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SIMULTANEOUS DIRECT AND INDIRECT CALORIMETRY ON NORMOXIC AND ANOXIC GOLDFISH

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Summary

Simultaneous direct and indirect calorimetry together with biochemical determinations of metabolite concentrations were used to compare the normoxic and anoxic energy metabolism of goldfish at 20°C. The normoxic and anoxic heat production levels determined by direct calorimetry were in agreement with previous results: 700 and 200 $J\,h^{-1}\,MW^{-1}$, respectively (where MW is metabolic weight, $kg^{0.85}$). Metabolite determinations during normoxia and after 3 and 8 h of anoxia showed that during anoxia a thermodynamic steady state is reached. By simultaneous calorimetry the amounts of oxidized substrates during normoxia and anoxia and the amount of excreted ethanol, the end product of incomplete anaerobic oxidation, as well as normoxic and anoxic carbon dioxide production were determined. During normoxia and anoxia the same substrates for oxidation are used (carbohydrate and protein) by small starving goldfish, but the end products are different. During normoxia oxidation is complete (to CO_2 and H_2O; protein oxidation also has ammonia as an end product, but this is considered physiologically as complete oxidation), whereas during anoxia oxidation is incomplete, with ethanol, which is excreted, and CO_2 as end products. From the indirect calorimetric calculations it appeared that anoxic goldfish also produce fat. Glycogen storage appears to be crucial in the anoxia survival strategy.

Introduction

Goldfish (*Carassius auratus* L.) are very tolerant of anaerobic conditions. They can survive up to 16 h of anoxia (Van den Thillart, 1977) by means of modified anaerobic metabolism. This anaerobic metabolic pathway results in the production of ethanol and carbon dioxide (Van den Thillart & Kesbeke, 1978; Shoubridge & Hochachka, 1980; Mourik *et al.* 1982; Van den Thillart *et al.* 1983; Van den Thillart & Van Waarde, 1985). The normoxic and anoxic heat production and the normoxic oxygen consumption at 20°C have recently been reported

Key words: direct calorimetry, indirect calorimetry, heat production, oxygen consumption, aerobic metabolism, anaerobic metabolism, goldfish.

(Van Waversveld *et al.* 1987) to be $700\,J\,h^{-1}\,MW^{-1}$, $200\,J\,h^{-1}\,MW^{-1}$ and $1{\cdot}6\,mmol\,O_2\,h^{-1}\,MW^{-1}$, respectively (where MW is metabolic weight, $kg^{0{\cdot}85}$).

According to Van den Thillart (1982) ethanol is produced by the combined actions of lactate dehydrogenase, pyruvate dehydrogenase and a special alcohol dehydrogenase (Mourik *et al.* 1982), which converts lactic acid into ethanol and CO_2. Therefore, ethanol and CO_2 are expected to be produced in a 1:1 ratio. Van den Thillart *et al.* (1983), however, found an anoxic CO_2/ethanol ratio greater than one. This phenomenon can only be explained by the activity of the tricarboxylic acid (TCA) cycle during anoxia. Van den Thillart & Van Waarde (1985) provided further evidence for this anaerobic TCA cycle activity. Since the TCA cycle is normally coupled to oxidative phosphorylation, anaerobic TCA cycle activity is only possible if a mitochondrial sink is available for reducing equivalents. Van den Thillart & Van Waarde (1985) proposed that lipid metabolism acts as a sink for the reducing equivalents in two ways: by chain elongation and by saturation of double bonds. They based this suggestion on the increased fat content after anoxia in crucian carp (Blazka, 1958).

The aim of this study is to investigate normoxic and anoxic energy metabolism in goldfish. Simultaneous direct and indirect calorimetry and biochemical determinations of metabolite concentrations are used to calculate the amounts of oxidized substrates and end products.

Materials and methods

Experiments were performed on 24 small healthy goldfish (weighing about 10 g), acclimated for several months to 20°C, a 16 h light period and high oxygen levels. The fish were divided randomly into six groups of four fish. To prevent rapid growth, the fish were kept on a low feeding regime (Trouvit pellets, Trouw, the Netherlands). Before each experiment they were placed in a vessel identical to the calorimeter vessel for about 4 days, to adapt them to fasting and the continuously dark experimental conditions. Before and after the experiment fish were weighed individually and the mean mass was used as the experimental mass.

