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Direct calorimetry of aquatic animals: effects of the combination of acidification and hypoxia on the metabolic rate of fish

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Abstract

The results of acidification on the metabolic rate of tilapia (*Oreochromis mossambicus* Peters) were studied in a differential 1-liter flow-through microcalorimeter (Setaram GF 108). After three days at pH 7.6 at normoxic conditions, the pH was slowly reduced to pH 4.0 at an acidification rate of 3.6 pH units over 240 min. Acidification during normoxia had no significant effects on the heat production ($\dot{Q}$), the oxygen consumption ($\dot{V}O_2$) and the oxycaloric equivalent. Furthermore, we investigated whether the combination of hypoxia and acidification works synergistically. Fish were exposed to a graded hypoxia load of respectively 40% air saturation (AS), 25% AS, 15% AS and 5% AS, 8 h per level, in combination with acidification at pH 4.0. No support for a synergism was found in this study. All tilapia survived severe hypoxia (5% AS) in combination with acidification (pH 4.0) due to a metabolic depression.

Keywords: Acidification; Biological system; Calorimeter; Fish; Hypoxia; Metabolic depression

1. Introduction

During the last 15 years, an increasing interest has developed in the role of acidification on the physiology of aquatic organisms, mainly fish. Studies concentrate on the ionic balance, endocrinological response and metabolism (substrate usage) reviews [1,2]. Little information is available on the energetic costs and the effects of acidification on the metabolic rate of fish.

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An approach to clarify this topic is the measurement of the standard metabolic rate (SMR) [3]. By comparing the SMR under control conditions with the situation in which environmental change is introduced, conclusions can be made about the energetic costs required to cope with the environmental stress factor. From the literature, it is well known that the SMR in fish may be affected by environmental factors like salinity [4–6] and temperature [7–10]. The effects of acidification on the SMR of fish are unclear and need therefore to be elucidated.

From microbiological calorimetry, some studies indicate that the metabolic rate is affected by external acidification. For the microorganisms *Saccharomyces cerevisiae* [11], *Debaryomyces hansenii* [12] and *Candida utilis* [13], the metabolic rate, measured directly via the heat production or oxygen consumption, increased after acidification. This can be ascribed to increased maintenance costs, e.g. for ion pumping [11,14]. Based on this information, it is reasonable to assume for multicellular organisms, like fish, that the metabolic rate also will be affected by environmental acidification. Moreover, other factors may play a role and affect the oxygen consumption rate, like the observed gill damage in fish after acute exposure to acidification [15]. Some information about the effects of acidification on the metabolic rate is available for fish from respirometry [16–18]. However respirometry alone may not give sufficient information, mainly because it does not measure the anaerobic component of the metabolic rate [19]. Furthermore it does not reveal whether the organism may save energy via the mechanism of metabolic depression, a reduction of the metabolic rate under the SMR [17,18]. This information can be acquired with direct calorimetry [19–21].

Moreover, acidification in combination with another stressor may work synergistically. This was observed for acidification in combination with handling stress [22] and exercise [23] and hypoxia [24].

In this study we want to test two hypotheses: (1) that water acidification affects the metabolic rate in fish and (2) that the combination of water acidification and hypoxia synergistically affects the metabolic rate and anaerobic capacity of fish.

In this study we examine the heat production and oxygen consumption of tilapia (*Oreochromis mossambicus*) gradually exposed to an acidic environment (pH 4.0) in combination with the stressor hypoxia.

2. Material and methods

2.1. Animals

Tilapia (*Oreochromis mossambicus* Peters) were obtained from the Catholic University Nijmegen. The animals were acclimated to 20°C, kept under normal laboratory conditions (14 h light, 10 h dark) and normoxic oxygen saturation values of 80%. The animals were fed with Trouvit pelleted food (Putten, The Netherlands). At least 3 weeks before the onset of the experiments, the fish were transferred to ten times diluted local tap water. To achieve this, 1 volume of copper-free tap water was mixed with 9 volumes of demineralized water. The end concentrations were: Na^+ 0.33, Cl^{-1} 0.31, Ca^{2+} 0.22,

