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Direct calorimetry of aquatic animals: automated and computerized data-acquisition system for simultaneous direct and indirect calorimetry

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Abstract

A system for computerized registration of heat production data and oxygen tension values of a differential 1 litre flow-through microcalorimeter (Setaram GF 108) is described. The linked values of oxygen concentration and heat production data were simultaneously stored on a computer. The aerobic and total heat production rates were thus measured of goldfish (*Carassius auratus* L.) during normoxia, hypoxia and anoxia exposure. Incidentally, a partial anaerobic response was observed in goldfish during normoxia. During severe hypoxia and anoxia, goldfish respond with a reduction of the metabolic rate and a strong increase of anaerobic heat production. From the linked oxygen consumption and heat production data, the oxycaloric equivalent was calculated.

The described system enables us to study continuously the effects of environmental factors on the metabolic rate of aquatic organisms.

Keywords: Anoxia; Calorimetry; Computer; *Carassius auratus* L.; Goldfish; Hypoxia; Living system; Oxygen

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1. Introduction

According to the laws of thermodynamics, all the available energy liberated by the oxidation of substrates is converted into heat, chemical and kinetic energy. The heat reflects the intensity not only of the cellular energy metabolism but also of the physiological processes necessary for the organization and functioning of the organism, in other words the liberated heat corresponds to the metabolic rate of the organism. Estimation of heat flow rate is carried out with a calorimeter. The heat flow rate can be detected directly, which requires expensive sophisticated equipment, or indirectly, based on gas exchange products which can be converted with a conversion factor into heat production. An advantage of calorimetry is that it is non-invasive and non-destructive so the animals can eventually remain stress free. A prerequisite for the calorimetric method is that a steady state situation is reached for heat production, gas exchange and metabolite levels. Furthermore, processes like locomotion and muscle activity produce heat. It was first assumed that the measurement of the heat production of fish using direct calorimetry was not possible because of their relatively low heat production and the high heat capacity of water [1]. However, some studies with fish have been published measuring heat production via direct methods [2–8].

In general, two factors have limited the development of calorimeters for direct measurement of the heat production of aquatic organisms. The first is the volume. Most chambers have a limited volume which limits the size of the organisms to be studied. Smith et al. [3] were the first to develop a system of appropriate size for the study of fish. Lowe [4] used a 100 cm^3 chamber. The second problem which had to be solved was the problem of constant experimental stress-free conditions. Most developed calorimeters are closed systems because the technical demands with respect to the thermal stabilization of the inflowing water for flow-through systems are high [1]. Recently a 1-liter differential flow-through system for direct calorimetric studies was developed [9] which enables the operator to perform long-term experiments under stress-free constant conditions [5–8,10]. In this way it can be used to monitor fluctuations in the metabolism, i.e. switching from aerobic to anaerobic or to intermediary forms of metabolism [10,11], or to study the effects of environmental factors like hypoxia [5,10], anoxia [5,6,7,8] and acidification [12] on the metabolic rate of aquatic organisms.

Because direct calorimetry is rather expensive, indirect methods are more common. In principle, there are two indirect methods. The first method is based on the oxygen consumption which can be converted with the oxycaloric equivalent (derived via bomb calorimetry of a mixture of substrates) to heat production. This method is common for aquatic animals because oxygen can easily be detected in the aquatic environment. In contrast to mammalian indirect calorimetry, the RQ ($\dot{V}(CO_2)/\dot{V}(O_2)$) for fish does not give sufficient information on substrate utilization. This is because protein oxidation by NH_3-excreting animals results in an RQ value of 0.96 which cannot be distinguished from carbohydrate oxidation with an RQ of 1.00 [13]. Therefore, another indirect method is applied based on CO_2 and NH_3 measurement. Based on the method of Brouwer [14] for ruminants, an

equation for fish has been derived [15] describing the relation between substrates, products and heat. This method has been used to determine an energy balance for anoxic goldfish. It was concluded that some fat synthesis occurred for goldfish under anoxic conditions [7]. The difference between both methods (direct and indirect) is that direct calorimetry measures total heat loss from the body while indirect calorimetry measures total heat production by the body [16]. So with direct calorimetry, the heat stored in the body by an increase of body temperature or accumulated in the form of energy-rich chemical bonds is not detected. However, during long term experiments, assuming there is no storage of heat in the body, there is considerable agreement between both methods.

The aim of this paper is to give a description of an automatic oxygen-registration system in a flow-through differential 1 litre calorimetric set-up for combined direct and indirect calorimetry. This system was developed because we wanted a continuous oxygen-registration system with which heat production and oxygen data could be linked in order to monitor continuously aerobic and total heat flow rate. In addition, it enables us to calculate the oxycaloric equivalent $\dot{Q}/\dot{V}(O_2)$ at any moment which gives information about the substrates used. Some values for the oxycaloric equivalent from the literature for the three main substrates for oxidation are given in Table 1.