All metabolic rate determinations were related to the metabolic weight (MW):

$$MW = \sum_{i=1}^{n} W_i^{0{\cdot}85},$$

where W (mass) is in kilograms (Beamish & Mookherjii, 1964; Ricker, 1973).

Experimental protocol

All groups of fish were first used for simultaneous direct and indirect calorimetric measurements during normoxia and 3 h of anoxia, according to the procedure described by Van Waversveld *et al.* (1987). The fish were then used a second time for simultaneous calorimetry combined with determinations of whole fish metabolite concentrations after normoxia, and 3 and 8 h of anoxia. As the

effects of handling the fish lasted for 2 days (stabilization of the calorimeter after introduction of the fish took only 12 h), normoxia measurements were made 3 days after the introduction of the fish into the calorimeter. Anoxia was reached after 3 h of nitrogen saturation of the inflowing water (zero oxygen concentration in the water flowing out of the reference vessel, 20 ml min^{-1}). A stable anoxic heat production level was reached after 1·5 h of anoxia. The fish were then kept anoxic for 3 or 8 h. Anoxia measurements were made during this period.

Direct calorimetry

Measurement of heat production by direct calorimetry was performed with the specially designed 1-l flow-through microcalorimeter (Sétaram GF108) described by Van Waversveld *et al.* (1987). As this is a differential calorimeter it eliminates all environmental influences. Base line stability (noise <2·5 μV) was established over the total length of an experiment (10 days or more): drift <10 μV. The sensitivity of the calorimeter depends on the flow through the measurement vessel, and was established before and after each experiment, with an electrical power of 10 (±0·1 %) mW. At a flow of 20 ml min^{-1}, the sensitivity is 93 ± 0·5 μV mW^{-1}. This value is twice that of the apparatus used by Pamatmat (1983): 46 μV mW^{-1}.

Indirect calorimetry

The application of indirect calorimetry under anoxic conditions has been described by Van Waversveld *et al.* (1988). It appears that the following indirect calorimetric formulae, equations 1–3, can be used under anaerobic conditions, if the fish reach a thermodynamic steady state (see Results on metabolite concentrations and Discussion on methodology). The heat production (H) and the amounts of oxidized carbohydrate (C) and fat (F) can be calculated from the oxygen consumption (O_2), the amount of oxidized protein (P) and the carbon dioxide (CO_2) and ethanol (E) production.

$$H = 0{\cdot}3565O_2 + 0{\cdot}1160CO_2 - 1{\cdot}8631P - 1{\cdot}4083E \text{ (J)}, \quad (1)$$

$$C = -0{\cdot}0673O_2 + 0{\cdot}0947CO_2 - 1{\cdot}0865P - 0{\cdot}2645E \text{ (mg)}, \quad (2)$$

$$F = 0{\cdot}0383O_2 - 0{\cdot}0383CO_2 - 0{\cdot}0728P + 0{\cdot}8314E \text{ (mg)}, \quad (3)$$

where O_2 and CO_2 are in μmol and P and E are in mg. The amount of oxidized protein (P) can be calculated from the ammonia production, on the assumptions that protein contains 16 % nitrogen (Brafield, 1985) and that the residual carbon skeletons break down after deamination. The determinations of metabolite concentrations were used to establish whether steady state was reached during anoxia. For example, at steady state the lactate and ethanol concentrations reach a stable level, after which there is no net lactate production and all the ethanol produced is excreted. These formulae can also be used during normoxia when $E = 0$.

The oxygen consumption measurements were carried out using an oxygen electrode (Radiometer, Denmark, E5046-0) mounted in a thermostatted electrode

cell (Radiometer, Denmark, D616) (Van Waversveld *et al.* 1987). The oxygen electrode was calibrated with air (100 % O_2)- and nitrogen (0 % O_2)-saturated water. The 100 % O_2 level was determined with a Winkler oxygen titration: 100 % O_2 = 8·86 p.p.m. O_2 at 101 kPa and 20°C. The linearity of the electrode was established with a gas-mixing pump (Wösthoff, West Germany, 2M301/a-f):

$$O_2\,\%\ \text{measured} = 1{\cdot}00 \times O_2\,\%\ \text{applied} + 0{\cdot}32,\ r = 1{\cdot}0000\ (N = 12).$$

