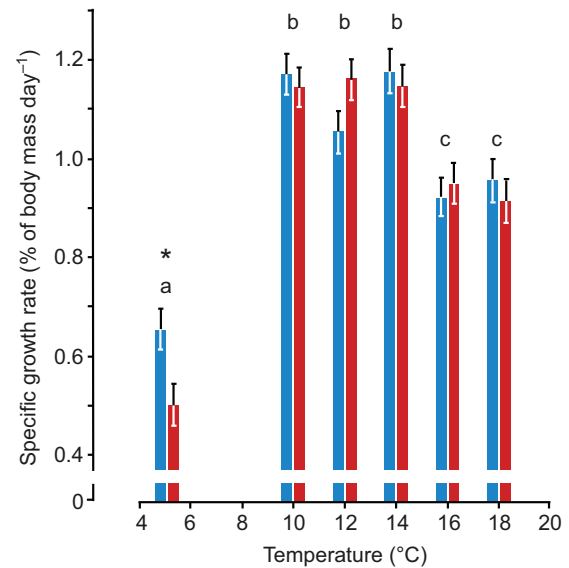


Fig. 3. Effects of temperature and CO₂ on specific growth rate. Specific growth rate in Atlantic halibut (*Hippoglossus hippoglossus*) after acclimation for 14–16 weeks to temperatures between 5 and 18°C in seawater with current ocean pH [blue (left) bars] and in seawater with pH reduced by 0.4 units using CO₂ [red (right) bars] ($N=34$ – 42). Data are presented as means \pm s.e.m. Different letters indicate significant differences between temperatures ($P<0.05$) and the asterisk indicates a significantly lower growth rate in the group acclimated to reduced ocean pH at 5°C ($P=0.008$).

2001; Jonassen et al., 1999); as well as the upper temperature (i.e. 18°C) where non-specific stress responses such as decreased number of circulating haematocytes and increased levels of serum lysozyme are observed (Langston et al., 2002).

Our results show a clear mismatch in the response to temperatures between growth rate (Fig. 3) and aerobic scope (Fig. 1A). In line with previous studies on optimal temperatures for growth in similar sized juveniles of Atlantic halibut fed *ad libitum*, the specific growth rate was highest at the three intermediate temperatures (10–14°C) (Imsland and Jonassen, 2001; Jonassen et al., 1999), while none of the variables related to oxygen transport (aerobic scope, maximum and standard metabolic rates, maximum flow-generating capacity, haematocrit or relative ventricular mass) were maximum at this temperature range. The absence of an optimal temperature above which the aerobic scope decreases goes against the prevailing hypothesis, which states that oxygen supply is limited at supra-optimal temperatures. The increasing aerobic scope with temperature is explained by positive effects of temperature on both maximum flow-generating capacity of the heart and haematocrit. Thus, oxygen transport capacity in the long-term acclimated Atlantic halibut does not decline at temperatures approaching the upper lethal temperatures as been reported for fish exposed to acute temperature increases (Eliason et al., 2011; Farrell, 2009). The aerobic scope–temperature profiles of thermally acclimated fish increase with temperature up to near-lethal temperatures, as previously reported for brown bullhead (*Americus nebulosus*), European sea bass (*Dicentrarchus labrax*) and turbot (*Scophthalmus maximus*) (Claireaux et al., 2006; Fry, 1947; Mallekh and Lagardere, 2002).

The fact that aerobic scope is highest at the two warmest temperatures while growth rate declines at these temperatures also contradicts another central assumption of the OCLTT hypothesis, which states that physiological performances such as growth rate should decline in concert with aerobic scope (Pörtner, 2008; Pörtner,