Originally, the heat production was measured in our laboratory with a differential 1 litre flow-through microcalorimeter (Setaram GF108, Lyon, France) with a microcomputer (Hewlett Packard 86B) for data registration [9]. The software was delivered by Setaram (Lyon, France). Oxygen concentration values were measured by perfusing samples of the outflowing water of the calorimeter along an oxygen electrode with a polypropylene membrane fitted in a thermostatted cell (Radiometer) and connected to a Radiometer Digital Oxygen Analyzer PHM 72 with a pO_2 module PHA 932. The oxygen consumption was calculated discontinuously by measuring the pO_2 of the water in the reference and the measurement vessel, and multiplying the obtained difference with the flow [5–8,20]. This procedure was carried out manually and the oxygen concentration values were registered graphically. We improved the system in three ways:

(1) MS DOS software was developed for automatic calibration and data registration. The use of MS DOS-applicable datasets enables us to apply the appropriate software to calculate the time constant of the system and to apply deconvolution techniques to correct for the lag time of the calorimetric set-up [21].

Table 1
Some oxycaloric equivalents (in kJ mol^{-1}) converted from table 10.1 of Ref. [15]

Substrates	Elliot and Davison [17]	Brafield and Llewelyn [18]	Gnaiger [19]
Carbohydrate	472.64	472.32	471.04
Fat	439.04	439.04	440.00
Protein to ammonia	428.48	427.52	447.04

(2) A rotating valve was installed which is controlled by timed-event protocol run by the computer, to replace the manual procedure.

(3) The oxygen tension values and heat production data were recorded simultaneously on the same microcomputer system so that both data sets could be linked. These improvements provide linked datasets of oxygen tension and heat production which gives information on the relation between aerobic and anaerobic heat production.

2. Materials and methods

The system described consists of three subsystems (Fig. 1): a measuring unit, containing the flow-through calorimeter set-up and the oxygen detection system; an interface unit with microprocessor system; and a registration unit with personal computer and software for communication and data collection.

2.1. Measuring unit

2.1.1. Flow-through system

The concept of continuous perfusion of the measurement vessel is applied to ensure constant experimental conditions for the animals. In this way, long-term monitoring of aquatic animals (1–50 g) under stress-free constant conditions can be applied [9], because waste products are flushed out. The flow-through concept

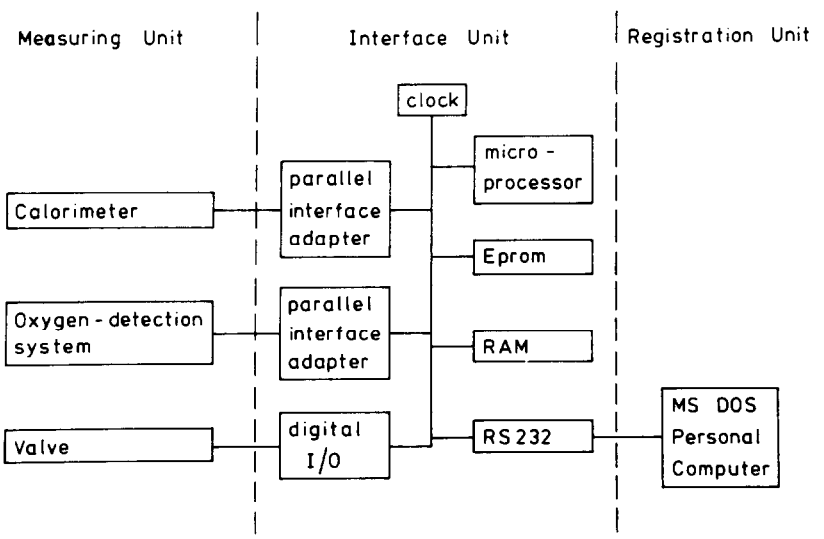

Fig. 1. Block diagram of the experimental set-up. The system consists of three units: a measuring unit, interface unit and registration unit.