K^+ 0.026, Mg^{2+} 0.058, NO_3^- 0.0019, HCO_3^- 0.375, SO_4^{2-} 0.094, SiO_2 0.015 mmol l^{-1} (data from water authority Rijnland 1992). $Al_{tot.}$ was below the detection limit of 13.0 nmol l^{-1}. The weight of the animals used for the acidification experiments in the calorimeter was 25.3 ± 1.28 g. The oxygen consumption ($\dot{V}O_2$) of the fish at a flow rate of 50 ml min^{-1} caused a PO_2 decline of approximately 10%. This is caused by a constant biomass kept in the vessel which consumes oxygen, while there is a constant refreshment of the water in the vessel by the water inflowing at a rate of 50 ml min^{-1}. Two days before the experiment, the animals were starved in an identical calorimetric vessel.

2.2. Calorimeter

The calorimetric system is described elsewhere [19,25]. Briefly, the heat production of the animals is measured in a differential flow-through calorimeter (Setaram GF 108, Caluire, Lyon, France) which measures continuously the rate of heat production of the fish in the 1-liter vessel. Before the fish were introduced, the sensitivity coefficient, which relates signal level to power input, was determined. The mean sensitivity coefficient of the 5 individual experiments was 84.59 ± 0.78 μV mW^{-1} ($n = 5$). The flow through the system was 50 ml min^{-1}. The baseline stability was 0.01 mW/24 h. The calorimetric set up was placed in a thermostatted room. The heat flux and oxygen tension signal (see below) were recorded on an IBM-compatible computer (Laser 386 SXE) with specially developed software for data acquisition and graphical presentation [19].

2.3. Oxygen registration

Oxygen is recorded on a digital oxygen analyzer, Radiometer Copenhagen type PHM 72c with a pO_2 module type PHA 932. A platinum–silver pO_2 electrode (Radiometer, type E5046, Copenhagen, Denmark) mounted in a thermostatted cell (Radiometer, type D616, Copenhagen, Denmark) is connected to the meter. An oxygen valve (Bürckert type 332-E-2-B-G-220/50-F-024) switches alternately the water from the measurement (m) or reference (r) vessel over the oxygen electrode. For each period of 4 h, the valve was 230 min in the c_m position and 10 min in the c_r position. Oxygen consumption ($\dot{V}O_2$) is calculated according to the Fick principle [26].

$$\dot{V}O_2 = v(c_r - c_m)\,mgO_2 \cdot h^{-1}$$

where v is the water flow through the vessels, 50 ml min^{-1}; c_r and c_m are respectively the oxygen concentration measured in the outflowing water of the reference and measurement vessel. The oxygen level of 100% air saturation (AS) (c_r) was measured with a Winkler titration and corresponded to 8.84 ± 0.062 ($n = 6$) mg l^{-1} [19].

2.4. Experimental protocol

The fish were placed in the calorimeter at pH 7.6 and normoxic oxygen conditions (100% AS). Because of the flow-through system [19,25], fresh oxygenated water is pumped to the vessel and waste products and CO_2 are flushed out. After three days, the pH was reduced to 4.0 at an acidification rate of 3.6 pH units over 240 min with H_2SO_4.

During the whole experiment, the pH was controlled by a pH stat (METROHM 605/614/655, Herisau, Schweiz) and 0.5 mmol l^{-1} H_2SO_4. Special pH electrodes were used (Russel CTL/LCW for low conductivity, Auchtermuchty, Scotland, UK). After 4 days, hypoxia was introduced with a gas-mixing pump (type 2M 301/a-F, Wösthoff, West Germany) with nitrogen gas and air. Fish were exposed to 4 periods of 8 h each of 40% AS, 25% AS, 15% AS and 5% AS. Using a marker system, the operator could pinpoint in the calorimetric data set when an experimental condition was changed [19].

2.5. Calculations and statistics

The sampling rate of the heat production signal and oxygen tension signal during the calorimetric and respirometric experiment was 1 sample per minute. Statistics were performed using a one-way ANOVA. $P \leqslant 0.05$ was considered as statistically significant. Normality of the data and homogeneity of variances were checked by Kolmogorov–Smirnov and F_{max} tests, respectively.