For the determination of ammonia and ethanol production, samples of the outflowing water from the measurement and reference vessels were collected in 100 ml glass vials with 0·5 ml of 0·5 mol l^{-1} phosphate buffer (pH 6) under paraffin oil. The phosphate buffer served to prevent loss of gaseous NH_3. Ammonia and ethanol concentrations were determined within a day using Boehringer-Mannheim enzymatic test combinations: 542946 (urea/ammonia) and 176290 (ethanol), respectively. These concentrations were corrected for the recovery of ammonia and ethanol using this sampling method, 90 ± 6 % in both cases. These recoveries were determined by adding a known amount of ammonium chloride and ethanol to the vial before sampling ($N = 4$). The concentration in the outflowing water from the reference vessel was taken as the concentration in the inflowing water to the measurement vessel. Ammonia and ethanol production were calculated as the difference between the concentrations in the inflowing and outflowing water of the measurement vessel, multiplied by the flow through the measurement vessel.

No determinations of carbon dioxide production were carried out. Carbon dioxide production was calculated by introducing real heat production, measured by direct calorimetry, into the indirect calorimetric heat production formula (equation 1) after the validity of these formulae had been established (see Results on metabolite concentrations). The amounts of oxidized carbohydrate and fat can then be calculated using the respective formulae (equations 2 and 3).

Determinations of metabolite concentrations

For the determination of metabolite concentrations in whole fish (by a modification of the method used by Van den Thillart *et al.* 1980) the calorimetric experiments were terminated at the end of normoxia, and 3 or 8 h of anoxia by anaesthetizing the fish with MS 222 (Sigma). After removal of the measurement vessel from the calorimeter, 2 l of MS 222 solution (200 mg l^{-1}) was passed through the vessel. After approximately 15 min the group of four fish was taken out, and the fish were ground in liquid nitrogen and mixed with one volume of 6 % (v/v) perchloric acid (0°C). Four samples of approximately 4 g from this mixture were homogenized in a high-speed mixer with three volumes of 6 % (v/v) perchloric acid (0°C). After approximately 15 min on ice, the perchloric acid extracts were centrifuged for 30 min at 30 000 *g*. 2 ml of the perchloric acid extract was taken for glycogen determinations. The remainder was neutralized to pH 6 with 1 mol l^{-1} K_2CO_3 in 0·5 mol l^{-1} triethanolamine. After 15 min on ice, the neutralized extracts were recentrifuged for 30 min at 30 000 *g*. The neutralized perchlorate extracts were kept at −20°C until metabolite determinations were made (during the

Table 1. *Some metabolite concentrations in goldfish at 20°C during normoxia and anoxia*

	Normoxia (μmol g^{-1})	3 h of anoxia (μmol g^{-1})	8 h of anoxia (μmol g^{-1})
Creatine phosphate	4·5 ± 0·5	1·5 ± 0·2	1·0 ± 0·0
Glycogen*	40 ± 5	25 ± 16	19 ± 14
Glucose	0·23 ± 0·23	3·2 ± 0·4	4·4 ± 0·3
Lactate	0·83 ± 1·03	4·8 ± 0·6	6·3 ± 0·9
Ethanol	0·33 ± 0·13	5·2 ± 0·7	6·5 ± 0·8

Values are means ± standard deviations of two determinations on four fish each.
*Measured in glycosyl units.
$\overline{W}$ (mass) = 11·1 ± 3·2 g.

following 2 days). For glycogen determinations, the 2 ml perchloric acid extracts were kept at room temperature after addition of 10 ml of 96 % ethanol, as described by Carrol *et al.* (1956). After 2 days the perchloric acid/ethanol extracts were centrifuged for 30 min at 30 000 ***g***. The pellet was dissolved in 2 ml of water and glycogen determinations were performed immediately.

Creatine phosphate concentrations were determined as described by Bergmeyer (1971). Determinations of ethanol, lactate, glucose and glycogen concentrations were performed with the following Boehringer-Mannheim enzymatic test combinations: 176290 (ethanol), 139084 (L-lactate), 139106 (glucose/fructose) and 207748 (starch). These concentrations were corrected for the respective recoveries of the extraction method: creatine phosphate, 96 ± 5 %; ethanol, 85 ± 6 %; lactate, 84 ± 3 %; glucose, 98 ± 3 % and glycogen 76 ± 1 %. The recoveries were determined by adding a mixture of the metabolites to the 4 g samples before homogenization ($N = 4$).

