

Metabolic rate and level of activity determined in tilapia (*Oreochromis mossambicus* Peters) by direct and indirect calorimetry and videomonitoring

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Abstract

In order to correlate the metabolic rate and locomotor activity level of fish, a calorimetric system (Sétaram GF 108) was supplemented with a video tracking and motion analysis system. The motion analysis system is based on digital image processing. Once every second, two images (with an interval of 120 ms) are digitized and subtracted. The difference between the two images is used as a measure of the animal's movement. The activity of different complex movements, like turning, accelerations, fin movements, and branchial ventilation, were thus quantified. The combination of motion analysis with calorimetry gave some interesting results. Firstly, during extreme hypoxia, tilapia does not become lethargic as has been described for the anoxia-tolerant crucian carp [1]. The locomotor activity level during severe hypoxia corresponded to the locomotor activity level during normoxia in the restricted area of the calorimetric vessel. This implies that the calorimetrically determined reduction of the heat flux by 50% under these conditions can be ascribed to a reduction in the cellular energy metabolism – metabolic depression. Secondly, the metabolic rate under constant light conditions was elevated from 11–18%, and the animals showed strong fluctuations in the heat flux; periods of aerobic metabolism alternated with periods of anaerobic metabolism. This was in contrast to the experiments under constant dark conditions in which the metabolic rate was around the standard metabolic rate (SMR). Under the applied conditions, no correlation was observed between heat production measurements and locomotor activity. This may possibly be ascribed to the limited size of the calorimetric vessel in which the animals' metabolic rates were around SMR. The observed oscillations in metabolic rate under light conditions could be another disrupting factor; oscillations in the circulation and ventilation could be responsible for this phenomenon.

Keywords: Activity measurement; Biological system; Calorimeter; Fish; Metabolic depression

1. Introduction

The standard metabolic rate (SMR) can be defined as the metabolic rate of postabsorptive, stress-free fish

with a locomotor activity of zero and acclimated at a particular temperature [2,3]. The SMR corresponds to the minimum rate of energy expenditure necessary to keep the organism alive [3]. In respirometry and calorimetry, the determination of the SMR is important because it can give important physiological infor-

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mation about some aspects of metabolism. Firstly, the SMR (or differences in SMR between experimental groups) may point to energetic differences in the cost of maintenance metabolism between individuals or strains. An example of this is a respirometric study of human subjects comparing an obese group with a control group [4]. Locomotor activity and oxygen consumption were determined by extrapolating to zero locomotor activity the basal metabolism could be estimated, giving a higher basal metabolism for the obese group. An example in which this method is applied for fish, (for example, in a comparison of two different strains at standard conditions) is not known. Secondly, the SMR can give information about the energetic costs for animals exposed to different environmental conditions like salinity [5–7] or temperature [2,8–10] or pH [11]. Thirdly, the SMR is an important 'reference point' needed to quantify the phenomenon of metabolic depression. Assuming that postabsorptive goldfish were kept under dark conditions at SMR, the animals showed a 70% reduction of heat production during anoxia [12]. Under adverse environmental conditions, some fish species are able to decrease their energy metabolism below the SMR (the minimal energetic costs for homeostasis under standard conditions), bringing about the so-called metabolic depression.

During respirometric and calorimetric measurements of free swimming undisturbed fish, the routine metabolism includes patterns of random physical activity. In general, when the excitement element due to handling is eliminated, the average routine metabolism approximates three times the SMR [1,3,10,13].

There are three ways to estimate the SMR for spontaneously moving, undisturbed fish: firstly, by exposing the fish in a flow-through respirometer to several levels of forced locomotor activity and extrapolating to zero activity [2,8,14,15]; secondly, by taking the lowest point of a daily cycle [2,16]; and thirdly, by measuring the metabolic rate of randomly moving animals in a restricted area (vessel) in combination with locomotor activity measurements [1,17,18] and extrapolating to zero activity. Compared to the first method, which allows only linear forward motion, the third method includes different complex patterns of locomotor activity, such as turning, accelerations, braking, etc. [18].

Several methods are described in the literature for detecting locomotor activity [2,18]. Some devices are based on the principle of mechanics [19], heat loss flowmeters [9,20–22], photocells [23,24], potentiometers [25], and echo sounder beams [26].

Recently it has become clear that video-imaging techniques are very useful for quantification of locomotor activity patterns of fish and other organisms [1,17,18,27–32].

We present here a video-imaging method in which complex patterns of locomotor activity in a calorimetric vessel can be recorded, as well as fine movements with little displacement of the body's center of gravity (e.g. fin movements).

The new aspect of the study described in this paper is the combination of locomotor activity measurement using a video-imaging system with calorimetric and respirometric measurements for the hypoxia-tolerant fish species tilapia (*Oreochromis mossambicus* Peters). In this study, we attempted to estimate the SMR in two ways – taking the lowest interval in a daily cycle and extrapolating to zero activity. Further, it is demonstrated that the locomotor activity during severe hypoxia (5% air saturation, 5% AS) corresponds to the normoxia locomotor activity, while the metabolic rate decreased by 50% in the fish species tilapia.