3. Results

A graphical presentation of the experiment is given in Fig. 1. The experiment begins and ends with a calibration procedure for the heat signal. This results in a heat production of approximately 10 mW. Then the fish were introduced into the

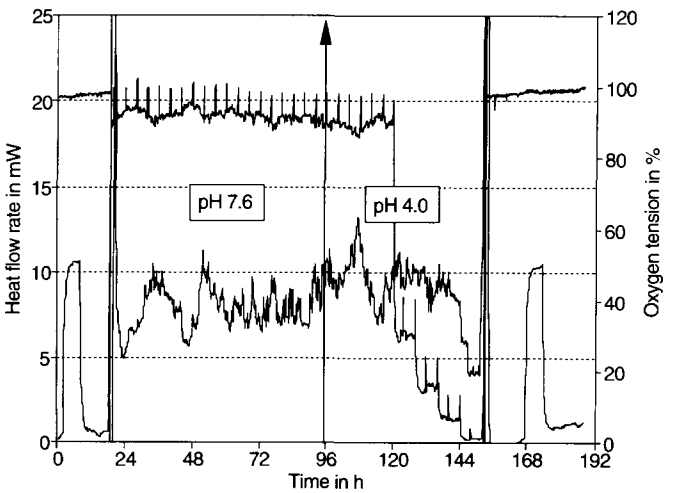

Fig. 1. Registration of an eight-day experiment with one single tilapia (*Oreochromis mossambicus* Peters). After 4 days the water was slowly acidified at an acidification rate of 3.6 pH units over 240 min. The fifth day the animal was exposed to a graded hypoxia load (40% AS, 25% AS, 15% AS and 5% AS) for 8 h per hypoxia level. The top signal alternating between reference and measurement position, is the oxygen tension signal. The irregular line is the heat production signal.

calorimeter. The top line corresponds to the oxygen tension signal, alternately measuring the measurement and reference vessels. The oxygen tension of the water flowing in the calorimeter is given by the top of the spikes in the pO_2 signal, switching every 4 h 10 min to the reference position. The difference between measurement and reference position is caused by the oxygen consumption of the fish and, if multiplied by the flow through the system, corresponds to the oxygen consumption.

The initial and final baseline positions of the heat production signal show almost no baseline drift (Fig. 1).

The mean heat production ($\dot{Q}$), the oxygen consumption ($\dot{V}O_2$) and the oxycaloric equivalent are given in Table 1. During the first day, the heat production is only slightly reduced.

Over the first two days, low oxycaloric equivalents (349–375 kJ mol^{-1}) were observed. If we assume that the effects of handling stress disappear within two days [21], we can test the effect of acidification by comparing the third (pH 7.6, normoxia) and fourth (pH 4.0, normoxia) day. Heat production and oxygen consumption were slightly elevated during acidification but were not significantly different (Table 1).

The combination of hypoxia and acidification gives the following results. At a hypoxia level of 40% AS and 25% AS (pH 4.0), the metabolic rate is not significantly higher than at day 3 (pH 7.6). At 15% AS, the metabolic rate is not significantly reduced (only 5.5%), while at 5% AS (pH 4.0) the metabolism is reduced by 44.1%. Clearly the reduction of the metabolic rate at 5% AS corresponds to a metabolic depression, a reduction in the metabolic rate below the SMR [3,19,20,25]. Apparently metabolic depression also occurs under severe hypoxic acidified (pH 4.0) conditions.

All animals survived the combination of severe hypoxia (5% AS) and acidification. No mortality was observed. The results of this study, therefore, indicate that acidification in combination with an additional stressor (hypoxia) does not work synergistically.

Table 1
Mean value and standard deviation of heat production, oxygen consumption and oxycaloric equivalent of 5 experiments with one individual tilapia (*Oreochromis mossambicus* Peters) exposed to the combination of water acidification and hypoxia. The fish was exposed in a Setaram GF 108 flow-through calorimeter for three days during normoxic periods at pH 7.6. The fourth day, the water was acidified to pH 4.0 at an acidification rate of 3.6 pH units over 240 min. After four days at normoxia, the animal was exposed to a graded hypoxia load (40% AS, 25% AS, 15% AS and 5% AS) for 8 h per hypoxia level

