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RESEARCH ARTICLE

Warm acclimation and oxygen depletion induce species-specific responses in salmonids

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ABSTRACT

Anthropogenic activities are greatly altering the habitats of animals, whereby fish are already encountering several stressors simultaneously. The purpose of the current study was to investigate the capacity of fish to respond to two different environmental stressors (high temperature and overnight hypoxia) separately and together. We found that acclimation to increased temperature (from 7.7±0.02°C to 14.9±0.05°C) and overnight hypoxia (daily changes from normoxia to 63–67% oxygen saturation), simulating climate change and eutrophication, had both antagonistic and synergistic effects on the capacity of fish to tolerate these stressors. The thermal tolerance of Arctic char (*Salvelinus alpinus*) and landlocked salmon (*Salmo salar* m. *sebago*) increased with warm acclimation by 1.3 and 2.2°C, respectively, but decreased when warm temperature was combined with overnight hypoxia (by 0.2 and 0.4°C, respectively). In contrast, the combination of the stressors more than doubled hypoxia tolerance in salmon and also increased hypoxia tolerance in char by 22%. Salmon had 1.2°C higher thermal tolerance than char, but char tolerated much lower oxygen levels than salmon at a given temperature. The changes in hypoxia tolerance were connected to the responses of the oxygen supply and delivery system. The relative ventricle mass was higher in cold- than in warm-acclimated salmon but the thickness of the compact layer of the ventricle increased with the combination of warm and hypoxia acclimation in both species. Char had also significantly larger hearts and thicker compact layers than salmon. The results illustrate that while fish can have protective responses when encountering a single environmental stressor, the combination of stressors can have unexpected species-specific effects that will influence their survival capacity.

KEY WORDS: Eutrophication, Temperature tolerance, Hypoxia tolerance, Cardiac system, Gill, Oxygen supply and delivery system

INTRODUCTION

Global climate is changing and polar regions are expected to experience severe warming in the future. Because arctic species are usually stenothermic, populations occurring near the higher end of tolerated temperatures will be affected most seriously. Temperature increase, however, is not the only stressor that the animals are subjected to. For example, areas where arctic fishes reside close to their upper thermal limit are eutrophic as a result of agricultural and industrial activities, and waste water from human settlements. Eutrophication is connected to hypoxic events as nutrient enrichment enhances the growth of phytoplankton. Oxygen is particularly depleted at night when photosynthesis does not take place but all organisms respire. These hypoxic and anoxic events have been associated with massive fish deaths worldwide; in the Baltic Sea area in particular, where the current research was performed, the situation is grave. Furthermore, overnight hypoxia is becoming more common with climate change: the formation of algal blooms is promoted, and the respiration of ectothermic organisms increases with increasing temperature (D'Avanzo and Kremer, 1994; Beck and Bruland, 2000; Vaquer-Sunyer and Duarte, 2008; Friedrich et al., 2014). Therefore, the purpose of the current study was to analyse the capacity of fish to respond to both environmental warming and overnight hypoxia separately and together.

The survival of fish under the pressure of environmental changes is dependent on their ability to respond appropriately to two different stressors: temperature increase and intermittent hypoxia. In view of this, it is very surprising that there are only a limited number of studies on the effects of multiple stressors on animals (Todgham and Stillman, 2013). This limitation is serious, as stressors can have synergistic or antagonistic effects (Nikinmaa, 2014). In the absence of studies in which both oxygen level and temperature are altered for a long period of time, temperature increase and hypoxia have been considered to have synergistic effects, meaning that a small change in one stressor could cause a large change in the capacity of animals to respond to either of the stressors, when the animals are simultaneously exposed to the two stressors (McBryan et al., 2013). The probability of synergistic effects of hypoxia and temperature on whole-animal physiology follows from observations that the maximum oxygen consumption of tissues increases with temperature more than the capacity of the circulatory system to supply oxygen to tissues, leading to anaerobic energy production at high temperatures (Fry, 1947; Farrell, 2009; Pörtner, 2010). This might result in acidaemia and hyperkalaemia of venous blood, which will further impair cardiac performance and set the limits for temperature tolerance (Farrell, 2009). As a result of environmental hypoxia, animals are already encountering the performance limits of the oxygen supply system at relatively low temperatures. The oxygen and capacity limited thermal tolerance (OCLTT) model was developed to accommodate such observations (Pörtner, 2010). In agreement with the model, there are findings that temperature tolerance decreases in hypoxic conditions (Verberk et al., 2013) and that high temperatures reduce hypoxia tolerance (e.g. Capossela et al., 2012; McBryan et al., 2013; Lapointe et al., 2014). However, there is ongoing debate about the suitability of the OCLTT model for all ectothermic animals (see Clark et al., 2013 and correspondence on that article).