2. Materials and methods

2.1. Animals

Tilapia (*Oreochromis mossambicus* Peters) were obtained from the Catholic University, Nijmegen. The animals were acclimated to 20°C and kept under normal laboratory conditions (14 h light, 10 h darkness) and normoxic oxygen saturation values of 80%. The animals were fed with Trouvit pelleted food (Putten, The Netherlands). The weight of the animals used was 24.9 ± 1.20 g for the dark experiments and 25.1 ± 0.29 g for the light experiments. The oxygen consumption (O_2) of the fish caused a P_{O_2} decline of 10–15% at a flow 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 [33,34]. In short, the heat production of the animals is measured in a differential flow through the calorimeter (Sétaram GF 108, Lyon, France), which measures continuously the rate of heat production of the fish in the vessel which has a volume of 1 l. Before the fish were placed in the vessel the sensitivity coefficient, which relates signal level to power input, was determined. The sensitivity coefficient of the four runs under dark conditions was $80.40 \pm 2.88 \mu\text{V mW}^{-1}$ ($n = 4$) and under light conditions $74.68 \pm 0.91 \mu\text{V Mw}^{-1}$ ($n = 4$). The flow through the system was 50 ml min^{-1} . The baseline stability is $\pm 0.005 \text{ Mw}$ per 24 h. The calorimetric apparatus was placed in a thermostatically controlled room set at $19.3 \pm 0.3^\circ\text{C}$. The operating temperature of the calorimeter was 20.0°C . The heat flux and oxygen tension signal (see below) are recorded on an IBM-compatible computer (Laser 386 SXE) with specially developed software for data recording and graphical presentation [34].

2.3. Oxygen registration

Oxygen was 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 Copenhagen E5046), mounted in a thermostatted cell (Radiometer Copenhagen D616), was connected to the meter. An oxygen valve (Bürckert type 332-E-2-B-G1/4 -220/50-F-024) alternated flow of the water from either the reference or the measurement vessel to the oxygen electrode. During a period of 4 h, the valve was in the c_m position for 230 min and in the c_r position for 10 min. Oxygen consumption (O_2) was calculated according to the Fick principle [35], $\text{O}_2 = v(c_r - c_m)\text{mgO}_2\text{h}^{-1}$, where v is the water flow through the vessels ($v = 50 \text{ ml min}^{-1}$) and c_r and c_m are the oxygen concentrations measured in the outflowing water of the reference and the measurement vessel, respectively. The oxygen level of 100% air saturation (c_r) was determined with a Winkler titration and corresponded to 8.84 ± 0.062 ($n = 6$) mg l^{-1} .

2.4. Mobility measurements

2.4.1. Hardware and software

The calorimetric vessels (both reference and measurement) were supplied with 90° side-view rigid endoscopes with glass fiber lighting (Classen, Hamburg, Germany). To maintain the twin detection system, the signal of the cold-light source (Endolux, Classen, Hamburg, Germany, supplied with 150 W halogen lamp) was split and introduced via the endoscopes in both vessels. In the cold-light source, a special water-cooled glass filter was built to eliminate any heat production. From blank runs without fish it became clear that the baseline remained stable (baseline stability $\pm 0.005 \text{ Mw}$ per 24 h) with the light source on.

The tip of the 90° endoscope was placed on a small window built into the lid of the calorimeter vessel, to prevent contact with the water solution. In this way, approximately 2/3 of the vessel could be observed. Only in the upper corners of the vessel was the animal out of sight. Stainless steel wire gauze was placed in the vessel to prevent the animal from occupying this area. The endoscope for the measurement vessel was supplied with a black and white video camera (CF 4/1 with 25 mm lens, Classen, Hamburg, Germany), which was positioned outside the calorimeter to prevent interference due to instrumental heat. The picture was looped through a black and white monitor (Panasonic WV-5410) for visual feedback to the experimenter. The signal was digitized subsequently via an eight-bit monochrome frame-grabber (PC-VISION-plus; Imaging Technology, Woburn, USA). The digitized images, including the difference image, were depicted on a monochrome 12" video monitor (Sony SSM-121CE, see Fig. 3).

Data for mobility were recorded on an IBM-compatible computer (486 DX2, 66 Mhz). The software used for video tracking and motion analysis was EthoVision version 1.30 with a special utility for recording mobility (Noldus Information Technology BV, Wageningen, The Netherlands).

2.4.2. The principle of the method

The assessment of mobility is based on the difference between two video images which are digitized at a fixed time interval. Each image is digitized with an image resolution of 256×256 pixels, with $2^8 = 256$

intensity levels per pixel. The two images in one sample are subtracted from each other, and the result is referred to as the difference image. Since the body of the fish contrasts with the background, movement results in a change of the intensity distribution across the picture. This change becomes visible in the difference image. By counting the number of pixels that have changed, the mobility which occurs during the inter-image interval can be quantified. Two variables influence this measurement: the noise threshold and the length of the inter-image interval.