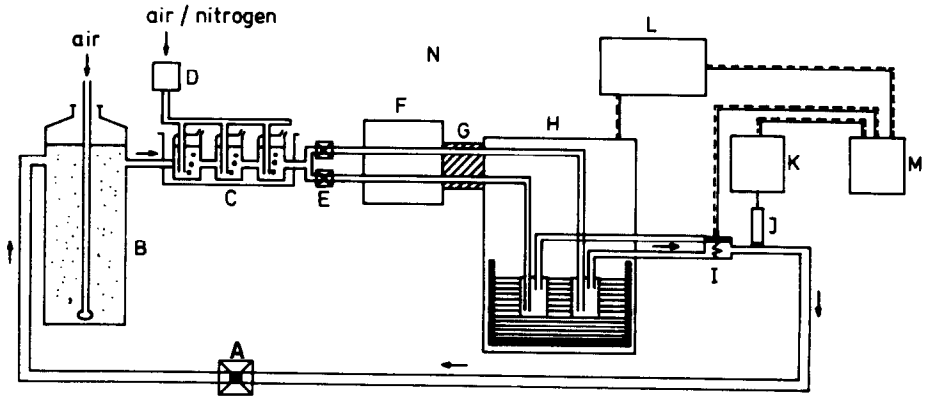

Fig. 2. Schematic drawing of the experimental set-up: A, pump; B, storage tank with aeration; C, glass bottles with gas exchangers in thermostatted waterbath (Tamson TMV 45) for aeration with air, nitrogen or a mixture; D, gas mixture pump (Wösthoff, West Germany, 2M301.a-F) to create hypoxic conditions; E, Verder pumps (Verder V006.10/2031); F, heat exchanger for temperature stabilization (Bioactivity monitor LKB2277); G, vacuum insulating tube to ensure thermal stability; H, 1 litre differential flow-through microcalorimeter (Setaram GF108), final temperature stabilization by means of three heat exchangers inside the calorimeter; I, automatic alternating Brückert three-way valve controlled by the computer; J, oxygen electrode (Radiometer, Denmark, E5046-0) which is fed by a 2 ml water flow drawn with a Gilson peristaltic pump; K, radiometer (Digital acid base analyzer PHM 72 with a pO_2 module PHA 932) connected with a specially developed interface to the computer; L, miscellaneous equipment for the calorimetric set-up, including amplifier (Setaram A85), voltmeter (Setaram VN), thermometer (Setaram TN2), temperature programmer and controller (Setaram), thermal safety unit (Setaram TS1); M, personal computer (Laser 386 SXE) for data registration; N, thermostatted room.

requires special demands for temperature stabilization of the water flowing through the calorimetric system. An overview of the set-up is given in Fig. 2. Water out of the storage tank flows through three glass gas-exchangers placed in a waterbath (Tamson TMV 45) with a temperature stability of ± 0.05°C. Depending on the exerimental protocol, the water can be saturated with air (normoxia) or nitrogen (hypoxia or anoxia). Using two magnetic Verder pumps (Verder V006.10/2031) which have an adjustable flow range from 0 to 100 ml min^{-1} and are supplied with carbon cogwheels for a stable water flow, the water is pumped through an isothermal heat exchanger (Bioactivity Monitor LKB2277) containing a 25 litre waterbath governed by a thermal sensing system (stability ± 0.0002°C). From the heat exchanger, the water flows through a vacuum-insulated tube to the calorimeter (Setaram GF108, Lyon, France) to avoid environmental disturbances by ventilation or radiation. Within the calorimeter the water is thermally equilibrated by 3 heat exchangers, two in contact with the temperature control system of the metal block (the internal and external thermostats) and one on top of the vessel, which is a counter-current heat exchanger [9]. The total experimental set-up is placed in a thermostatted room which is set at a temperature of 19.3 ± 0.3°C. The described devices and the thermostats ensure a temperature stability within the calorimetric vessels of $\pm 1 \times 10^{-4}$°C.

2.1.2. Twin-detection system

The differential twin-detection system is applied to suppress environmental disturbances. Two identical vessels are positioned in the thermostatted calorimetric block. The experimental signal is the difference between the signals of the measuring vessel containing the animals and the identical reference vessel. This principle eliminates any disturbances of the heat sink and guarantees a stable baseline of 0.005 mW per 24 h (Fig. 3).

2.1.3. Analog/digital conversion and amplification

Two symmetrical thermal fluxmeters (each composed of 40 basic heat fluxmeters connected in series) surround each of the experimental vessels. The heat flow rate from the vessels to the calorimetric block is transformed to an electrical signal (thermopile voltage). The recorded signal is proportional to the temperature difference between the measurement and reference vessels (100 $\mu V\ mW^{-1}$). Amplification and analog-to-digital conversion is applied by the amplifier (Setaram A85), which has ten selection ranges for the input signal and a maximum output signal of 1 V. The different amplification factors are incorporated in the software. The amplifier contains a digital output on TTL level (Transisitor Transistor Logic) which is connected to the microprocessor interface unit.

2.1.4. Oxygen-registration system

The oxygen recorder is a digital oxygen analyzer, Radiometer Copenhagen type PHM 72c with pO_2 module, type PHA 932. A platinum–silver pO_2 electrode