Results

Metabolite concentrations

The results of the determinations of metabolite concentrations are summarized in Table 1. The values at normoxia and 8 h of anoxia are of the same order of magnitude as those found by Johnston & Bernard (1983) for crucian carp at normoxia and 6 h of anoxia at 15°C. It appears that the major changes in the concentrations of creatine phosphate, lactate and ethanol take place during the hypoxic period and the first hours of anoxia. After this there are only minor changes. Glycogen concentration decreases during anoxia, whereas glucose concentration increases (mainly during the hypoxic period and the first hours of anoxia). These results will be compared with the calculated levels of carbohydrate oxidation.

The results for lactate and creatine phosphate are in agreement with those of Van den Thillart *et al.* (1976): a stabilization of the concentration after the first

Table 2. *Simultaneous direct and indirect calorimetry on goldfish at 20°C during normoxia and anoxia*

	Normoxia ($N = 12$)	3 h of anoxia ($N = 8$)	8 h of anoxia ($N = 2$)
Heat production ($J\,h^{-1}\,MW^{-1}$)	709 ± 120	203 ± 30	206 ± 30
Oxygen consumption ($mmol\,h^{-1}\,MW^{-1}$)	1·51 ± 0·22	0	0
Ammonia production ($mmol\,h^{-1}\,MW^{-1}$)	0·12 ± 0·07	0·15 ± 0·12	0·09 ± 0·01
Ethanol production ($mmol\,h^{-1}\,MW^{-1}$)	0	1·13 ± 0·24	1·53 ± 0·28
Carbon dioxide production ($mmol\,h^{-1}\,MW^{-1}$)	1·64 ± 0·44	2·62 ± 0·38	2·76 ± 0·43
Protein oxidation ($mg\,h^{-1}\,MW^{-1}$)	10·5 ± 6·9	12·8 ± 10·7	8·0 ± 1·3
Carbohydrate oxidation ($mg\,h^{-1}\,MW^{-1}$)	43 ± 29	221 ± 32	234 ± 36
Fat oxidation ($mg\,h^{-1}\,MW^{-1}$)	−5·9 ± 12	−58 ± 13	−48 ± 6
Oxycalorific value ($kJ\,l^{-1}\,O_2$)	20·9 ± 0·9		
Respiratory quotient (CO_2/O_2)	1·08 ± 0·19		
CO_2/ethanol		2·4 ± 0·4	1·8 ± 0

Values are means ± standard deviations of N experiments with four fish each.

$\overline{W}$ (mass) = 10·9 ± 3·2 g; MW (metabolic weight) = $\sum_{i=1}^{n} W_i^{0.85}$ (W in kg).

hours of anoxia. ATP can also contribute to energy metabolism if its concentration is reduced. The ATP determination in whole goldfish did not work, because ATP disappeared during the extraction (recovery = 0 %). As the method did work on muscle tissue, the ATP in the whole fish must have disappeared because of a high ATPase activity during the extraction procedure. The ATP concentrations of white and red muscle, however, were not affected by anoxia (Van den Thillart *et al.* 1980). Therefore, it seems reasonable to assume that the ATP concentration of whole goldfish was also stable during anoxia. The ammonia concentration of whole goldfish could not be determined; it also disappeared during the extraction. However, as ammonia excretion is not influenced by anoxia (Table 2 and Van den Thillart & Kesbeke, 1978; Johnston & Bernard, 1983), ammonia concentration should not be affected. This was also found by Johnston & Bernard (1983), who measured ammonia concentrations of whole crucian carp during normoxia and anoxia.

It is concluded that after the first hours of anoxia a thermodynamic steady state is reached with respect to the intermediates of energy metabolism. This implies that the indirect calorimetric formulae described above can be used (see Discussion on methodology).

Simultaneous direct and indirect calorimetry

Table 2 summarizes the calorimetric results. The normoxic and anoxic heat production levels are similar to those found previously (Van Waversveld *et al.* 1987): 700 and 200 $J\,h^{-1}MW^{-1}$, respectively. There appear to be no differences between the results at 3 and 8 h of anoxia. Mean normoxic oxygen consumption is slightly less than that found previously (Van Waversveld *et al.* 1987): 1·6 $mmol\,h^{-1}MW^{-1}$, resulting in a raised mean oxycalorific value: 21 $kJ\,l^{-1}O_2$ (instead of 20 $kJ\,l^{-1}O_2$), which indicates the use of carbohydrate as the main substrate for oxidation. The mean respiratory quotient is just above 1, also indicating the use of carbohydrate, with a small production of fat. These indications are confirmed by the calculated amounts of substrates oxidized during normoxia (Table 2) (a negative value for fat indicates production instead of oxidation).