2.4.2.1. Noise threshold. Pixels in the difference image are only counted if the change in intensity exceeds a preset noise level. Without the threshold, noise in the video signal could also be interpreted as mobility. In the current experiments, the noise threshold was set at 35. The operator could see at the video screen that in this way no background noise could be recorded as mobility, and the intensity difference between fish and background was maximal.

2.4.2.2. Length of the inter-image interval. The amount of mobility that is measured also depends on the time interval between the two subsequent images. In this study, best results were obtained with an inter-image interval of 120 ms. The mobility measurement was repeated every second (i.e. a sampling rate of 1 Hz).

2.5. Experimental protocol

Two types of experiments were carried out with fish under either constant dark conditions or constant light conditions. Firstly, a calibration procedure was performed to determine the sensitivity coefficient of the calorimeter. An experiment lasted three days and consisted of an initial baseline of 2 h and a calibration procedure of 6 h, after which the fish was introduced into the vessel and the calorimeter was left to stabilize for 6 h. This was followed by a normoxic period of 24 h, in which mobility measurements were performed, and a hypoxic period of 32 h (four intervals of 8 h including mobility measurements). After the period of hypoxia exposure the fish was taken out of the calorimetric vessel. Thereafter, the calorimeter

was left to stabilize for 6 h before a calibration procedure was applied, and the experiment was finished with a final baseline of 2 h. Hypoxic conditions were created using a gas-mixing pump (Wösthoff, Germany, 2M, 301/a-F) with nitrogen gas and air. During four periods of 8 h, hypoxia levels of 40% AS, 25% AS, 15% AS and 5% AS were created. The data sets of heat production and oxygen consumption vs. mobility measurements were synchronized by marking the points in the calorimetric data set at which the mobility measurements were switched on [34].

2.6. Calculations and statistics

The sampling rate of the heat production and oxygen tension signal during the calorimetric and respirometric experiment of three days was one sample per min. Over a 24 h normoxic period and four hypoxic periods of 8 h, mobility measurements of the fish in the vessel were performed. These mobility measurements, with a sampling rate of one sample per second, were averaged over one min intervals. This resulted in 1440 data points during the normoxic period for heat production, oxygen consumption, and mobility; there were 480 data points for the three parameters during each hypoxia level.

The data from the normoxic period were used to determine the SMR by taking the lowest interval of 1 h in a daily cycle and by extrapolating to zero activity. The ventilation frequency was determined by observing the fish on the video screen and counting the number of beats per time period. Results were statistically tested using a two sample *t*-test. $P \leq 0.05$ was considered statistically significant.

3. Results

A typical calorimetric experiment with tilapia under constant dark conditions is presented in Fig. 1a, while a typical experiment with this species under constant light conditions is shown in Fig. 1b. The experiment starts with a calibration procedure for the heat signal. This results in a heat production of approximately 10 mW. Thereafter, the fish is introduced in the vessel. The oxygen consumption and heat production are recorded for 24 h.