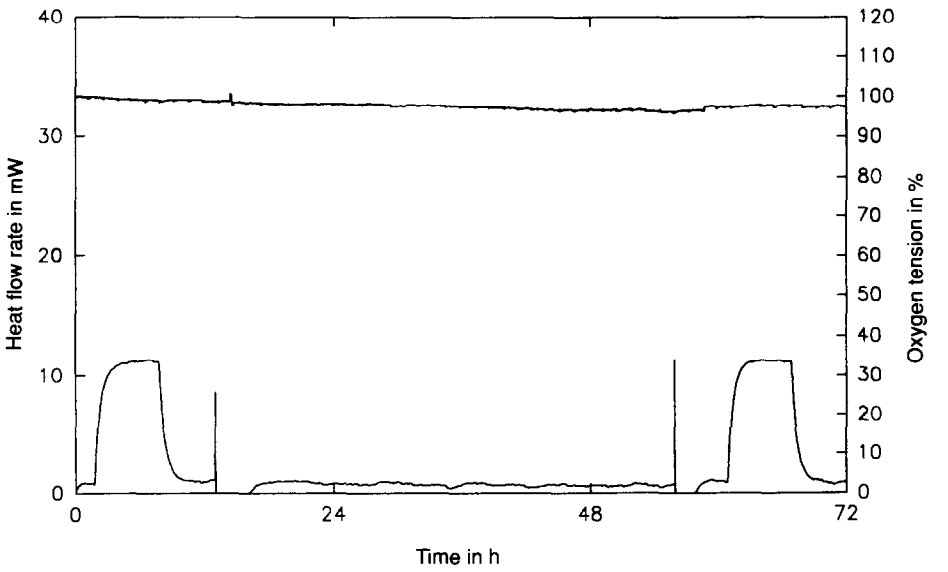

Fig. 3. Registration of a 3-day control experiment (no fish in measurement vessel). The lower line is the oxygen tension signal. The upper line is the heat production signal.

(Radiometer Copenhagen E5046) is mounted in a thermostatted cell (Radiometer Copenhagen D616) and connected to the meter. All parts from calorimeter to oxygen electrode are in stainless steel to prevent oxygen diffusion. From the outflowing water of the calorimeter, a flow of 2 ml min^{-1} is drawn over the electrode using a Gilson peristaltic pump. This is sufficient to neglect the small internal blank oxygen consumption of the electrode. The digital oxygen analyzer contains a 25-pins Binary Coded Decimal (BCD, Cannon DB 25 S) output socket. The output signal of the oxygen connector is a 4.5-digit BCD signal on TTL level so it could be connected to the microprocessor interface in the same way as the amplifier of the calorimeter.

2.1.5. Switching valve

The valve alternating between measurement and reference outlet is a bi-stable Bürckert valve (type 332-E-2-B-G$\frac{1}{4}$-220/50-F-024). This valve switches position by a short current pulse. This is a preferred alternative to continuously powered valves which generate heat, thus disturbing the oxygen tension signal.

2.1.6. Oxygen consumption

Rates of oxygen consumption were calculated following the Fick principle [20]

$$\dot{V}(O_2) = v(c_r - c_m) \quad (\text{in mg } O_2\ h^{-1})$$

where v is the water flow through the vessels (for both vessels $v = 50$ ml min^{-1}) and c_r and c_m are respectively the oxygen concentration measured in the outflowing water of the reference and measurement vessels. Using a Winkler titration, the oxygen concentration of the air-saturated water flowing in the calorimeter was detected which corresponded to an oxygen concentration of 8.94 ± 0.063 ($n = 6$). After passage through the calorimeter the oxygen concentration corresponded to a value of 8.84 ± 0.062 ($n = 6$) mg per litre. In the calculations this latter value corresponded to c_r.

2.2. Interface unit

The interface unit consists of a stand-alone microprocessor system which records the signal of the two instruments (calorimeter and oxygen detection system). In addition, it can control the Bürckert oxygen valve and communicate with the computer via an RS232 port. The settings, the sampling time of the instrument and the duty cycle of the valve, can be changed by communication via the computer. Normally the computer samples via the interface to the instruments and stores the values internally in memory; if another operation is applied, e.g. when the operator checks the experimental data, the microprocessor of the interface automatically takes over data-acquisition and valve control. If the computer switches back to record mode, it copies immediately all the new data from the microprocessor memory to the hard disk and then continues sampling.

2.3. Registration unit

The computer used for the data collection is a Laser 386 SXE microcomputer. To avoid electronic noise the interface stores ten measured values with a sample frequency of 6 s in a buffer and sends the average value for storage to the computer, resulting in an effective sampling rate of one per minute. The sampling rate can be set by the operator. Software was developed in the language Qbasic; a flowchart is given in Fig. 4. The following items are implemented in the program.

2.3.1. Calibration procedure

Heat flow rate calibration is performed by electrical power (Setaram EJ 2 Joule calibrator). The aim of the calibration procedure is to define the response of the calorimeter under the prevailing conditions by detecting its sensitivity coefficient. During a defined time an electrical current of 0.315 A and a voltage of 3.164 V is applied to a resistor of 1000 Ω mounted inside the measurement vessel. This results in a power output signal of 9.97 mW. The quantity of heat dissipated in the calorimeter is calculated by integration, while corrections are made for the initial and final baselines. The ratio of the measured signal and the applied power results in the sensitivity coefficient. A typical value for a calibration coefficient at a flow of 50 ml min^{-1} and a temperature of 20°C is 88.79 ± 0.44 μV mW^{-1} ($n = 4$). Before starting an experiment, the derived sensitivity coefficient of the calibration procedure is introduced. With this factor, recorded voltage signals are directly transformed to power (mW).

2.3.2. The selection menu

It is possible to change the settings of the system and to specify the experimental variables, such as the length of the experiment, amplification factor, sampling rate, and directions for valve position, i.e. measurement or reference. Furthermore, a marker can be added to the data by the operator, which is recorded in an extra column of the datafile and enables us to indicate exactly when the variable condition, e.g. hypoxia, was introduced during the experiment.