In previous studies related to the OCLTT hypothesis, two confounding factors have not been taken into account. First, it has not been considered that acclimating to two simultaneously occurring stressors may elicit responses that are different from the responses to a single stressor. Environmental changes commonly

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occur simultaneously and, thus, such studies are required in order to evaluate the responses that may occur in the wild. Second, earlier studies have insufficiently considered the possibility of species-specific responses to the different stressors and their interactions, including effects of acclimation. We addressed both of these questions by studying the responses of two salmonid species, Arctic char [*Salvelinus alpinus* (Linnaeus 1758)] and landlocked salmon (*Salmo salar* m. *sebago* Girard) to conditions mimicking naturally occurring temperature changes and diurnal changes in oxygen level. These species were chosen as they are known to have different thermal tolerances, Arctic char being more cold stenothermal than salmon (e.g. Baroudy and Elliott, 1994) and, therefore, possibly more vulnerable to climate change. The influence of population origin and natural environmental conditions on thermal/hypoxia tolerance (Eliason et al., 2011) were minimised, as the two species originate from the same lake (Lake Saimaa) and were reared in similar conditions, following natural temperature and photoperiod, at a fish hatchery at Lake Saimaa (62°04′N, 28°33′E). In addition to measuring the thermal and hypoxia tolerance, morphological features of the heart and gills (i.e. oxygen supply and delivery system) were assessed in order to evaluate whether the plasticity in these characters could be behind the changes in the thermal and hypoxia tolerance of the animals. In order to address these questions, 1 year old char and salmon were acclimated to four different conditions: (i) warm temperature, normoxia; (ii) warm temperature, daytime normoxia and overnight hypoxia; (iii) cold temperature, normoxia; and (iv) cold temperature, daytime normoxia and overnight hypoxia, for 4 weeks before testing the hypoxia and thermal tolerance of the animals.

RESULTS

The species differed markedly in both thermal and hypoxia tolerance. The critical thermal maximum (CT_{max}) of salmon was on average 1.2°C higher than that of char. With regard to hypoxia tolerance, all the salmon lost equilibrium (indicating that the maximal tolerance to hypoxia had been exceeded) at 13% oxygen saturation (1.4 mg O_2 l^{-1}) within 2 h (during that time the oxygen saturation was kept constant). At this oxygen concentration the char did not show any signs of stress. They lost equilibrium at 6.8±0.08% oxygen saturation (0.7 mg O_2 l^{-1}) on average (Fig. 1).

The CT_{max} of both char and salmon changed substantially after different acclimation conditions. CT_{max} was slightly reduced by long-term overnight hypoxia at both acclimation temperatures and in both species (by 0.2 and 0.4°C, for char and salmon, respectively) (Fig. 1A). However, the increase in CT_{max} upon warm acclimation was similar in the absence and presence of overnight hypoxia, being 1.3 and 2.2°C for char and salmon, respectively.

In char, the increase in acclimation temperature did not influence hypoxia tolerance when the fish were reared in normoxia. Similarly, overnight hypoxia did not change the hypoxia tolerance at the cold acclimation temperature. However, on average char lost equilibrium at 22% lower oxygen level when the fish were reared in a combination of warm temperature and overnight hypoxia (Fig. 1B).

In salmon, warm acclimation and overnight hypoxia more than doubled the hypoxia tolerance. The group acclimated to warm temperature and overnight hypoxia were able to tolerate the reduction in water oxygen content more than nine times longer than the group having the lowest tolerance (cold, normoxia) (Fig. 1C).

The oxygen supply and delivery system also showed species- and acclimation-specific responses to different conditions. The cold-acclimated salmon had heavier ventricles than warm-acclimated individuals, while no such difference was seen in char (Fig. 2A). In both species, however, acclimation to a combination of warm temperature and overnight hypoxia increased the thickness of the compact layer of the ventricles by 17% and 26% for char and salmon, respectively (Fig. 2B, Fig. 3A,B). The ventricles of char were 13% heavier than those of salmon when related to the mass of the fish (Fig. 2A) with 48% thicker compact myocardium (Fig. 2B). Both these structural properties are compatible with higher hypoxia tolerance of char than salmon. Furthermore, warm acclimation alone increased the capillary density in the compact layer by 8% on average and even more so in normoxic char (16%) (Fig. 2C, Fig. 3C,D). Acclimation to overnight hypoxia increased the height of the secondary gill lamellae in salmon (Fig. 2D, Fig. 3E,F).

The *F*- and *P*-values of MANCOVA (fish mass as covariate as warm-acclimated fish were 8.7% heavier and 2.8% longer than cold-acclimated fish, and the normoxia-acclimated fish were 4.6% heavier and 1.9% longer than overnight hypoxia-acclimated fish; Table 1) are presented in Table 2.

DISCUSSION

The present results clearly show that, first, acclimation to different temperatures and to overnight hypoxia can have both antagonistic and synergistic effects, and, second, the responses to the stressors and the changes during acclimation can be species-specific.