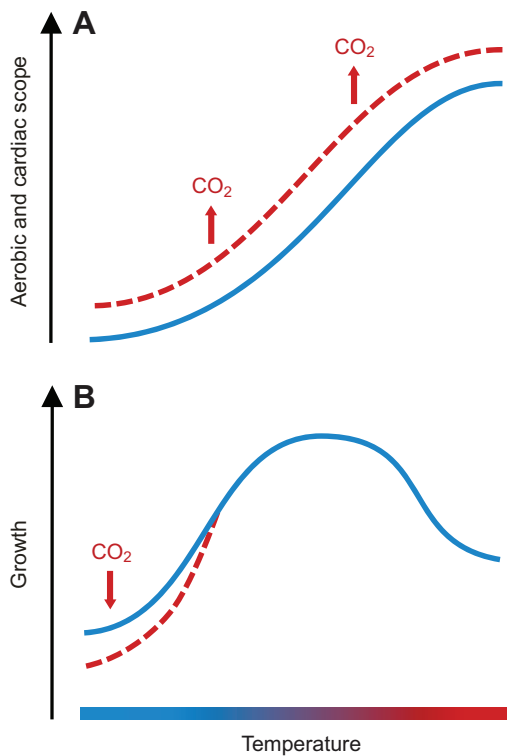


Fig. 4. Thermal performance mismatch between aerobic scope and growth. Conceptual figure of the mismatch in optimal temperatures for different performances in a marine fish. Aerobic and cardiac scope (A) fails to explain the detrimental effects on growth (B) resulting from the climate change stressors warming and CO₂. This illustrates the complex interaction between future elevated temperatures and ocean acidification (red dotted line), and highlights the problem of inferring fitness from measurements of a single performance.

2010). The hypothesis further predicts that temperature limitations on aerobic scope can be used to explain tolerance, performance and distribution of wild populations (Pörtner and Farrell, 2008; Pörtner and Knust, 2007). The present study suggests that different performance indices, such as growth rate and aerobic scope, have different optimal temperatures and that neither temperature optima necessarily reflect the temperatures at which the species is most abundant in nature. This is in agreement with models on thermal adaptation in terrestrial ectotherms, which demonstrate that natural selection can favour selection of a body temperature where specific performance traits are sub-optimal, particularly in species inhabiting environments with large temperature fluctuations (Asbury and Angilletta, 2010; Deutsch et al., 2008; Martin and Huey, 2008).

Based on the OCLTT hypothesis, previous reviews have also predicted that low seawater pH, when interacting with suboptimal temperature, will decrease aerobic scope (Pörtner, 2010; Pörtner and Farrell, 2008). However, again contrary to these predictions, yet in agreement with recent findings in coral reef fish (Rummer et al., 2013), we show that aerobic scope is higher in fish acclimated to elevated water CO₂. Consistent with the positive effect of low pH on aerobic scope, the maximum cardiac flow-generating capacity also increases after acclimation to low pH, at least at 12 and 18°C. While the exact mechanisms for the enhanced pumping capacity of the low pH-acclimated hearts remain unknown, heart performance was assessed *in situ* using a standardized Ringer's solution with the same pH in both the normal and high CO₂ treatments, suggesting that the improved maximum flow-generating capacity was intrinsic to the

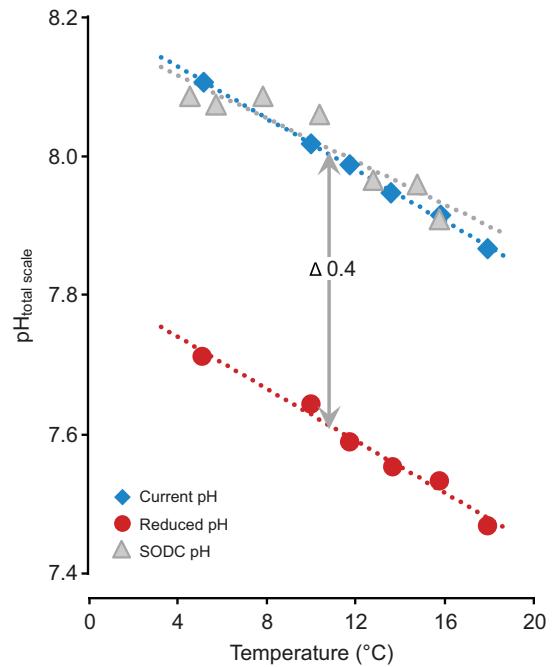


Fig. 5. Temperature dependence of pH_{total scale} in seawater. Data are given as mean values of pH_{total scale} from the six different temperature manipulation treatments in either seawater with the current ocean pH or in seawater with pH reduced by 0.4 units with CO₂ gas, together with seven measurements from the Swedish Oceanographic Data Centre (SODC) recorded in the Gullmarsfjord. A linear trend line is fitted to each data set for illustrative purposes only.

heart and independent of perfusion fluid pH. Tropical fish exposed to increased CO₂ levels display increased spontaneous swimming activity (Munday et al., 2012). If the halibut in the present study had a similar behavioural response to the low pH treatment, an interesting possibility is that the improved heart performance was a training effect, as observed in many other fish species (Davison, 1997).

The negative effects of long-term acclimation to low pH on growth rate at 5°C further supports that aerobic scope and growth rate may not necessarily be causally linked. The concept of aerobic scope as the unifying physiological principle of thermal tolerance therefore needs revision, and should include the complex interaction between future elevated temperatures and ocean acidification (Fig. 4). Given the negative effect of low pH observed in growth rate at a temperature where juvenile Atlantic halibut typically occur in nature, this finding raises concerns in relation to future climate scenarios. While increasing temperatures have been shown to decrease distribution ranges of various fish species at the warm edge of their distribution ranges (Perry et al., 2005), the present study suggests that decreased ocean pH may instead limit distribution and productivity at the cold edge of a species' distribution range. Nevertheless, further studies are needed to reveal the exact mechanisms of this response, as it was not caused by limitations in aerobic scope.