2.3.3. Software for a graphical presentation on the computer screen

The datafile can be monitored during an experiment. The heat production data (mW) and oxygen tension values (%) are depicted in a graph (see Fig. 5).

2.4. Experimental protocol

2.4.1. Control experiment

To derive the sensitivity coefficient, an initial stable baseline was measured for 20 min ('waiting time'); thereafter a power of 9.97 mW was applied for 360 min to the vessel without fish ('impulsion time'). The return time was 240 min and the input range was 2.5 mV. The experiment lasted 3 days. No fish were placed in the vessel. The sampling rate was once a minute (average value of 10 data points) resulting in 4320 data points each for oxygen tension and heat production. The oxygen tension

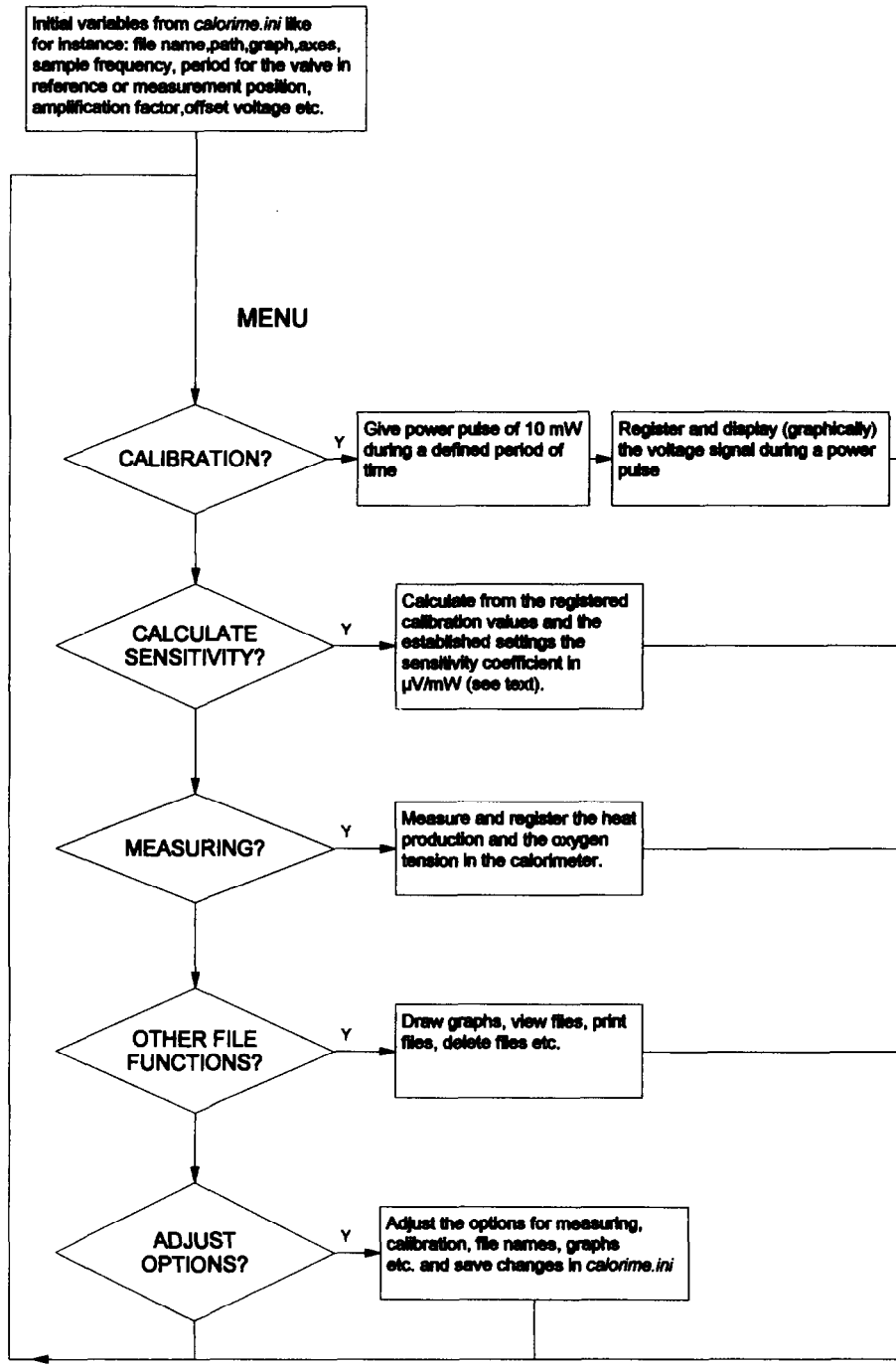

Fig. 4. Flow chart diagram of the developed software.