A major difference observed between char and salmon was the higher CT_{max} and lower hypoxia tolerance of salmon. The observed difference in temperature tolerance between char and salmon is

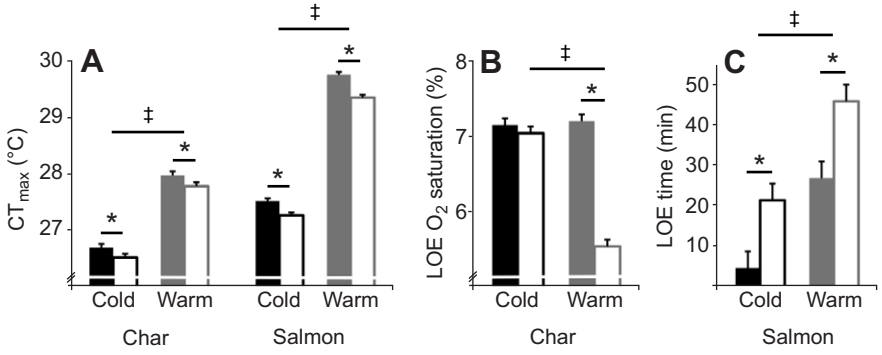

Fig. 1. Influence of temperature and overnight hypoxia acclimation on temperature and hypoxia tolerance of char and salmon. (A) The critical thermal maximum (CT_{max}) of char and salmon acclimated to cold (7.7°C) and warm (14.9°C) temperature with either normoxic conditions (filled bars) or overnight hypoxia (open bars). CT_{max} differed significantly between species ($P<0.001$). (B) The oxygen saturation level at which the char lost equilibrium (LOE) during the hypoxia tolerance test. (C) The time when the salmon lost equilibrium after 13% oxygen saturation was reached. *Significant difference between normoxia and overnight hypoxia acclimation; ‡significant difference between cold- and warm-acclimated fish. Values are means±s.e.m., N=15 per group.

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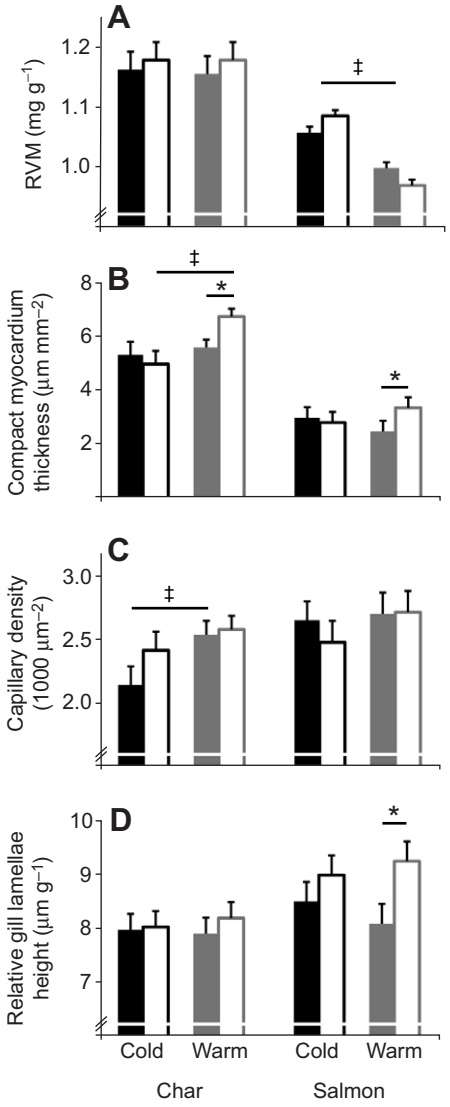

Fig. 2. Influence of temperature and overnight hypoxia acclimation on the oxygen supply and delivery system of char and salmon. (A) The relative ventricle mass (RVM) of char and salmon acclimated to cold (7.7°C) and warm (14.9°C) temperature with either normoxic conditions (filled bars) or overnight hypoxia (open bars). RVM differed significantly between species (P<0.001). (B) The relative thickness of the compact myocardium when compared with the cross-section of the ventricle. Char had a significantly thicker compact myocardium than salmon (P<0.001). (C) The capillary density of the compact myocardium. Warm-acclimated char had a significantly higher capillary density than cold-acclimated char (P<0.05). (D) The relative height of the secondary gill lamellae when compared with the mass of the fish. Acclimation to overnight hypoxia significantly increased the height of the lamellae in contact with water in salmon (P<0.05). *Significant difference between normoxia and overnight hypoxia acclimations; ‡significant difference between cold- and warm-acclimated fish. Values are means±s.e.m., N=10 per group.