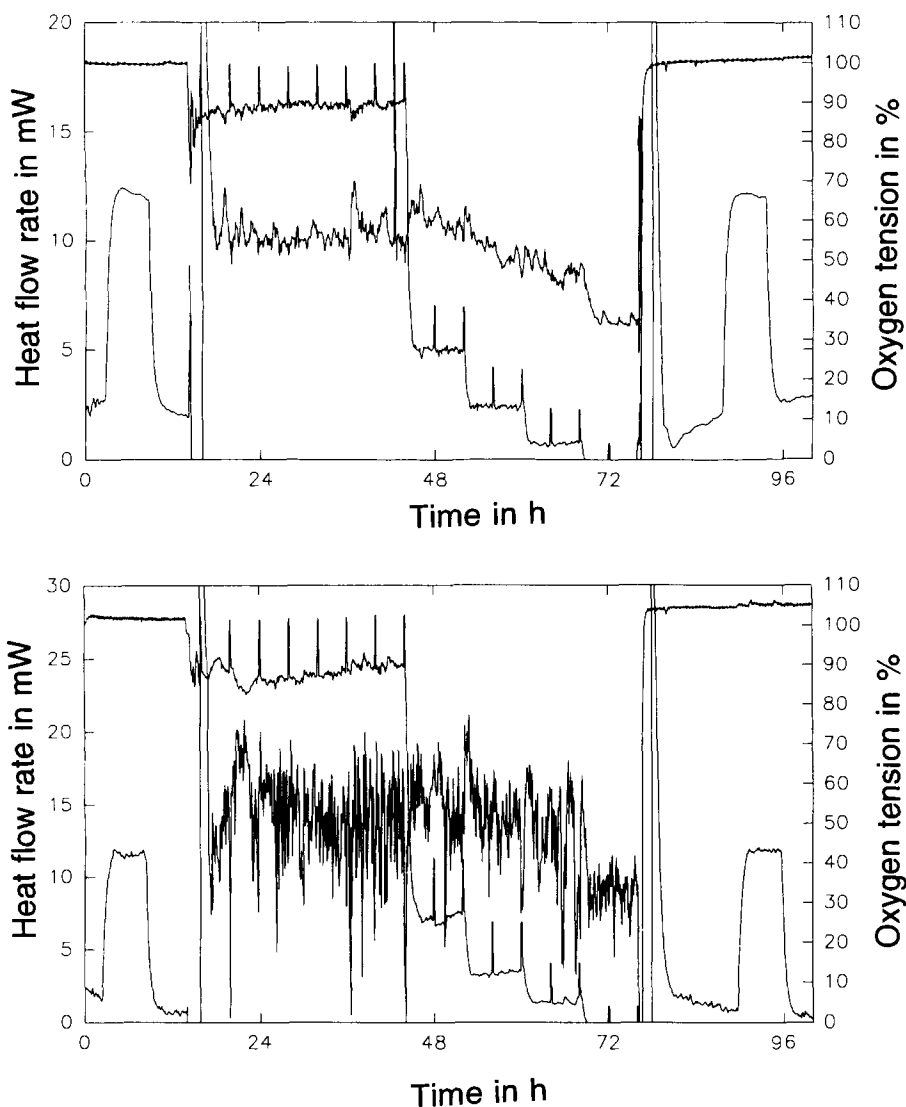


Fig. 1. Time course of a calorimetric experiment with tilapia (*Oreochromis mossambicus* Peters) under constant (a) dark and (b) light conditions for 4 days. After 24 h of normoxia the animal is exposed to a graded hypoxia load of 40% AS, 25% AS, 15% AS and 5% AS, with each hypoxia period lasting for 8 h. The top signal, alternating between reference and measurement vessel, is the oxygen tension signal. The irregular line is the heat production signal.

The oxygen tension of the inflowing water is indicated by the top of the spikes in the pO_2 curve, which switch every 250 min to the reference position. After 24 h, hypoxic water is introduced for 8 h per level.

The experiment under constant light conditions differs on two points from the 'dark' experiment. Firstly, the heat production of the fish oscillates under

light conditions and almost reaches zero mW for very short periods. Estimations of the time constant under light conditions, especially the few cases where the heat production rate approaches the zero line (Fig. 1b) revealed that it was < 5 min. Secondly, the metabolic rate under normoxia, based on direct calorimetry, is 18.8% higher in the light than in the dark, while when

calculated via the technique of indirect calorimetry, this value is 11.1%. The mean heat production over a 24 h period, measured by direct calorimetry, was $1681 \text{ mJ h}^{-1} \text{ g}^{-1} \text{ ww}$ in the light vs. $1415 \text{ mJ h}^{-1} \text{ g}^{-1} \text{ ww}$ in the dark, while by indirect calorimetry this was $1596 \text{ mJ h}^{-1} \text{ g}^{-1} \text{ ww}$ vs. $1437 \text{ mJ h}^{-1} \text{ g}^{-1} \text{ ww}$ (Table 1).

For the normoxic period of 24 h the mean oxycaloric equivalents (per interval of 1 h) are given in Table 1. For the 'dark' experiments, the mean value is $427.7 \text{ kJ (mol O}_2\text{)}^{-1}$, while for the light experiment this value is $456.8 \text{ kJ (mol O}_2\text{)}^{-1}$. There is no significant difference between these two values (Table 1). Assuming an oxycaloric value for mixed substrates of $433.6 \text{ kJ (mol O}_2\text{)}^{-1}$ [36] and converting oxygen consumption to heat production there is large agreement between the direct and indirect calorimetric methods. For the dark experiments, comparing the indirect with the direct calorimetric method, the maximal difference was +6.4%, while for the light experiments the maximal difference was -8.7% (Table 1).

In Table 2, the mean heat production, oxygen consumption and oxycaloric equivalents during the whole normoxic (24 h) and hypoxic periods (8 h per period) are given. For the 40% AS, 25% AS, 15% AS and 5% AS hypoxia levels the heat production under dark conditions is respectively, 103.5%, 97.3%, 81.1% and 54.6% of the mean normoxic heat production,

while for the light experiments this is 104.3%, 96.2%, 70.3% and 48.0%. This indicates that metabolic depression occurs at 15% AS and 5% AS. With increasing hypoxia levels, the oxycaloric equivalent is concomitantly increasing for the 'light' as well as the 'dark' experiments (Table 2). At 5% AS, this increase is significantly higher, which is indicative of an activation of anaerobic heat production.

This becomes clear from Fig. 2a and b in which typical experiments for dark and light conditions are depicted. The heat production measured by direct calorimetry is compared with the heat production based on indirect calorimetry. The black areas correspond to an 'anaerobic overshoot' and the hatched area to an 'aerobic overshoot'. During the 5% AS hypoxia level, the metabolism of the fish was significantly anaerobic over the whole period (Fig. 2a-Fig. 2b). The anaerobic contribution is significantly higher under light than under dark conditions (Fig. 2a-Fig. 2b).