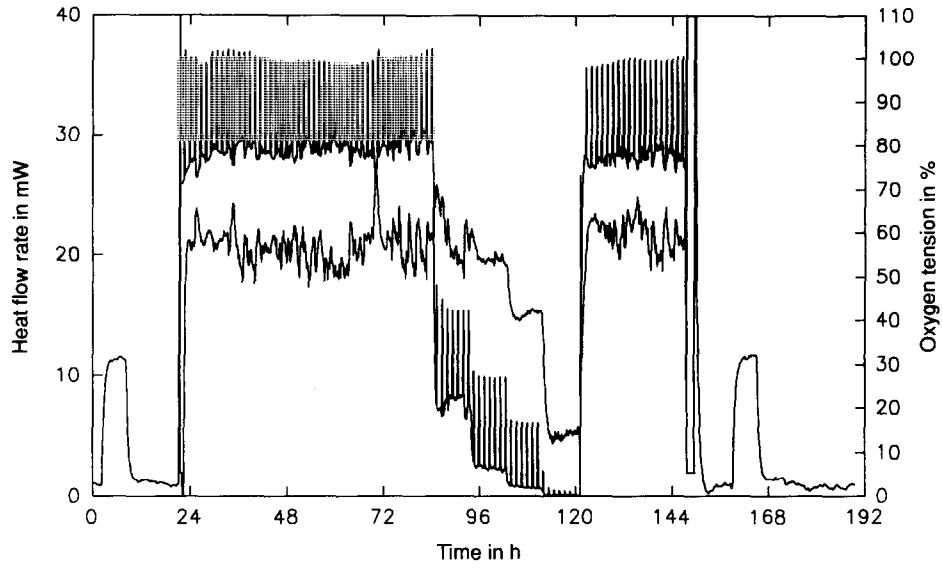

Fig. 5. Registration of an 8-day experiment with 4 goldfish (*Carassius auratus* L.) exposed after 84 h to three levels of hypoxia (42.5%, 27.1% and 16.7% air saturation) followed by anoxia. The top signal, alternating between reference and measurement position, is the oxygen tension signal. The irregular line is the heat production signal.

of the outflowing water from the measurement vessel was measured during 70 min, followed by detection of the water from the reference vessel over a period of 10 min. Results are depicted in Fig. 3.

2.4.2. Experiment with animals

Goldfish (*Carassius auratus* L.) were kept at 20°C under normal conditions (10 h dark, 14 h light, oxygen values >80% air saturation) and fed with Trouvit pelleted food (Putten, The Netherlands). Four individuals with a weight of 9.6, 8.1, 9.5 and 6.4 g were selected. For standardization and reduction of the circadian rhythm, the fish were starved under dark conditions for one week in an identical calorimetric vessel which was also perfused at a flow rate of 50 ml min^{-1}. The sensitivity coefficient was derived as described under the blank experiment. The experiment lasted 8 days. The sampling rate was once a minute resulting in 11 520 data points each for oxygen tension and heat production. Calibration of the oxygen electrode was performed in a 10% sodium sulfite solution (0% air saturation) and air-saturated water (100%). The position of the valve was 70 min in the measurement position followed by 10 min in the reference position. After 84 h normoxia, hypoxia was introduced by equilibrating the water with a gas mixture of nitrogen and air produced by a gas-mixing pump (Wösthoff, Germany, 2M, 301/a-F). Anoxic conditions were created by saturating the inflowing water with pure nitrogen gas. The animals were exposed to three levels of hypoxia (42.5%, 27.1% and 16.7% air saturation), each for a period of 9 h, followed by a 9 h period of anoxia.

3. Results

A graphical presentation of a control experiment (no fish in measurement vessel) is given in Fig. 3. The experiment is initiated and finished with a calibration procedure. The baseline instability of the heat flow rate signal is less than 0.005 mW per 24 h. The top line corresponds to the oxygen tension signal, alternating measuring the measurement and reference vessel. No differences were observed in oxygen tension between both positions of sampling, the measurement or the reference vessel.

The experiment with goldfish is depicted in Fig. 5. The oxygen signal shows the switching of the oxygen tension value between the reference value (100% air saturation) and the measurement value (lower value). The difference between these two values multiplied by the flow corresponds to the oxygen consumption. The initial and final baseline positions show almost no baseline drift (Fig. 5).

The mean heat production ($\dot{Q}$) during normoxia was 2080 mJ h^{-1} g^{-1}ww; the oxygen consumption ($\dot{V}(O_2)$) was 5.15 μmol h^{-1} g^{-1}ww (interval 1–46 h, Fig. 5). The oxycaloric equivalent is 407.6 kJ mol^{-1}. When the upper level of hypoxia is introduced (42.5% air saturation), a peak in heat production is observed and an increased oxygen consumption, possibly due to increased locomotory activity. The heat production ($\dot{Q}$) during this hypoxia level was 2152 mJ h^{-1} g^{-1}ww and the oxygen consumption ($\dot{V}(O_2)$) was 5.54 μmol h^{-1} g^{-1}ww (interval 48–53 h, Fig. 5). At 27.1% air saturation hypoxia level, the heat production ($\dot{Q}$) was 2001 mJ h^{-1} g^{-1}ww and the oxygen consumption ($\dot{V}(O_2)$) was 5.33 μmol h^{-1} g^{-1}ww (interval 54–60 h, Fig. 5). At the hypoxia level of 16.7% air saturation, the fish responded with a clear reduction in the heat production. The heat production ($\dot{Q}$) during this hypoxia level was 1517 mJ h^{-1} g^{-1}ww and the oxygen consumption ($\dot{V}(O_2)$) was 3.95 μmol h^{-1} g^{-1}ww (interval 61–67 h, Fig. 5). This may be due to a reduced mobility or a reduction of the metabolic rate below the level of the standard metabolic rate (SMR), the so-called "metabolic depression" [5–8,10,18,22]. Metabolic depression is observed during the anoxic period (interval 68–74 h, Fig. 6). The heat production ($\dot{Q}$) is depressed to a level of 473 mJ h^{-1} g^{-1}ww. The heat production at the 16.7% air saturation hypoxia level corresponds to 73% of the heat production during normoxia and, at the anoxic period, to 23% of the normoxic heat production.