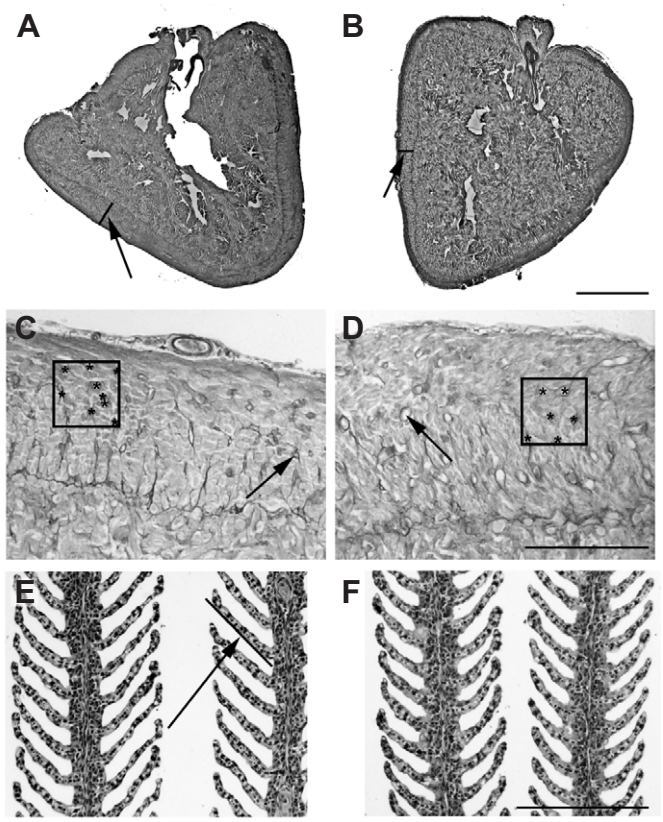

Fig. 3. Histological sections of ventricles and secondary gill lamellae. (A,B) Cross-section of ventricles of (A) warm- and overnight hypoxia-acclimated char and (B) warm- and normoxia-acclimated char. Line and arrow indicate the compact layer; scale bar is 1000 µm. (C,D) Cross-section of the compact layer of (C) warm- and normoxia-acclimated char and (D) cold- and normoxia-acclimated char. Arrows point to capillaries; scale bar is 100 µm. Inside the 2500 µm^2 squares, capillaries are marked with an asterisk. (E,F) The sagittal sections of secondary gill lamellae of (E) warm- and overnight hypoxia-acclimated salmon and (F) warm- and normoxia-acclimated salmon. Line and arrow in E show the point where the height of the secondary lamellae in contact with water was measured. Scale bar is 100 µm.

supported by other studies (e.g. Baroudy and Elliott, 1994) and the values were similar to those observed previously. In the current study, values of CT_{max} were 29.8±0.05 and 28.0±0.07°C for warm- and normoxia-acclimated salmon and char, respectively, compared with 32.6 and 28.7°C for salmon and char reported by Beitinger et al. (2000). It appears that char are not able to acclimate to changing environmental conditions as well as salmon, which has been suggested by earlier studies. For example, incipient lethal temperatures did not change in char nearly as much as in salmon when fish were reared in different acclimation temperatures (Baroudy and Elliott, 1994). The lower capacity of char to respond to a temperature increase possibly makes them more vulnerable to climate change. However, it should be noted that longer term acclimation might change their thermal tolerance. In the current study, we were, for example, able to see acclimatory capacity in cardiac tissue of char (compact myocardium thickness and capillary density). Furthermore, what was interesting was that in both species the CT_{max} increased in both the presence and the absence of overnight hypoxia with warm acclimation even though the change was lower in char than in salmon. Salmon, indeed, have been observed to have significant cardio-physiological capabilities to respond to changes in environmental temperature. For example, in a previous study by Anttila et al. (2014) the maximum heart rate and the temperature at which the heart rate collapsed changed from 21–23°C to 27.5°C for Atlantic salmon (*Salmo salar*) acclimated to 12 and 20°C, respectively, independent of the origin of the fish. Such plasticity makes them highly capable of tolerating an increase in temperature. The population-independent acclimation capacity in Atlantic salmon is also different from Pacific salmon (*Oncorhynchus nerka*), in which populations differ substantially in temperature tolerance in relation to the their environmental

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Table 1. The size of fish in the different acclimation groups

		Mass (g)	Length (cm)
Char			
Warm	Normoxia	27.5±1.0	14.8±0.2
	Overnight hypoxia	23.7±1.0*	14.1±0.2*
Cold	Normoxia	25.0±1.0‡	14.5±0.2
	Overnight hypoxia	25.5±1.0	14.6±0.2
Salmon			
Warm	Normoxia	28.7±1.5	13.8±0.3
	Overnight hypoxia	25.8±1.1*	13.3±0.2
Cold	Normoxia	22.3±1.1‡	12.7±0.2‡
	Overnight hypoxia	23.7±1.2	12.7±0.2

The acclimation temperature and acclimation to overnight hypoxia/normoxia had a significant (P<0.001) effect on both the mass and length of the fish. Warm-acclimated fish were 8.7% heavier and 2.8% longer than cold-acclimated fish, and normoxia-reared fish were 4.6% heavier and 1.9% longer than overnight hypoxia-reared fish. *Significant difference between normoxia and overnight hypoxia acclimations; ‡significant difference between cold- and warm-acclimated fish in their respective hypoxia acclimation conditions. Values are means±s.e.m., N=15 per group.