Typical examples of mobility registration are illustrated in Fig. 3, photoseries 1 to 3. The series A to B are made with an interval of 1 s, while photos C are the difference images. The white area in C reflects the changed pixels due to the movement of the fish. In photoseries 1, there is only movement of the pectoral fins of the fish. In photoseries 2 and 3, the fish is moving resulting in an overall displacement of the

Table 1

Comparison of the indirect vs. the direct calorimetric method based on an oxycaloric equivalent of $433.6 \text{ kJ mol}^{-1}$ [36] for four individual experiments with tilapia under constant dark and light conditions over a period of 24 h

Dark	Ox. equivalent mean per 24 h/(kJ mol ^{±1})	Heat direct mean per 24 h/(mJ h ⁻¹ (gww) ⁻¹)	Heat indirect mean per 24 h/(mJ h ⁻¹ (gww) ⁻¹)	Comparison mean indirect vs. mean direct
Exp. 1	439.9	1390	1375	-1.1%
Exp. 2	410.2	1142	1215	+6.4%
Exp. 3	441.4	1740	1715	-1.5%
Exp. 4	419.3	1389	1442	+3.8%
MEAN	427.7	1415	1437	
STD	13.4	213	181	
<hr/>				
Light				
Exp. 1	453.2	1713	1645	-3.9%
Exp. 2	472.6	1701	1554	-8.7%
Exp. 3	449.6	1695	1637	-3.4%
Exp. 4	451.8	1614	1549	-4.0%
MEAN	456.8	1681	1596	
STD	9.2	39	45	

Table 2

Mean value and standard deviation of heat production, oxygen consumption and oxycaloric equivalents of four experiments with one individual tilapia under constant dark ($n = 4$) and under constant light ($n = 4$) conditions. The fish is exposed in a Sétaram GF 108 flow-through calorimeter for a normoxic period of 24 h followed by a graded hypoxia load (40% AS, 25% AS, 15% AS and 5% AS) for 8 h per hypoxia level

Dark	Heat production/ ($\text{mJ h}^{-1} (\text{gww})^{-1}$)	Oxygen consumption / ($\mu\text{mol h}^{-1} (\text{gww})^{-1}$)	Oxycaloric equivalent/ (kJ mol^{-1})
Normoxia	1415±213	3.3±0.37	427.7±13.3
Hyp-40%	1465±172	3.3±0.36	450.5±13.4
Hyp-25%	1376±193	3.1±0.35	448.0±25.7
Hyp-15%	1147±164	2.5±0.30	460.7±38.7
Hyp-5%	774±149 ^a	1.0±0.12 ^a	816.0±141.1 ^a
Light	Heat production/ ($\text{mJ h}^{-1} (\text{gww})^{-1}$)	Oxygen consumption / ($\mu\text{mol h}^{-1} (\text{gww})^{-1}$)	Oxycaloric equivalent/ (kJ mol^{-1})
Normoxia	1680±39	3.7±0.09	456.8±9.2
Hyp-40%	1753±65	3.3±0.22	553.9±35.1
Hyp-25%	1616±100	3.3±0.24	503.8±54.9
Hyp-15%	1182±163	2.6±0.18	457.5±40.7
Hyp-5%	807±100 ^a	0.8±0.14 ^a	873.7±337.8 ^a

^a Denotes significant difference ($P \leq 0.05$).