	Heat production/ ($mJ\,h^{-1}\cdot gww^{-1}$)	Oxygen consumption/ ($\mu mol\,h^{-1}\cdot gww^{-1}$)	Oxycaloric equivalent/ ($kJ\,mol^{-1}$)
Day 1, pH 7.6	984.9 ± 139.2	2.86 ± 0.40	348.9 ± 42.9
Day 2, pH 7.6	1040.8 ± 128.8	2.82 ± 0.35	375.0 ± 55.8
Day 3, pH 7.6	1182.7 ± 104.8	2.66 ± 0.15	446.5 ± 37.3
Day 4, pH 4.0	1275.2 ± 201.1	2.78 ± 0.28	458.3 ± 40.8
Hyp 40%, pH 4.0	1302.2 ± 199.9	3.44 ± 0.38	381.0 ± 26.0
Hyp 25%, pH 4.0	1220.5 ± 226.9	3.14 ± 0.45	389.1 ± 47.9
Hyp 15%, pH 4.0	1116.3 ± 218.8	2.58 ± 0.23	432.7 ± 78.8
Hyp 5%, pH 4.0	661.1 ± 217.2	1.07 ± 0.23	626.9 ± 180.1

4. Discussion

If we assume that the effects of handling stress last two days [21], we can use the metabolic rate of the third day as a reference value. This result corresponds to former calorimetric studies. In an earlier study with a group of four goldfish, we observed a normoxic metabolic heat production ($\dot{Q}$) of 2080 mJ h^{-1} gww^{-1} and an oxygen consumption ($\dot{V}O_2$) of 5.15 μmol h^{-1} gww^{-1} [19]. In another study with goldfish ($n=4$), the normoxic metabolic heat production ($\dot{Q}$) corresponded to 2709 mJ h^{-1} gww and the oxygen consumption ($\dot{V}O_2$) was 6.48 μmol h^{-1} gww^{-1} corresponding to an oxycaloric equivalent of 422 kJ mol^{-1}[20]. The heat production data with groups of fish are a little higher compared to studies with a single fish in the calorimetric vessel. In a comparable study with a single tilapia, the mean normoxic heat production ($\dot{Q}$) was 1415 mJ h^{-1} gww, the oxygen consumption ($\dot{V}O_2$) was 3.31 μmol h^{-1} gww^{-1} and the oxycaloric equivalent was 428 kJ mol^{-1} ($n=4$) [3].

In this study, on day 3 (pH 7.6) we observed a metabolic rate of 1182.7 mJ h^{-1} gww^{-1} (0.329 mW gww^{-1}) which corresponds to 297.1 J h^{-1} $kg^{-0.8}$ (Table 1). In a former study, we observed for tilapia a value of 1415.3 mJ h^{-1} gww^{-1} (0.393 mW gww^{-1}) which corresponds to 355.5 J h^{-1} $kg^{-0.8}$ [3]. Osman [27] observed for starving tilapia (*Oreochromis niloticus*) of this size class (20 g) at a temperature of 26.5°C, a routine metabolic rate of 1050 J h^{-1} $kg^{-0.8}$ which is 3.5 times higher than the value observed in this study (Table 1). Hepher et al. [28] cited in Ref. [27] observed in red tilapia of approximately 75 g at 20.9°C, a metabolic rate of 52.6 kJ $kg^{-0.8}$ d^{-1} which corresponds to 2192 J h^{-1} $kg^{-0.8}$. The value of Hepher et al. [28] is 7.4 times higher than the value observed in our laboratories [3]. However, the effect of temperature on the metabolic rate may be drastic. Becker and Fishelson [29] observed a 50% rise in the routine metabolic rate (RMR) in *Oreochromis niloticus* from 26 to 30°C. These authors measured an oxygen consumption of *Oreochromis nilotus* at 26°C of 44 mg O_2 h^{-1} $kg^{-0.8}$ which can be converted with an oxycaloric equivalent of 433.6 kJ mol^{-1} [30] to 596.2 J h^{-1} $kg^{0.8}$ [29]. This value is 2.0 times higher than the observed 297.1 J h^{-1} $kg^{-0.8}$ in this study. Finally, Caulton [31], cited in Ref. [27], calculated for starving *Oreochromis niloticus* at $T=20$°C a routine metabolic rate of 385.3 J h^{-1} $kg^{-0.8}$ which is 1.3 times higher than the observed value in this study.