During anoxia, ammonia production, and therefore also protein oxidation, is unchanged, whereas carbon dioxide production is raised. The ammonia production levels are in agreement with those measured by Van den Thillart & Kesbeke (1978), who also found that oxygen availability had no influence on ammonia production (0·1 $mmol\,h^{-1}MW^{-1}$). Johnston & Bernard (1983) report similar findings for crucian carp. The increase in carbon dioxide production during anoxia agrees with the results of Van den Thillart & Kesbeke (1978) and Van den Thillart *et al.* (1983) (1·4 and 2·1 $mmol\,h^{-1}MW^{-1}$ during normoxia and anoxia, respectively) although the present results are approximately 20% higher. The anoxic ethanol production rates are similar to those found by Van den Thillart *et al.* (1983), 1·2 $mmol\,h^{-1}MW^{-1}$, with a tendency to increase with time. The CO_2/ethanol ratio calculated by them is also in agreement with the present results. However, with longer anoxic periods (8–14 h) they found that the ratio decreased to approximately 1. Johnston & Bernard (1983) and Holopainen *et al.* (1986) found the same ethanol excretion rates for crucian carp at 15 and 18°C. For comparison, the results of Van den Thillart & Kesbeke (1978) and Van den Thillart *et al.* (1983) are given in Table 3.

Anoxia does not influence the level of protein oxidation (see above). Carbohydrate oxidation and fat production (a negative value indicates production instead of oxidation) are raised during anoxia.

Discussion

Methodology

The validity of the applied indirect calorimetric formulae was established by the determination, in whole fish, of concentrations of metabolites involved in energy metabolism. These show that the major change in concentrations occurs during the hypoxic period and the first hours of anoxia. After this there is only a minor accumulation of lactate and ethanol and breakdown of creatine phosphate (Table 1; ATP and ammonia concentrations are considered to be unaffected by anoxia). It is concluded that goldfish reach a thermodynamic steady state with

Table 3. *Indirect calorimetry on data from the literature on goldfish at 20°C during normoxia and anoxia*

	Normoxia	Anoxia
Oxygen consumption (mmol h^{-1} MW^{-1})	1·8	0
Carbon dioxide production (mmol h^{-1} MW^{-1})	1·4	2·1
Ammonia production (mmol h^{-1} MW^{-1})	0·1	0·1
Ethanol production (mmol h^{-1} MW^{-1})	0	1·2
Heat production (J h^{-1} MW^{-1})	787	149
Protein oxidation (mg h^{-1} MW^{-1})	9	9
Carbohydrate oxidation (mg h^{-1} MW^{-1})	2	175
Fat oxidation (mg h^{-1} MW^{-1})	15	−35
Oxycalorific value (kJ l^{-1} O_2)	19·6	
Respiratory quotient (CO_2/O_2)	0·78	
CO_2/ethanol		1·8

$\overline{W}$ (mass) = 100 g; MW (metabolic weight) = $\sum_{i=1}^{n} W_i^{0.85}$ (W in kg).

Data are taken from Van den Thillart (1977), Van den Thillart & Kesbeke (1978) and Van den Thillart *et al.* (1983).

respect to the intermediates of energy metabolism, which justifies the use of the presented indirect calorimetric formulae. However, according to Van Waversveld *et al.* (1988), the formulae can also be adjusted for lactate as end product and for the breakdown of creatine phosphate. These extended formulae, used with the results of Table 1 on lactate, ethanol and creatine phosphate, give a slightly lower (1·5 %) value for carbon dioxide production and a slightly higher (3 %) value for carbohydrate breakdown, but the calculated fat production is lower (13 %). The pattern of substrate utilization, however, remains the same. Holopainen *et al.* (1986) found that crucian carp excrete not only ethanol but also acetic acid. This may also be excreted by goldfish, a closely related species. As the rates of acetic acid excretion are reported to be only 10 % or less of that for ethanol, however, it is to be expected that this will have little or no influence on the pattern of substrate utilization described here.

Patterns of substrate utilization

The results shown in Table 2 can be translated to patterns of substrate utilization (Fig. 1). It appears that during both normoxia and anoxia the same substrates are used, but the end products are different. The most striking aspect of anaerobic metabolism is fat production (see Introduction). Of the two possibilities for lipid metabolism acting as a sink for reducing equivalents (chain elongation and saturation of double bonds), only the first can explain the fat production. This can be achieved by a reversal of the normal process of β-oxidation, making it possible for the tricarboxylic acid to be active during anoxia. In view of this, the fa produced should be seen as a byproduct.