Table 2. The effects of acclimation temperature, overnight hypoxia and species on thermal and hypoxia tolerance and the oxygen supply and delivery system of char and salmon

Factor	F-value	P-value
CT_{max}		
Acclimation temperature	1171.4	<0.001
Acclimation O_2 level	31.1	<0.001
Species	669.8	<0.001
Mass (covariate)	2.2	0.14
Temperature×O_2×species	0.6	0.45
Hypoxia tolerance char		
Acclimation temperature	27.5	<0.001
Acclimation O_2 level	49.2	<0.001
Mass (covariate)	0.7	0.41
Temperature×O_2	38.2	<0.001
Hypoxia tolerance salmon		
Acclimation temperature	13.6	<0.001
Acclimation O_2 level	8.2	<0.01
Mass (covariate)	0.3	0.58
Temperature×O_2	0.01	0.91
Relative ventricle mass		
Acclimation temperature	27.0	<0.001
Acclimation O_2 level	2.9	0.09
Species	210.0	<0.001
Temperature×O_2×species	0.53	0.47
Compact myocardium thickness		
Acclimation temperature	10.6	<0.01
Acclimation O_2 level	0.01	0.91
Species	168.5	<0.001
Mass (covariate)	19.8	<0.001
Temperature×O_2×species	2.2	0.14
Capillary density at compact layer		
Acclimation temperature	6.2	<0.05
Acclimation O_2 level	0.03	0.87
Species	2.1	0.15
Mass (covariate)	3.0	0.09
Temperature×O_2×species	0.5	0.48
Relative secondary gill lamellae height		
Acclimation temperature	0.004	0.95
Acclimation O_2 level	4.1	<0.05
Species	7.7	<0.01
Temperature×O_2×species	0.19	0.66

The variability of critical thermal maximum (CT_{max}) and hypoxia tolerance, i.e. loss of equilibrium (LOE) oxygen saturation level for char and LOE time for salmon, relative ventricle mass, compact myocardium thickness, capillary density at the compact layer and relative gill lamellae height were tested with MANCOVA, with acclimation temperature, acclimation to overnight hypoxia/normoxia and species as factors (including interactions) and mass of the fish as covariate.

Note: as the hypoxia tolerance was measured with different endpoints for char and salmon, the factorial effect of species was not tested for hypoxia tolerance. The mass of the fish was not used as a covariate for relative ventricle mass and relative gill lamellae height as the mass of the fish is already included in these variables, i.e. relative ventricle mass is ventricle mass divided by the mass of the fish, and relative gill lamellae height is lamellar height divided by the mass of the fish. These two variables were tested using MANOVA, with species, acclimation temperature and acclimation to overnight hypoxia/normoxia as factors.

temperatures (Eliason et al., 2011). However, in the current study, hypoxia and temperature tolerance differences between char and salmon could possibly be related to the preferred environmental conditions of the species, similar to the findings of Eliason et al. (2011) with Pacific salmon populations. Char prefer cold temperatures (Larsson, 2005), which during summer are found at the bottom of big lakes, which may be hypoxic (e.g. Hawley et al., 2006). However, such speculations require further field research about the species' preferred habitats.

A major aim of the present study was to examine whether acclimation to the two stressors, temperature and overnight hypoxia, results in synergistic (i.e. critical temperature and hypoxia tolerance increase more after acclimation to both stressors than if only one stress is applied) or antagonistic (acclimation to both stressors causes smaller changes in critical temperature and hypoxia tolerance than acclimation to only one stressor) effects. Previously, acute thermal stress has been shown to reduce hypoxia tolerance at high temperatures in killifish (*Fundulus heteroclitus*). This reduction is more severe than expected based on the OCLTT model (McBryan et al., 2013). Acute thermal stress also increased the oxygen tension, in that a significant reduction in metabolic rate was observed (critical oxygen tension) in summer flounder (*Paralichthys dentatus*) and striped bass (*Morone saxatilis*) (Capossela et al., 2012; Lapointe et al., 2014). In contrast, moderate, acute heat shock increased hypoxia tolerance in tidepool sculpins (*Oligocottus maculosus*), but hypoxia tolerance was reduced with a more severe thermal shock (Todgham et al., 2005). These studies did not, however, taken into consideration the acclimation capacity of the animals. Here, the increase in acclimation temperature increased the hypoxia tolerance considerably, especially in salmon but also in char after warm temperature was combined with overnight hypoxia. The effects are species specific and depend upon the ability of species to acclimate to a particular stressor. Similarly, as in the stenothermal cold water char, in stenothermal tropical fish, acclimation to a change in temperature hardly affects hypoxia tolerance (at a given temperature). When the acclimation temperature was initially increased, the critical oxygen tension of *Ostorhinchus doederleini* and *Pomacentrus moluccensis* decreased as expected based on the OCLTT model (Nilsson et al., 2010). Maintenance of the fish at the elevated temperature did not affect the critical oxygen tension significantly, although there was a tendency towards an increase of hypoxia tolerance compared with fish acutely transferred to the higher temperature (Nilsson et al., 2010). In other species, both an effect and no effect of acclimation to one stressor on the tolerance of the other stressor have been described. For example, the hypoxia tolerance of paddlefish (*Polyodon spathula*) decreased when the acclimation temperature was increased from 18 to 26°C (Aboagye and Allen, 2014). In contrast, both warm and hypoxia acclimation increased hypoxia tolerance of Southern flounder (*Paralichthys lethostigma*) (Del Toro-Silva et al., 2008). Our results show that merely an increase in acclimation temperature increased the hypoxia tolerance in salmon, but in char, in normoxic conditions, temperature did not have any effect