The effects of acidification alone during normoxia can be observed by comparing the third with the fourth day (Table 1). Although the metabolic rate during acidification (pH 4.0) is higher, this is not significantly different. Results in the literature, using acidification as a single stressor, are conflicting. Calorimetric and respirometric studies with microorganisms showed an increase in the maintenance metabolism. For *Saccharomyces cerevisiae* the heat yield increased from 47 to 72 kJ g^{-1} at pH 2.8 [11]. For *Debaryomyces hansenii*, the heat yield was significantly increased at an external pH below 3.7, while at a pH of around 3 the energy expenditure for growth was more than doubled [11,12]. In *Candida utilis*, lowering the culture pH from 6.9 to 5.1 resulted in a progressive increase in the respiration rate while there was a collapse of respiration at pH 4.8 [13]. In contrast, for crayfish (*Cherax destructor*), a hypometabolism was observed when exposing the animals to pH 4.5 (Ca^{2+} 500 μmol l^{-1}); the metabolic depression based on the oxygen consump-

tion was reduced by 79% compared to control animals (pH 7.1, Ca^{2+} 500 μmol l^{-1}) [32].

From respirometric studies with fish by Ultsch et al. [16], Ye et al. [18], and van Dijk et al. [23], two conclusions can be made. First, exposure of fish to acidified (pH 4.0) soft water impairs the O_2 transport. Second, it does not limit resting O_2 consumption but reduces the scope for activity [16–18]. However, exposure of fish to more severe acid conditions in the pH range of 3.0–3.5 does impair resting oxygen uptake and may result in mortality [16–18]. Furthermore, van Dijk et al. [17] reported that tilapia decreased the SMR, its average oxygen consumption and the maximum metabolic rate in acid water (pH 4.0) while the animal became motionless.

The results presented in this study with tilapia show no reduction of the metabolic rate (heat production and oxygen consumption) after acidification of the water.

The second hypothesis postulated in the introduction of this study, that the combination of acidification and hypoxia leads to synergism, was not confirmed. This is in contrast to an earlier in vivo ^{31}P NMR study by van Ginneken et al. [24]. In the latter study the results with the fish species tilapia indicated that acidification alone to pH 4.0 had no effect on phosphocreatine concentration [PCr], intracellular pH or oxygen consumption. The combination of acidification with hypoxia, however, resulted in 50% mortality, while PCr depletion was more severe in the acutely acidified groups (from pH 7.6 to 4.0 over a period of 6 h) and PCr restoration during reoxygenation was retarded in the survivor group [24]. However, the experimental protocol of both studies was different. Firstly, in the in vivo ^{31}P NMR study, the animals were exposed to a graded hypoxia load of one hour per level. In this calorimetric study, the animals were exposed for 8 h to a certain hypoxia level enabling the animals to adapt to the environmental conditions. Secondly, the anaerobic metabolism was more activated in the ^{31}P NMR study because the minimal hypoxia level was 3% AS, in contrast to this calorimetric study in which the level was higher, 5% AS. Thirdly, in the experimental set up used in the ^{31}P NMR study, the fish were fixed by an inflatable bag in a flow-through cell, while in the calorimetric study the fish were freely swimming in the vessel. Probably a third stressor, confinement stress, may have had an impact in the ^{31}P NMR study.

During severe hypoxia at 5% AS under acidified conditions, metabolic depression is observed. This was also observed in our control hypoxia experiments without any acidification [3]. This indicates that the metabolic depression is caused by the hypoxic conditions. Furthermore, it can be concluded that even under acid conditions, animals are able to depress their metabolism. The reduction of the metabolic rate is 44.1% compared to the normoxic value, which corresponds to the earlier observed metabolic depression of 50% of the SMR at 5% AS (pH 7.6) in this species [3].

Finally, it can be concluded that the oxycaloric equivalent of 627 kJ mol^{-1} at 5% AS is clearly indicative of activation of the anaerobic metabolism.

In conclusion, acidification (pH 4.0) as a single stressor does not affect the heat production and oxygen consumption of tilapia. No synergism was observed during the combination of the stressors hypoxia and acidification. Tilapia is able, under severe hypoxia in combination with acidification (pH 4.0), to exploit the mechanism of metabolic depression.

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