Conclusions

The present study shows that aerobic scope and cardiac performance in Atlantic halibut are not compromised following long-term acclimation to temperature and pH conditions predicted for the near future. The absence of an optimal temperature above which aerobic scope decreases, and the mismatch between aerobic scope and growth, are findings that are difficult to reconcile with the hypothesis that oxygen supply is limiting whole-animal performance and fitness at

Table 1. Environmental data measured twice a week for 14–16 weeks at six different temperatures in either seawater with the current ocean pH or in seawater with pH reduced through bubbling with CO₂ gas

Acclimation temperature	CO ₂ treatment	Replicate	Temperature (°C)	Alkalinity (μmol kg ⁻¹)	pH _{total scale} ^{in situ}	pH _{total scale} ^{STP}	P _{CO₂} ^{in situ} (μatm)	P _{CO₂} ^{STP} (μatm)	P _{CO₂} ^{N5} (μatm)	P _{CO₂} ^{STP×N5} (μatm)
5	Current pH	1	5.20±0.11	2190±25	8.10±0.01	8.19±0.01	337±10	258±8	331±10	388±10
		2	5.11±0.10	2204±24	8.12±0.01	8.20±0.01	323±9	248±7	320±10	376±9
	Reduced pH	1	5.10±0.11	2159±31	7.71±0.03	7.79±0.03	896±52	708±42	888±51	1031±51
		2	5.11±0.11	2158±40	7.71±0.02	7.79±0.02	881±35	695±28	872±35	1015±34
10	Current pH	1	9.97±0.02	2199±25	8.02±0.01	8.19±0.01	422±12	257±7	260±7	371±7
		2	10.04±0.02	2223±29	8.01±0.01	8.18±0.01	432±10	261±6	264±6	376±6
	Reduced pH	1	10.02±0.01	2143±43	7.67±0.02	7.83±0.03	1007±51	639±35	617±32	898±32
		2	10.02±0.02	2219±33	7.61±0.02	7.76±0.03	1219±71	782±49	748±44	1029±44
12	Current pH	1	11.73±0.02	2244±51	7.99±0.01	8.19±0.01	468±11	262±6	243±6	373±6
		2	11.69±0.02	2266±34	7.99±0.01	8.18±0.01	478±13	268±8	249±7	379±7
	Reduced pH	1	11.71±0.02	2224±24	7.61±0.02	7.78±0.02	1223±52	727±34	636±27	964±27
		2	11.71±0.03	2234±26	7.57±0.02	7.74±0.02	1382±71	831±47	720±37	1048±37
14	Current pH	1	13.56±0.07	2226±27	7.95±0.01	8.18±0.01	516±12	266±6	224±5	374±5
		2	13.58±0.07	2257±39	7.94±0.01	8.17±0.01	537±15	277±8	233±7	383±6
	Reduced pH	1	13.60±0.08	2236±37	7.53±0.02	7.72±0.03	1540±86	863±53	666±37	1047±37
		2	13.63±0.08	2230±23	7.58±0.02	7.78±0.02	1347±51	742±30	581±22	963±22
16	Current pH	1	15.80±0.04	2254±30	7.91±0.01	8.18±0.01	579±12	270±6	202±4	377±4
		2	15.82±0.04	2271±42	7.91±0.01	8.18±0.01	583±13	272±6	203±4	378±4
	Reduced pH	1	15.72±0.04	2244±31	7.54±0.02	7.76±0.02	1527±77	780±44	538±27	978±27
		2	15.72±0.04	2222±28	7.53±0.03	7.75±0.03	1570±94	809±53	553±33	994±33
18	Current pH	1	17.86±0.03	2226±27	7.87±0.01	8.16±0.01	642±14	276±6	184±4	381±4
		2	17.88±0.02	2170±26	7.86±0.01	8.15±0.01	643±15	276±6	183±4	381±4
	Reduced pH	1	17.86±0.07	2233±25	7.47±0.02	7.73±0.02	1791±85	854±45	513±25	1013±24
		2	17.90±0.06	2242±22	7.46±0.02	7.71±0.03	1861±105	892±58	532±31	1033±30