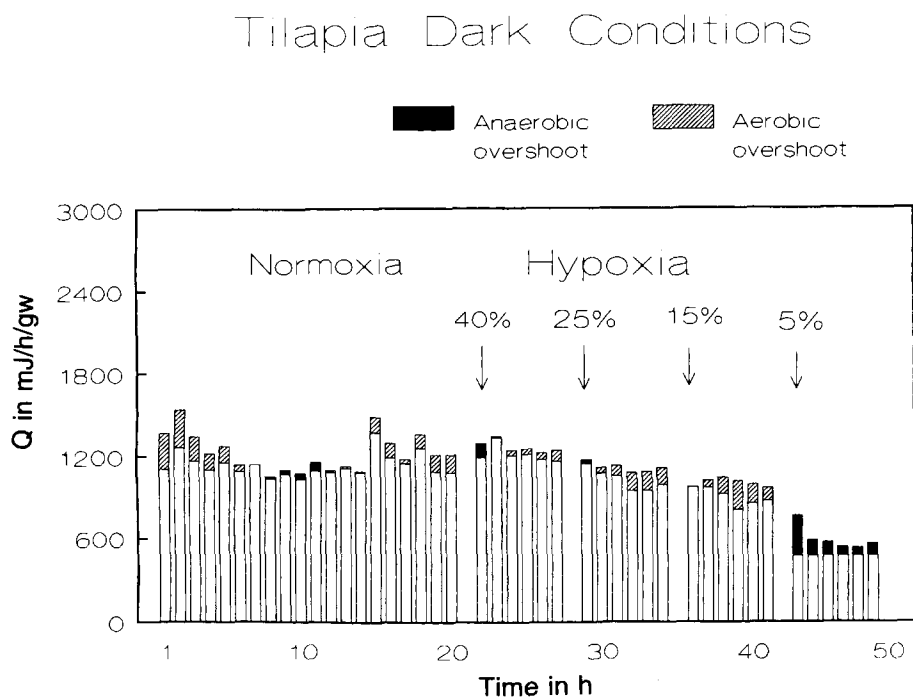


Fig. 2. Experiment of tilapia (*Oreochromis mossambicus* Peters) under constant (a) dark and (b) light conditions. The heat production (open columns), based on indirect and direct calorimetric data is expressed per gram incubated wet weight per interval of 1 h. The hatched columns indicate an aerobic overshoot (open + hatched columns: heat production based on indirect calorimetry). The black columns indicate an anaerobic overshoot (open + black columns: heat production based on direct calorimetry).

Tilapia Light Conditions

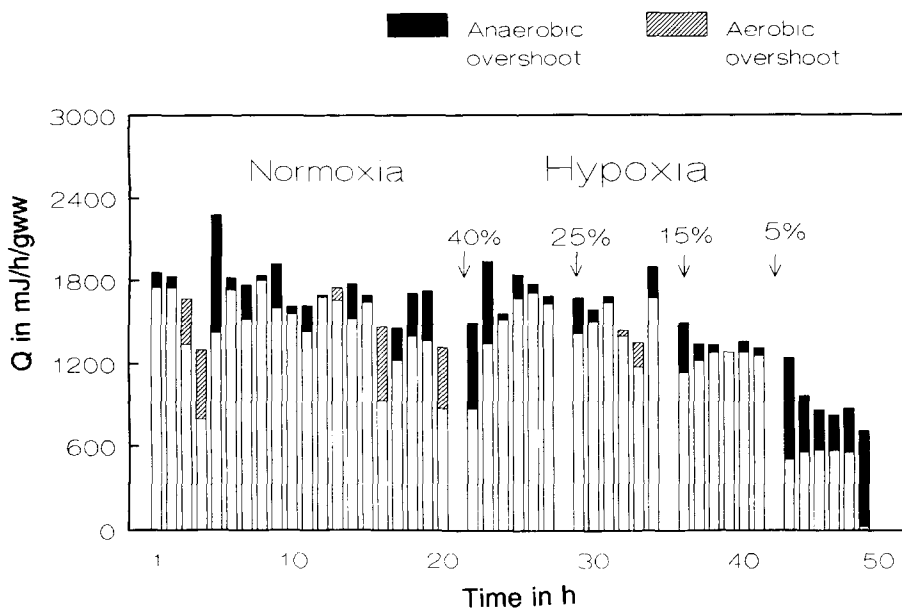


Fig. 2. (Continued).

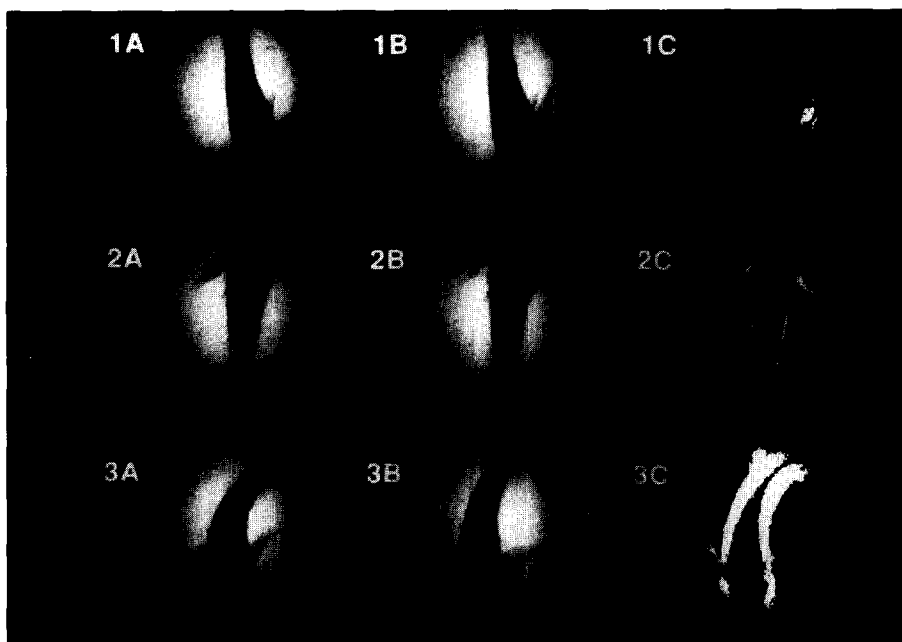


Fig. 3. Photo series for which the inter-image interval between image A and B is 120 ms. Image C is the difference image where the white area reflects the changed pixels due to the difference between image A and B. From this white area (composed of individual pixels) mobility can be quantified (see text). Photoseries 1 depicts only mobility due to movement of the pectoral fins of the fish. Photoseries 2 and 3 depict mobility of the fish with a displacement of the body's center of gravity.