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on the hypoxia tolerance, possibly because the thermal acclimation capacity is lower in this species. However, our crucial finding, when considering the survival capacity of fish, was that the combination of warm acclimation and overnight hypoxia increased hypoxia tolerance in both species.

The observed changes in the whole-animal thermal and hypoxia tolerance were associated with changes in the structure of the gills and the heart (i.e. oxygen supply and delivery system). In animals, the oxygen consumption of tissues increases with temperature. This can be associated with structural and functional responses at the organ and cellular level to increase the capacity to supply oxygen, as observed previously (Aho and Vornanen, 2001; Hassinen et al., 2008; Klaiman et al., 2011; Anttila et al., 2014). The compact myocardium thickness has, for example, been shown to increase after warm acclimation in rainbow trout (*O. mykiss*) (Klaiman et al., 2011) similar to the current study. With regard to hypoxia, both circulatory rearrangements (Soivio and Tuurala, 1981) and gill remodelling (Dhillon et al., 2013) can take place to increase the amount of oxygen diffusing from water to blood. Further, both the blood oxygen affinity and oxygen-carrying capacity increase in hypoxia in salmonids (Soivio et al., 1980). Thus, both temperature and hypoxia acclimation can lead to significant changes in the oxygen supply and delivery system. In the current study, we observed that during simultaneous temperature increase and overnight hypoxia the structural changes in the gills and the heart were exacerbated. For example, in char the proportion of the compact layer, which receives coronary blood circulation, increased as a result of exposure to the combination of stressors. Moreover, warm acclimation increased the capillary density in the compact layer. This is similar to the finding of Egginton and Cordiner (1997), who showed that warm acclimation increased the capillary density of the compact myocardium of rainbow trout. The compact myocardium indeed seems to be highly responsive to changes in environmental conditions. Overall, the results highlight the importance of cardiac morphology and function for oxygen supply. Furthermore, the secondary gill lamellae were highest after acclimation to a combination of stressors in salmon, probably indicating lamellar pressure changes (Soivio and Tuurala, 1981).

Importantly, although the organ-level changes were connected to increased hypoxia tolerance after acclimation to a combination of stressors, these changes were not connected to increased CT_{max}. In fact, the effects of temperature and overnight hypoxia acclimation were antagonistic when considering the thermal tolerance. Consequently, char had higher hypoxia tolerance and larger ventricles (possibly indicating larger stroke volume; Franklin and Davie, 1992) with a thicker compact layer, but lower temperature tolerance than salmon. Although it is proposed that temperature tolerance is oxygen and capacity limited (Pörtner, 2010), there can be different physiological factors limiting temperature and hypoxia tolerance (Sokolova, 2013). Our results support this notion. It appears that char have a limited capacity for thermal acclimation, while their responses to hypoxia can be profound. Schulte (2014) has suggested that the cost of responding to one stressor may decrease the capacity of an animal to respond to another stressor. Such a trade-off can also partly explain the current results concerning simultaneous antagonistic and synergistic effects of temperature and overnight hypoxia.

In conclusion, the current study demonstrates how environmental stressors that fish encounter in their natural habitats can have unexpected effects on their ability to tolerate these conditions. Eutrophication-induced overnight hypoxia seems to increase the hypoxia tolerance in warm-acclimated fish. However, the CT_{max} of both salmon and char is reduced when the environment is hypoxic overnight. Therefore, the survival capacity of the fish depends on which of the environmental stressors is more serious and on the species. According to our results, char tolerate low oxygen levels well, while an increase in temperature can be detrimental for this species, while salmon seem to respond in the opposite way. The results also show that the acclimation capacity of char is lower than that of salmon, which, overall, makes char more vulnerable to environmental changes. The results also point out that the complexity of the natural environment needs to be taken into account more in future studies.