Salinity during the acclimation period was 32.0±0.14 ppm (mean ± s.e.m.) and was measured for all 24 treatments simultaneously directly from the incoming seawater. Superscripts for pH_{total scale} and P_{CO₂} are as follows: *in situ*, values at ambient temperature; STP, values as if to be measured at standard temperature (0°C) and pressure (1 atm); N5, P_{CO₂} values normalized to 5°C according to the isochemical temperature effect on seawater of 4.23% according to Takahashi et al. (Takahashi et al., 1993); STP×N5, P_{CO₂}^{N5} values as if to be measured at standard temperature (0°C) and pressure (1 atm).

supra-optimal temperatures in fish. Whether this species is an exception or represents the rule awaits further experiments. Nonetheless, a cause for concern is that the growth rate of a commercially and ecologically important species such as the Atlantic halibut is reduced by CO₂ levels predicted for the end of the century at temperatures that this species normally encounters in nature.

MATERIALS AND METHODS

Animals

A total of 493 fully metamorphosed, juvenile Atlantic halibut (16.4±0.2 g) were transported by air-freight from a commercial halibut supplier (Fiskey Ltd, Hjalteyrri, Iceland) to the experimental facilities at the Sven Lovén Centre for Marine Sciences, Kristineberg, on the Swedish west coast. All animal husbandry conditions and experimental protocols were approved by the Ethical Committee of Gothenburg (permits 221-2010 and 329-2010). The fish were kept in tanks with flow-through seawater (32.0±0.14 ppt) at 6°C, under a 12 h:12 h light:dark photoperiod, and were fed once a day with 2.5% of body mass of commercial fish feed. This represents an excess feeding regime as the ration given exceeded the consumption. Leftover food was automatically flushed out of the tanks within 2 h.

Experimental design

At the start of the experiment (14 days after arrival), approximately 20 animals were placed in each of twenty-four 100 litre tanks supplied with flow-through seawater (2.5 l min⁻¹), each from its own aerated 200 litre header tank, where temperature and pH were controlled. Over a period of 13 days, the temperature was adjusted to create six temperature regimes of 5, 10, 12, 14, 16 and 18°C, with quadruple tanks per temperature, and continuous temperature logging. For each temperature regime, two tanks were supplied with water at current ocean pH, while two tanks were supplied with water with pH reduced by 0.4 units. The reduction in pH was accomplished by bubbling of pure CO₂ into the header tank (controlled by Aqua Medic computers, Bissendorf, Germany). Twice weekly, the pH was measured (WTW pH 3310 with a SenTix 41

electrode, Weilheim, Germany) and alkalinity was calculated (Eppendorf BioPhotometer, Hamburg, Germany) (Sarazin et al., 1999). The pH was measured in the scale adopted for seawater (normally referred to as the pH_{total scale}), and this was done using TRIS (2-amino-2-hydroxy-1,3 propanediol) and AMP (2-aminopyridine) buffers (Riebesell et al., 2010). The overall difference in pH_{total scale} was maintained between temperatures (-0.4±0.02 pH_{total scale} units). The temperature dependence of seawater pH_{total scale} corresponds both with the natural temperature dependence measured in the Gullmarsfjord (Fig. 5) and with that reported in the literature (Hunter, 1998). The pH_{total scale} in the setup corresponds to a pH_{total scale} at STP (i.e. as if all measurements were taken at standard temperature and pressure) of 8.2 for the control tanks and 7.8 in the low pH treatments. The partial pressure of CO₂ (P_{CO₂}) was calculated using pH_{total scale} and alkalinity, using the dissociation constant for KHSO₄ from Dickson (Dickson, 1990) and the CO₂ equilibrium constants from Roy et al. (Roy et al., 1993). All P_{CO₂} values were normalized to 5°C according to the isochemical temperature effect on seawater of 4.23% (P_{CO₂}^{N5}) (Takahashi et al., 1993). This relationship between P_{CO₂}^{N5} and temperature in the setup corresponded well with the relationship reported for the northern parts of the North Atlantic between 1981 and 2001 (Olsen et al., 2003). The P_{CO₂}^{N5} in the setup corresponded to P_{CO₂}^{N5} at STP of 380 μatm for the control tanks and 1000 μatm for the low pH tanks. All measured environmental variables are summarized in Table 1.

Analytical procedures

At the start of the experiment, the fish were weighed and marked individually with Visible Implant Elastomer (Northwest Marine Technology Inc., Tumwater, WA, USA). Following the 14–16 week temperature/pH exposure, each fish (N=35–42 per treatment, adding up to a total of 463 fish) was identified and weighed for calculation of individual specific growth rate, expressed as percentage increase in body mass per day (Imslund and Jonassen, 2001).