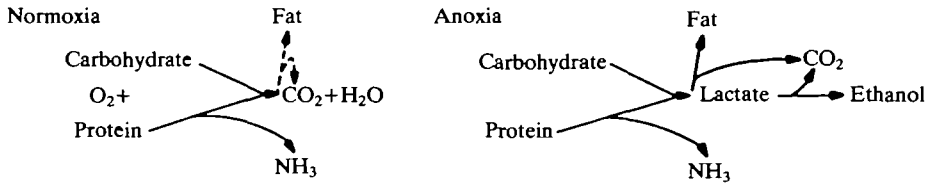

Fig. 1. Patterns of normoxic and anoxic substrate utilization by starving small goldfish at 20°C (derived from Table 2).

Using the results of Van den Thillart (1977), Van den Thillart & Kesbeke (1978) and Van den Thillart *et al.* (1983), normoxic and anoxic heat production and substrate utilization can be calculated. The results of these calculations are summarized in Table 3. The pattern of anoxic substrate utilization shown in Table 3 is very similar to that shown in Fig. 1. However, the anoxic heat production level shown in Table 3 is approximately 25 % lower than that of the directly measured heat production (Table 2). This is due to the 20 % lower value for carbon dioxide production. Van den Thillart & Kesbeke (1978) reported one carbon dioxide production level similar to that of the present results. This value was estimated after approximately 8 h of anoxia.

The normoxic situation shown in Table 3 differs from the present results (Table 2 and Fig. 1) with respect to the amounts of oxidized substrates: mainly protein and fat instead of protein and carbohydrate. This difference in normoxic substrate utilization could be caused by a difference in the condition of the fish. The fish (100 g) used by Van den Thillart (Table 3) were fed optimally and could store fat. The small fish (10 g) used in the present study were kept on a low feeding regime, a situation which probably prevented the storage of fat. They therefore had no stored fat which could be used during starvation and they had to use their stored carbohydrate.

The glycogen pool after approximately 1 week of starvation is $40\,\mu mol\,g^{-1}$ (Table 1). With a normoxic carbohydrate oxidation rate of $43\,mg\,h^{-1}\,MW^{-1}$ (Table 2), this glycogen store is sufficient for only another 3 days, assuming that glycogen stores are completely depleted. This is not the case according to Van den Thillart *et al.* (1980). Davies (1966), however, found that goldfish at 20°C could be starved for several weeks at least. The fish used by Davies (1966) were fed optimally and probably therefore had fat stores allowing them to survive extended starvation periods. To survive long starvation periods, goldfish without fat stores have to switch to protein oxidation after their carbohydrate stores are reduced to minimal levels. It would be interesting to study whether goldfish can cope with anaerobic situations after their glycogen store is exhausted and, if so, how they solve this problem energetically.

During anoxia the glycogen pool ($40\,\mu mol\,g^{-1}$, Table 1) is sufficient for approximately 15 h, if metabolized at $225\,mg\,h^{-1}\,MW^{-1}$, Table 2. The anaerobic limit is probably less than this because Van den Thillart *et al.* (1980) found, after an almost lethal anoxic period, only a 50–70 % reduction of the muscle and liver

glycogen stores. This is in agreement with the median lethal time of 16 h found by Van den Thillart (1977). The fish used by Van den Thillart (1977) were fed optimally and starved for only 3 days. They might, therefore, have had higher glycogen stores. The reduction of the glycogen level by 50 % after 8 h of anoxia (Table 1) is in agreement with the 15 h survival time calculated above. The glycogen level after 3 h of anoxia (25 μmol g^{-1}) appears to be too low with respect to the 15 h survival time. However, the raised glucose level (3 μmol g^{-1}) should also be taken into account. This brings the carbohydrate level after 3 h of anoxia into agreement with the carbohydrate oxidation rate.

These considerations show that the glycogen store in goldfish plays a crucial role in the anoxia survival strategy. The importance of carbohydrate storage in wintering survival strategy of crucian carp was reported by Hyvärinen *et al.* (1985). They stated that a large glycogen store is necessary for life in water with prolonged anoxic periods (up to 6 months a year). Furthermore, anoxic ethanol production is an elegant solution to the problems of surviving in extreme anaerobic situations, as ethanol is excreted and cannot do the fish any harm. However, because of this excretion, the solution is energetically very expensive, as only a fraction of the chemical energy of the substrates is used.

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