MATERIALS AND METHODS

The experiment was carried out at the Finnish Game and Fisheries Institute Saimaa from 1 July to 10 August 2013. All procedures were approved by the Finnish Animal Experiment Board (ESAVI/4068/04.10.07/2013). The populations of Arctic char and landlocked salmon used in the experiments originated from Lake Saimaa (62°04′N, 28°33′E). Char and salmon were reared at a fish hatchery (Finnish Game and Fisheries Institute Saimaa) for three and one generation, respectively. Fish were reared at the hatchery in natural conditions until the experiments (at the time of experiments the water temperature was ∼15°C, sunrise was at 03:00 h and sunset was at 23:00 h). Before the experiments, juvenile (1+ year) char and landlocked salmon were divided into four acclimation groups: (i) warm temperature (14.9±0.05°C), normoxia (10.0±0.2 mg O_2 l^{-1}); (ii) warm temperature, daytime normoxia and overnight hypoxia (6.3±0.4 mg O_2 l^{-}, 63% oxygen saturation); (iii) cold temperature (7.7±0.02°C), normoxia (11.9±0.1 mg O_2 l^{-1}); (iv) cold temperature, daytime normoxia and overnight hypoxia (8.0±0.6 mg O_2 l^{-1}, 67% oxygen saturation). The total number of fish in identical circular tanks (diameter 90 cm, water depth 50 cm, volume of water 320 l) was 40 (different fish were used in different sets of experiments; 15 individuals for CT_{max} measurements, 15 individuals for hypoxia tolerance measurements and 10 individuals for morphological measurements; all the tanks had similar biomass). The tanks received filtered and aerated water from nearby Lake Pahkajärvi (62°04′N, 28°33′E). Fish were fed with commercial fish pellets (Raisio Group; www.raisiogroup.com) *ad libitum*. During the experiment, the natural photoperiod of the region (65°N) was followed and overnight hypoxias were matched to dusk and dawn: oxygen level started to decrease at 23:00 h and increase at 03:00 h each day. The water flow to tanks was ∼4 l min^{-1}. Overnight hypoxia was achieved by reducing the water flow to the tanks to 80 and 250 ml min^{-1} for cold and warm tanks, respectively, and by allowing the oxygen level of the water to decrease through fish respiration. The flow was high enough to allow removal of nitrogen waste. To avoid the diffusion of oxygen from the atmosphere, most of the water surface was covered with bubble wrap. The oxygen level of the water was measured with a temperature-compensated PreSens oxygen dipping probe connected to a fibre optic oxygen monitor (Fibox 3; PreSens, Regensburg, Germany). Fish were acclimated for 4 weeks before their CT_{max} and hypoxia tolerance were measured.

CT_{max} measurements

Each of the acclimation groups was tested separately (total number of fish, N=120). For the experiment, fish (N=15 per acclimation condition and species) were transferred from the acclimation tank into an experimental tank (volume 100 l) where the water temperature was the same as the acclimation temperature of fish (either 8 or 15°C). The measurements followed the protocol of Fangue et al. (2006). Because of possible diurnal light cycle effects on the temperature tolerance of fish (Healy and Schulte, 2012), all the experiments were performed between 10:00 h and 13:00 h. After 1 h acclimation period in the experimental tank, the temperature of the water was increased at a constant rate of 0.3°C min^{-1} up to 24°C and by 0.1°C min^{-1} thereafter until the fish exhibited loss of equilibrium (LOE). Water temperature was controlled with a circulating 2500 W heater (RC6, Lauda, Lauda-Königshofen, Germany) and two submersible aquarium heaters [Theo 100 W, Hydor, Bassano del Grappa (VI), Italy]. Water homogeneity and oxygenation was assured by bubbling air vigorously into the tanks and using aquarium pumps to circulate the water, keeping the

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oxygenation level above 80% saturation throughout the experiments. Once a fish lost equilibrium, it was quickly removed from the tank and placed in a recovery tank (all the fish had their own 10 l recovery tanks) at the acclimation temperature. After the experiment, all the fish were killed with 200 ppm MS-222 buffered with sodium bicarbonate. The mass and length of the fish were recorded. The ventricle was quickly removed and the mass of the ventricle was measured. The relative ventricle mass was calculated by dividing the ventricle mass by the mass of the fish.