In Fig. 6, the heat production based on indirect and direct calorimetric data is given. The oxygen consumption data in mmol h^{-1} g^{-1}ww were transformed to a theoretical heat production in mJ h^{-1} g^{-1}ww using an oxycaloric equivalent for mixed substrates of 433.6 kJ mol^{-1} O_2 [15]. The calculated heat production was compared with the measured heat production. The total area of the unshaded and hatched columns corresponds to the heat production based on indirect calorimetry. The total area of the unshaded and solid columns corresponds to the heat production based on direct calorimetry. The hatched area indicates, therefore, the storage of (chemical) energy and the solid area the liberation of chemical (anaerobic) energy.

During normoxia, the animals respond in incidental cases with an anaerobic overshoot. This process of a fluctuating metabolism can be ascribed to a heat

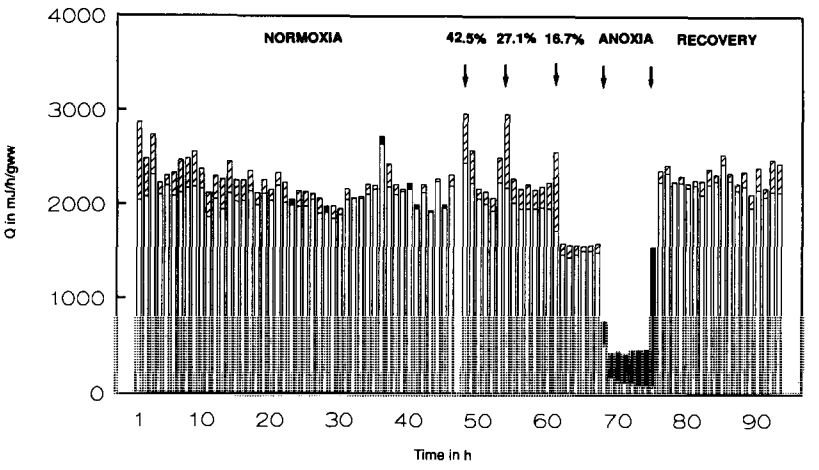

Fig. 6. Heat production (unshaded columns) based on indirect and direct calorimetric data expressed per gram incubated wet weight per interval of 1 h. The hatched columns indicate an aerobic overshoot (unshaded + hatched columns represent heat production based on indirect calorimetry). The solid columns indicate an anaerobic overshoot (unshaded + solid columns represent heat production based on direct calorimetry).

production which is temporarily higher than the oxygen consumption. As shown in Fig. 6, no anaerobic response was observed for goldfish during the hypoxia levels. Probably the lowering of the metabolic rate during the exposure to 16.7% hypoxia was sufficient for the animals to remain aerobic. During the anoxic period the heat production must originate, of course, from anaerobic metabolism, as indicated by the black bars.

During the first hour of recovery, there is a large anaerobic overshoot. The heat production ($\dot{Q}$) during recovery was 2163 mJ h^{-1} g^{-1}ww and the oxygen consumption ($\dot{V}(O_2)$) was 5.09 μmol h^{-1} g^{-1}ww (interval 76–93 h, Fig. 5). No oxygen debt was observed.

4. Discussion

We preferred the system of one oxygen electrode over measuring the twin system with two separate electrodes, because this would introduce the problem of electrode drift in two separate systems. In practice it is possible to use two electrodes by alternately switching (from measurement to reference and vice versa) to correct for electrode drift in both systems [23]. However our system with the alternating valve appears to be a good alternative for measuring oxygen tension in a twin system. Electrode drift is a factor which can introduce severe errors in oxygen consumption data. During the initial period when measuring with a new membrane this drift can be 15% over a period of 170 h. In our system with the alternating oxygen valve, we have eliminated this source of error by frequent calibration (each hour) of the reference vessel.

When animals are absent there is no difference in oxygen consumption between reference and measurement vessel (Fig. 3). This implies that we can calculate the oxygen consumption via the Fick principle [20], see Section 2.

The oxygen concentration of the air-saturated water detected via a Winkler titration was lower, 8.94 ± 0.063 mg l^{-1}, than the literature value of 9.2 mg l^{-1} at 20°C [24]. Regarding the endogenous oxygen consumption of the system, the oxygen concentration at the outlet of the calorimeter is 8.84 ± 0.062 mg l^{-1}, which corresponds to a blank oxygen consumption of 0.1 mg l^{-1} which corresponds at a flow of 50 ml min^{-1} to 0.3 mg h^{-1}.