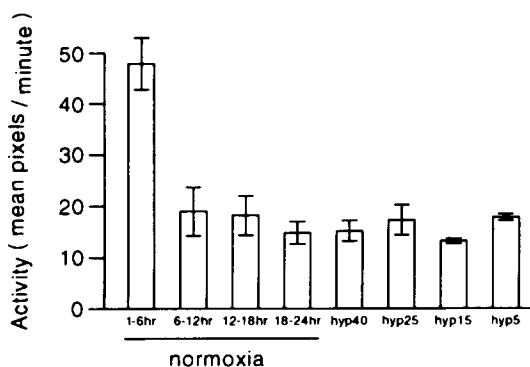


Fig. 4. Mobility of the animal during a graded hypoxia load. Depicted is the mean \pm SD of four individual experiments with one animal.

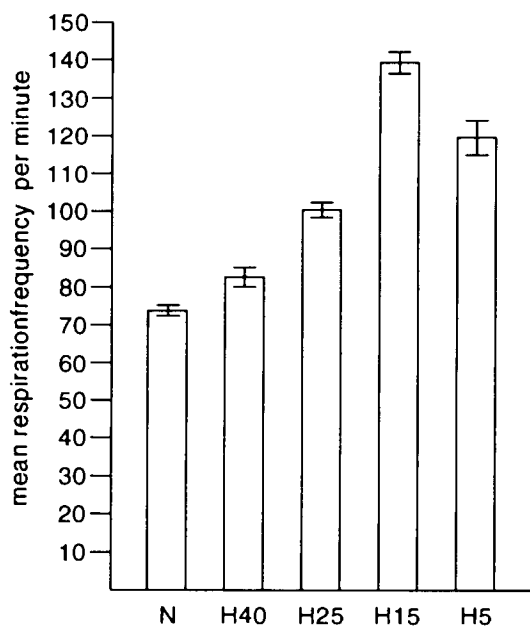


Fig. 5. Ventilation frequency of the animal during a graded hypoxia load. Depicted is the mean \pm SD of four individual experiments with one animal.

body. The results of the locomotor activity measurements are given in Fig. 4. From this figure it becomes clear that the activity of the fish in the vessel increases during the first 6 h. The ventilation frequency, based on the branchial ventilation, shows a similar picture in

all experiments. The frequency increases from 74 beats per min to a maximum of 139 beats per min, which is reached at 15% AS (Fig. 5). No correlation was observed between locomotor activity and heat production (the correlation coefficients for the four individual experiments between heat production and activity was 0.004, 0.054, 0.003, and 0.099), so that estimation of the SMR based on an extrapolation to zero activity is not possible.

4. Discussion

From Table 1, it can be observed for the dark experiments that the minimal heat production is $1142 \text{ mJ h}^{-1} \text{ g}^{-1} \text{ ww}$ via direct calorimetry and $1215 \text{ mJ h}^{-1} \text{ g}^{-1} \text{ ww}$ via indirect calorimetry, while for the light experiments these values are 1614 and $1549 \text{ mJ h}^{-1} \text{ g}^{-1} \text{ ww}$ respectively, corresponding to 141% and 127% of the dark experiment values. Brett and Groves [3] summarized the results of '57 adequate experiments' to determine the SMR and found a value of $0.29 \pm 0.11 \text{ kcal kg}^{-1} \text{ h}^{-1}$ which corresponds to $1213.4 \text{ mJ h}^{-1} \text{ g}^{-1} \text{ ww}$. This value corresponds with the values observed in this dark study. A point of criticism on the value for the SMR of Brett and Groves [3] may be that they neither defined 'adequate experiments' nor elucidated their criteria.

Under light conditions, tilapia occasionally reach very low heat production values (Fig. 1b). In our opinion, these low values do not correspond to the SMR because the animals are not able to maintain this metabolic rate level for a sustained period of time. The mechanism is puzzling. A possible explanation may be a temporary decrease or even discontinuity of ventilation and/or blood circulation. Such behavior would immediately block both heat flux and gas exchange. In a previous paper [37] we calculated a time constant via the technique of system identification for the Sétaram GF 108 calorimeter with 1 l vessel of 33.2 min. In this study we observed a time constant of $< 5 \text{ min}$ (Fig. 1b). This can probably be explained by the approach used to calculate the time constant and the position of the heat generating object in the vessel. In case of the time constant of 33.2 min this was based on electrical calibration with a resistor placed on top of the calorimetric vessel. In this study the fish was kept with a stainless wire gauze at the

bottom of the vessel to be able to perform mobility measurements. More research is needed to elucidate this matter.

Comparing the dark experiment (Fig. 2a) with the light experiment (Fig. 2b), it becomes clear that, under normoxia, the animals in constant light conditions experience periods of both aerobic overshoot and anaerobic overshoot. This indicates that the anaerobic metabolism is activated under normoxia from time to time. Under dark conditions, the animals are closer to the SMR, the metabolism does not show so many fluctuations, and the activation of the anaerobic metabolism is diminished.