Hypoxia tolerance measurements

To measure hypoxia tolerance, each acclimation group was tested separately in 40 l experimental tanks (N=120). After transfer to the experimental tank, fish (N=15 per acclimation condition and species) were allowed to adjust to the new conditions for 1 h. The water temperature in the tank was kept constant at 12°C for all acclimation groups with a circulating chiller/heater (RC6, Lauda). After the adjustment period, the level of oxygen in the water was decreased at a constant rate of 1.5% O_2 saturation min^{-1} until 13% oxygen saturation (1.4 mg O_2 l^{-1}) was reached by bubbling nitrogen into the tank. The water surface was covered with bubble wrap to prevent atmospheric gas exchange. The time until salmon exhibited LOE (after the 13% oxygen saturation level was reached) was measured. Once a fish lost equilibrium, it was removed from the tank and placed into a recovery tank before sampling, similar to the CT_{max} experiment. Char did not exhibit LOE 2 h after 13% oxygen saturation was reached nor did they show any signs of stress. Thus, to reach the hypoxia tolerance limits of char, after 2 h at 13% oxygen saturation, the oxygen level was reduced at a rate of 0.2% O_2 saturation min^{-1} until fish lost equilibrium (~6.8±0.08%), and the oxygen level and time were recorded. As for salmon, after LOE, char were immediately removed from the tank and placed in the recovery tank before sampling. The relative ventricle mass was calculated by dividing the ventricle mass by the mass of the fish.

Organ-level measurements

Ten additional fish from each species and acclimation condition were killed with 200 ppm MS-222 buffered with sodium bicarbonate in order to collect organ samples from fish that had not gone through hypoxia or temperature tolerance tests. The mass and length of the fish were measured and the ventricle and the first gill arch from the left side were removed. The ventricle was weighed and then halved mid-sagittally. The tissue samples were fixed in 4% formalin in phosphate-buffered saline for histological analyses. The relative ventricle mass was calculated by dividing the ventricle mass by the mass of the fish as above.

The organ samples were dehydrated in alcohol and UltraClear (Mallinckrodt Baker Inc., Center Valley, PA, USA) series (70% EtOH 1 h, 94% EtOH 1 h, 100% EtOH 3×1 h, UltraClear 2×1 h) before embedding in paraffin wax. Serial sections (5 µm) cut with a microtome (301-268.001, Ernst Leitz GmbH, Wetzlar, Germany) were mounted on glass slides, de-waxed with UltraClear and rehydrated with an alcohol series and distilled water.

The ventricle samples were stained with amylase–periodic acid–Schiff, which stains carbohydrates, giving a dark red stain in, for example, capillary walls (Andersen, 1975). Briefly, the sections were incubated for 1 h in 1% amylase at 37°C, rinsed with distilled water and oxidised with 1% periodic acid at room temperature for 20 min. Schiff's reagent was used for staining (20 min) before rinsing and dehydrating sections with alcohol and UltraClear series. The sections were examined under a Leica DM RXA microscope (Leica Microsystems, Wetzlar, Germany). The thickness of the compact layer was measured from each ventricle at intervals (~300 µm) around the perimeter by measuring the distance from the distal edge of compact layer to the spongy myocardium (see Fig. 3A,B). From each ventricle at least 30 measurements were taken. The average compact thickness was divided by the cross-sectional area of the ventricle. The capillary density of the compact layer was measured by counting the number of capillaries in an area of ~4000 μm^2 (Fig. 3C,D) and calculating the capillary density in 1000 μm^2. From each ventricle, at least six different measurements were taken around the perimeter.

Sagittal sections along the primary gill filament were used to provide cross-sections of the secondary lamellae, which were stained with Mayer's haematoxylin, which stains nuclei dark purple (RAL Diagnostics, Martillac, France). From these sections it was possible to measure the height of the secondary lamellae in contact with water (i.e. total height of the lamellae minus height buried in interlamellar cell mass) (Fig. 3E,F). Lamellar height was measured in at least six sections for each individual and the average was used to calculate the relative lamellar height by dividing it by fish mass.

Statistical analyses

The variability of CT_{max} and hypoxia tolerance LOE time for salmon and LOE oxygen saturation level for char, compact myocardium thickness and capillary density of the compact layer were tested with MANCOVA, with species, acclimation temperature and acclimation to overnight hypoxia/normoxia as factors and mass of the fish as covariate. MANCOVA was followed by the Holm–Sidak *post hoc* test (for hypoxia tolerance, the species were not compared against each other because of differences in determining the LOE; see above). The variability of relative ventricle mass and relative gill lamellae height was tested with MANOVA, with species, acclimation temperature and acclimation to overnight hypoxia/normoxia as factors (mass of the fish was not a covariate as the mass of the fish is included in these variables), followed with the Holm–Sidak *post hoc* test. The mean mass and length of the groups were also compared with MANOVA, with species, temperature and overnight hypoxia as factors, followed by the Holm–Sidak *post hoc* test. The analyses were done with IBM SPSS Statistics, version 22 (Armonk, NY, USA). Statistical significance for comparisons of mean values was set at α=0.05. The values are presented as means±s.e.m.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

K.A. designed the study with M.N., K.A. carried out the hypoxia and thermal tolerance measurements together with M.L., J.M.P. and M.K. K.A. carried out the lab work, data analyses and statistical analyses, and drafted the manuscript. K.A., M.L., E.S. and I.K. participated in setting up the acclimation groups and performed preliminary experiments. All authors revised the manuscript critically for important intellectual content and gave final approval for publication.

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