As far as we know, this is the first study where, using the technique of direct and indirect calorimetry, a partial anaerobic response is observed in fish during normoxia. This phenomenon has been demonstrated in the marine worm (*Sipunculus nudus* L.) [10] and in bivalves (*Modiolus demissus*) [11]. The anaerobic overshoot may be explained because the heat production is larger than the oxygen consumption so the animal is partly anaerobic. These fluctuations in net metabolism can be ascribed to a temporarily higher energy demand than is produced by aerobic metabolism which may be due to increased activity of the animals in the vessel. For exercising fish, anaerobic glycolysis is the main anaerobic metabolic route [25]. Only during the hypoxia level of 16.7% and anoxia is a reduction of the metabolic rate observed, to a level of 73% and 23% respectively of the normoxic rate. Reduction of the metabolic rate may be caused by two processes: a reduction of the mobility to a level of the standard metabolic rate (SMR); or a reduction of the cellular energy metabolism below the level of SMR, the metabolic depression [23]. A problem is the distinction between these two processes. Studies were performed to detect the SMR via a video tracking system; the SMR should then be found at zero activity.

A comparison between the direct and indirect calorimetric methods is given in Table 2. For both methods, the values for the lowest and highest rates match each other very closely. The observed higher heat production during the highest rate level can be ascribed to anaerobic processes. For the mean value over the 61 h period, there is a difference of +6.7% which follows from the comparison of indirect with direct calorimetry. Lowe [4] observed a value of −3.34% for both methods.

Comparing the indirect (respirometric) with the direct calorimetric data (Fig. 6), there are some remarkable features. Eliminating the first hour of the 61 h normoxic

Table 2
Oxycaloric equivalents and comparison between indirect and direct calorimetry (mean ± SD)

	Oxycaloric equivalent in kJ mol^{-1}	Direct heat in mJ h^{-1} $(gww)^{-1}$	Indirect heat in mJ h^{-1} $(gww)^{-1}$	Comparison of indirect and direct in %
Lowest rate	363.7	1850.0	1850.5	+0.03
Highest rate	448.6	2646.5	2562.2	−3.2
Mean over 61 h	407.6 ± 23.6	2081 ± 135.6	2221 ± 191.8	+6.7

period (because of stabilization of the system), a regression coefficient of 0.73 was calculated between the measured heat flow rate and the oxygen consumption rate ($\dot{Q} \times \dot{V}(O_2)$). Differences can be ascribed to: heat dissipation due to anaerobic processes; mixing effects affecting the oxygen tension signal as a result of locomotory activity; and small differences in the time constants of the two signals, namely oxygen tension and heat production. Studies were performed to elucidate this using a mathematical approach to the lag time characteristics of the calorimetric system [21,26].

The main aim of the linkage of both datasets, heat production and oxygen consumption, was to calculate the oxycaloric equivalent at any given moment. This value gives information about substrate usage. In this study, comparing the observed value of 407.6 ± 23.6 kJ $(\text{mol } O_2)^{-1}$ with literature data (Table 1), it can be remarked that this value is rather low. Some factors may affect the oxygen consumption, such as changes in maximal saturation degree, endogenous oxygen consumption of the system and electrode drift. There has probably been an overestimation of the oxygen consumption. In another study with the same set-up [21], an oxycaloric equivalent of 422.1 ± 50.33 kJ $(\text{mol } O_2)^{-1}$ was observed, which does correspond to the values of Table 1. It is however very difficult to obtain data so accurate that conclusions can be made about the oxycaloric equivalent and substrate usage. Gnaiger et al. [27] 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^{-1}, which is in the range of the differences between substrates (Table 1). Calculating the oxycaloric equivalents per hour, we observed large fluctuations, with a minimal value of 363.7 kJ mol^{-1} and a maximal value of 448.6 kJ mol^{-1} (Table 2). Brafield [15] also observed considerable fluctuations in the hourly means of the oxycaloric equivalent during a 49 h experiment. He ascribed this to the fluctuations in activity of the fish. Fluctuations in the metabolism of aquatic animals due to partial anaerobioses probably affect the oxycaloric equivalent to a large extent. In human calorimetry/respirometry, there is in general a good agreement between direct and indirect calorimetry [28]. In six 24 h measurements of energy balance there was an agreement of $\pm 3\%$ which is in the range of experimental error. However, in seven other 24 h experiments, there was a disagreement of 8%–23% [28]. The reason for this disagreement is so far unknown, and further studies are required to elucidate this problem.

5. Conclusions

We have developed a computerized data registration system to perform continuous calo-respirometric studies in aquatic animals. Using an automatically alternating valve, the oxygen signal is calibrated hourly resulting in accurate and reliable oxygen consumption data. In combination with the direct calorimetry system, this is a powerful tool for studying the metabolic rate of large aquatic animals in long term experiments under a range of environmental conditions.

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