The second method for determination the SMR, based on an extrapolation to zero locomotor activity, did not work for two possible reasons. Firstly, the fish had limited space and, consequently, could not generate a high level of activity. Secondly, very low levels of heat production were achieved by temporarily blocking the exchange of heat production via ventilation and/or circulation. Several calorimetric studies have observed a correlation between locomotor activity and heat production via indirect calorimetric methods. This was observed for oxygen consumption and locomotor activity for a single goldfish [19], a group of roaches (*Rutilus rutilus*) [18], mice at different environmental temperatures [38], rats at different environmental temperatures [39], and humans [4]. In our study, we did not observe any correlation between locomotor activity level and heat production. An explanation may be that we measured via the difference image method a variety of patterns of locomotor activity. Some may have a high energy output (like burst activity patterns of swimming) but others (like branchial ventilation) may have a low energy expenditure. Besides, the limited space in the vessel restricts the animal in the ability to generate high levels of energy expenditure.

The mean oxycaloric equivalents of the dark and light experiments are 428 ± 13.35 and 457 ± 9.21 kJ (mol O₂)⁻¹, respectively. This is in the range of an oxycaloric equivalent of a mixed substrate. As stated earlier [34], it is our opinion that the accuracy of heat flux and oxygen consumption measurements do not allow conclusions about substrate usage. Firstly, Gnaiger et al. [40] assumed an experimental error of 5% for both methods (direct and indirect) which gives an error in the oxycaloric equivalent of

$\pm \sqrt{(5^2 + 5^2)} = \pm 7\%$ corresponding to ± 32 kJ (mol O₂)⁻¹ which is within the difference range of substrates [34]. Secondly, we observed large fluctuations in the oxycaloric equivalents (unpublished results), which were also observed by Brafield [36]. This can probably be explained by large fluctuations in the locomotor activity of the fish; also, we observed in this experiment that an immobile fish can fluctuate its metabolic rate to a large extent. As a result of these fluctuations, the metabolism of the animals may become partly anaerobic (Fig. 2b, black area) during normoxia.

The metabolic rate under constant light conditions was elevated by 18% based on the heat production measurements and 11% based on the oxygen consumption data. Ross and McKinney [41] summarize some hormones which may contribute to this response of elevated levels under light conditions. A correlation was observed between high day serotonin levels and swimming activity in Gulf killifish, *Fundulus grandis* [42]. ACTH peaks have been observed in goldfish (*Carassius auratus*) after the onset of light [43]. Blood cortisol levels show the same pattern [44,45]. Both serum prolactin and growth hormone show a diurnal variation linked to photo period in Sockeye salmon, *Oncorhynchus nerka* [46]. The hormone which has the largest contribution to an elevated metabolic rate under constant light conditions is unclear.

As stated by Nilsson et al. [1], two cooperating strategies for saving energy, one physiological and one behavioral, are utilized by anoxia-tolerant animals [47]. The behavioral strategy was recently observed, recording locomotor activity measurements of goldfish with a video-imaging system and the animals reduced the mobility under conditions of anoxia [1]. The second energy saving strategy of metabolic depression was measured in anoxic goldfish in our laboratories with the same calorimetric system and was estimated to be a 70% reduction, compared to the normoxic metabolic rate [12]. However, no locomotor activity measurements were performed at that time, so that the SMR level could not be estimated. Because measurements were performed in the dark with stress-free, postabsorptive, undisturbed animals, it was assumed that the measured level during normoxia corresponded to the SMR. In this study, we did measure the activity level of tilapia under light conditions; therefore we can conclude the extent to which

the reduction of the metabolic rate can be ascribed to either a behavioural strategy or the cellular process of metabolic depression. Therefore, we have to consider the locomotor activity measurements given in Fig. 4. The mean locomotor activity pattern at a hypoxia level of 5% AS is not significantly different from normoxia (interval 12–24 h). From this observation, it can be concluded that the drop in energy expenditure is the result of a reduction of the energy consumption and the metabolic depression and not due to a reduction of locomotor activity.

From this study it becomes clear that the animals under severe hypoxia do not become lethargic or show a reduction of locomotor activity as is observed with anoxia-tolerant species during anoxia [1]. The frequency of branchial ventilation increases sharply combined with heavy bouncing movements of the body due to increased ventilation. This may be in contrast to the observed reduction of mobility observed in anoxic crucian carp [1]. The observed reduction of the metabolic rate can only be ascribed to a reduction of energy consumption, the metabolic depression [48]. The strategy of metabolic depression has been previously observed in a variety of fish species: tilapia (this study), the crucian carp [1], goldfish [12,34,37], sole [49], eel (van den Thillart et al. unpublished results). This may be an important strategy for a number of fish species to cope with conditions of low oxygen.

In conclusion, we determined the heat production and oxygen consumption, in combination with locomotor activity measurements for the fish species tilapia exposed to hypoxic conditions. The experiments under constant dark and light conditions give some new information about the normoxic metabolic rate and the process of metabolic depression under conditions of hypoxia.

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