Sixteen identical custom-made respirometers (1.2 litres), supplied with water from the respective holding tank, were used to measure the oxygen consumption rate of individual fish. Fibre-optic oxygen meters calibrated in

accordance with the supplier's manual (Oxy-4 Micro with oxygen Micro-optode PSt1, PreSens GmbH, Regensburg, Germany) and connected in line with individual circulation pumps in each respirometer were used to record water oxygen levels. By switching off a time-controlled flush pump, the oxygen consumption rate was calculated from the decrease in oxygen content in the respirometers over a given time (over 10 min at the warmer temperatures and 20 min at the colder temperatures, yielding two to three readings per hour). Eight fish from each temperature/pH regime (four from each of the two replicate tanks, $N=8$) were placed in individual respirometers and allowed to adjust for 24 h. The fish were unfed for 40 h prior to their introduction into the chambers to reduce the risk of residual specific dynamic action. Oxygen consumption rate of the undisturbed fish was then determined for 22 h and the standard metabolic rate was calculated as the average of the three lowest oxygen consumption measurements recorded. Fish were then removed from the respirometers, subjected to encouraged exhaustive exercise for 5 min by manually chasing the fish, and then quickly returned to the respirometers (within 1 min) according to the methods described in Clark et al. (Clark et al., 2013). The oxygen consumption rates were recorded at short intervals for another 2 h. Maximum metabolic rate was defined as the period with the highest oxygen consumption after exercise. Aerobic scope was calculated as the difference between standard and maximum metabolic rates (Farrell et al., 2009; Fry, 1947).

Blood was sampled from the caudal vessels of a subset of fish ($N=13-23$ per experimental group) using a heparinized syringe, and haematocrit was determined using a haematocrit centrifuge and haematocrit capillary tubes.

To determine the relative ventricular mass, the ventricle was dissected out, blotted dry and emptied of blood. The bulbus and atrium were trimmed away for calculation of the relative ventricular mass, expressed as percent of body mass ($N=21-31$ per experimental group, a total of 315 fish).

From the remaining fish, an *in situ* perfused heart preparation modified from Farrell et al. (Farrell et al., 2007) was used to assess the maximum cardiac flow-generating capacity at 5, 12 and 18°C (three fish from each of the two replicate tanks, $N=6$ per experimental group). A halibut Ringer's solution (Rønnestad et al., 2000) containing isoprenaline (200 nmol l⁻¹, for maximum adrenergic stimulation) was used to perfuse the heart preparation, which was immersed in a saline-filled thermostat-controlled bath. All hearts were tested at their treatment temperature. Maximum flow-generating capacity of the heart was assessed by increasing the input pressure until no further increase in cardiac output was observed. Flow was continuously monitored using an in-line 1.0 mm Transonic flow probe connected to a flow-meter (T206, Transonic Systems, Ithaca, NY, USA).

Statistical methods and data presentation

The objective was to investigate the effect of temperature and pH on several variables that were expected to show significant acclimation to the experimental treatments. The following general linear model was used to describe the experimental setup:

$$y = \text{temp} + \text{pH} + (\text{temp} \times \text{pH}) + [\text{tank} (\text{temp} \times \text{pH})] + \text{error}, \quad (1)$$

where the measured variable (y) is explained by two main factors, temperature (temp) and pH, and the interaction effect of these two factors. In addition, to control for tank effects, each group was divided into two replicate tanks and included in the model as a nested factor [tank (temp × pH)]. Also, for the metabolic variables, mass was used as a regressor in the model in order to reduce the error term:

$$y = \text{temp} + \text{pH} + \text{temp} \times \text{pH} + \text{tank} (\text{temp} \times \text{pH}) + b \times x + \text{error}. \quad (2)$$

In this increased model, the mass (x) and the regression parameter (b) are without any apparent interactions and sufficient after model checks. Maximum cardiac flow capacity and relative ventricular mass were natural log transformed before analysis as these parameters did not meet the assumptions for a normal distribution and constant variance. P -values were adjusted for multiple testing using the Holm–Bonferroni method (Holm, 1979). Note that because of the complexity of the study and the high number of tests performed, the risk for type II error was relatively large in some of the more detailed comparisons. Calculations were carried out in SAS 9.2 (SAS Institute, Cary, NC, USA). Data are presented in the text as means ± s.e.m.

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Competing interests

The authors declare no competing financial interests.

Author contributions

All authors conceived and designed the experiment. A.G., F.J., E.J., H.S., O.O.-M., I.E. and M.A. performed the experiments. A.G., F.J., E.J., K.W., H.S. and M.A. analyzed the data. A.G., F.J. and E.S. wrote the paper.

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