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Calorimetric and biochemical studies on the effects of environmental hypoxia and chemicals on freshwater fish[1]

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Abstract

The aim of this study was to assess the acute, sublethal effects of chemicals on fish from freshwater habitats. To this end goldfish (*Carassius auratus*) were exposed for up to 48 h to 2,4-dinitrophenol at concentrations well below the LC_{50} (96 h). In some experiments, fish were exposed to 2,4-dinitrophenol plus hypoxia/anoxia as an additional metabolic stress in order to enhance the effects of the chemical. Microcalorimetry proved very sensitive for detecting metabolic changes induced by 2,4-dinitrophenol. At 6 mg · l^{-1} (corresponding to ¼ LC_{50}) the chemical had no significant effects on behaviour, motor activity and ventilation frequency, whereas the heat flow rate was markedly increased. Zebrafish *Brachydanio rerio* were unable to tolerate severe hypoxia, whereas goldfish proved very tolerant of hypoxia/anoxia. In goldfish, hypoxia induced a marked decrease in heat production, to less than 30% of the normoxic rate. Postanoxic recovery after 3–7 h of anoxia was rapid and complete; it was characterised by a transient period of excess heat. 2,4-Dinitrophenol affected the rate of aerobic heat production. It had no significant effect during hypoxia/anoxia but it markedly increased the heat flow rate during postanoxic recovery. As an adaptation to anoxia, goldfish produce and excrete ethanol during anaerobiosis. This process was not affected by 2,4-dinitrophenol. Microcalorimetry appears to be a sensitive method to detect metabolic effects of environmental chemicals.

Keywords: Hypoxia; Anoxia; *Carassius*; *Brachydanio*; Microcalorimetry; 2,4-Dinitrophenol; Ethanol

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1. Introduction

Chemicals used in industry, agriculture or in households can have adverse effects if they are released into the environment. It is hence important to assess the risks of chemicals in the environment, for instance on animals in aquatic habitats. Traditionally, and often legally required, the acute toxicity of substances in water is determined by LC_{50} tests. In these tests animals are exposed to successively increasing concentrations of a substance in order to find the concentration at which 50% of the animals will die in a given time span (usually 96 or 48 h). Fish tests require large numbers of test animals (100–120 per chemical, a total of 36 922 fish during 1986 in the Federal Republic of Germany, Bundesministerium für Forschung und Technologie, Bonn, 1988), relatively high concentrations of chemicals, and they do not take into account sublethal effects although these are common in polluted habitats. In those habitats, environmental chemicals are usually present at concentrations much below the LC_{50}. Effects of chemicals at low concentrations are difficult to demonstrate, especially if it is not known by which mechanisms the chemicals act. In any case, one would expect environmental chemicals to affect metabolic processes of organisms. Microcalorimetry is a sensitive method for detecting metabolic effects provided they are accompanied by changes in the rate of heat dissipation.

By means of microcalorimetry, the combined effects of chemicals and hypoxia on metabolic rate can be studied. The advantages of such an approach are as follows: (1) combined with hypoxia the effect of chemicals might be enhanced by the additional metabolic stress and thus easier to detect. This would increase the sensitivity of the method; (2) chemicals polluting aquatic habitats tend to promote hypoxia in these habitats, for instance by inhibition of photosynthesis of aquatic plants, or by inducing the oxygen-consuming decomposition of organic matter. Hence the combined effects of chemicals and hypoxia is not unlikely in aquatic habitats, and using this combination may help to simulate realistic chemical pollution.

We have chosen goldfish (*Carassius auratus*) as a "model fish" (see Section 4) and the uncoupler 2,4-dinitrophenol as a "model substance", to examine whether microcalorimetry can be used for testing acute effects of environmental chemicals on freshwater fish.

2. Materials and methods

2.1. Animals

Goldfish (*Carassius auratus*) of 5–7 cm body length and 6–9 g body weight were purchased from Aquaristik Glaser (Rodgau, Germany) and kept in a 200 l aquarium at about 20°C. Zebrafish (*Brachydanio rerio*) were purchased from West-Aquarium (Bad Lauterberg, Germany) and kept in a 60 l aquarium at 25°C. Goldfish were fed on Pondsticks (Tetra Pond Teichfutter, Tetra-Werke, Melle, Germany) and zebrafish were fed on Tetra Min (Tetra).

2.2. Behavioural studies

For these studies goldfish were kept in a tank holding 12 l water. The tank was part of

an apparatus made of Plexiglas [1]. The tank was connected to a water-filled cylinder (total volume 19.3 l) through which gas could be bubbled. Water was circulated through the system by means of an aquarium pump (Eheim), and the oxygen content was constantly recorded using an oxygen electrode (TriOximatic® EO 200, WTW, Weilheim, Germany) connected to an oxygen-meter (Oxi 530, WTW) and a plotter (Servotrace, Sefram, Paris, France). The fish were transferred to the apparatus on the day before the experiment in order to reduce handling stress. Hypoxia/anoxia was produced by bubbling pure nitrogen instead of air through the water, at a rate of 4 l per min thus reducing oxygen content less than 1% within 40 min (see Fig. 1).

Ventilation frequency was determined by counting a fixed number of gill movements (usually between 20 and 40) of individual fish while the corresponding timespan was recorded.

When in these experiments the effects of 2,4-dinitrophenol were to be tested, the chemical was added to the water 24 h before hypoxia was initiated. The concentration of 2,4-dinitrophenol was monitored as described in Section 2.4.

2.3. Calorimetry

A Calvet MS 80 twin calorimeter from Setaram (Lyon, France), equipped with 100 ml circulation cells, was used. It was calibrated by "Joule cells", and the sensitivity was 53 μV mW^{-1} (cf. [2]). In experiments with goldfish the calorimeter was tilted to an angle of 75°. The experimental and reference cell were each filled with 80 ml tap water.

Normoxic conditions were maintained by passing through the calorimeter a flow of "synthetic air" at a constant rate of 500 ± 5 ml h^{-1} per cell. Hypoxia/anoxia was produced by changing the gas flow from air to pure nitrogen. As a consequence the O_2 content in the water of the calorimeter cells was rapidly decreased; it reached 50% of the normoxic value within less then 10 min and 1% within about 60 min. In experiments with zebrafish a flow rate of 250 ml h^{-1} per cell was used. At this rate a flow of pure N_2 reduced the O_2 content to 50% of the normoxic value within 13 min, to 6% within about 50 min. Calorimeter signals were recorded and processed by a computer using software provided by Rosenberg Elektronik (Nieder-Olm) and Multigraf from Weka-Software (Frankfurt-Main).

2.4. Chemical/biochemical analyses

2,4-Dinitrophenol (Sigma, Deisenhofen/Germany) was dissolved in Sörensen phosphate buffer (50 mM, pH 7.2) with addition of a gram equivalent of NaOH. The concentration of 2,4-dinitrophenol was determined by means of spectrophotometry (Ultrospec III, Pharmacia, LKB) at 400 nm using a standard curve.

Ethanol produced during anaerobiosis by goldfish was measured enzymatically by recording its oxidation to acetaldehyde by the NAD$^+$-dependent alcohol dehydrogenase, using the Ultrospec III at 340 nm. In these experiments a single goldfish was kept in a small Plexiglas tank that was completely filled with 1 l water. Hypoxia/anoxia was brought about as described in Section 2.2. by bubbling pure N_2 instead of air through a water-filled cylinder (1 l volume) connected to the tank at a rate of 4 l min^{-1} for 60 min.

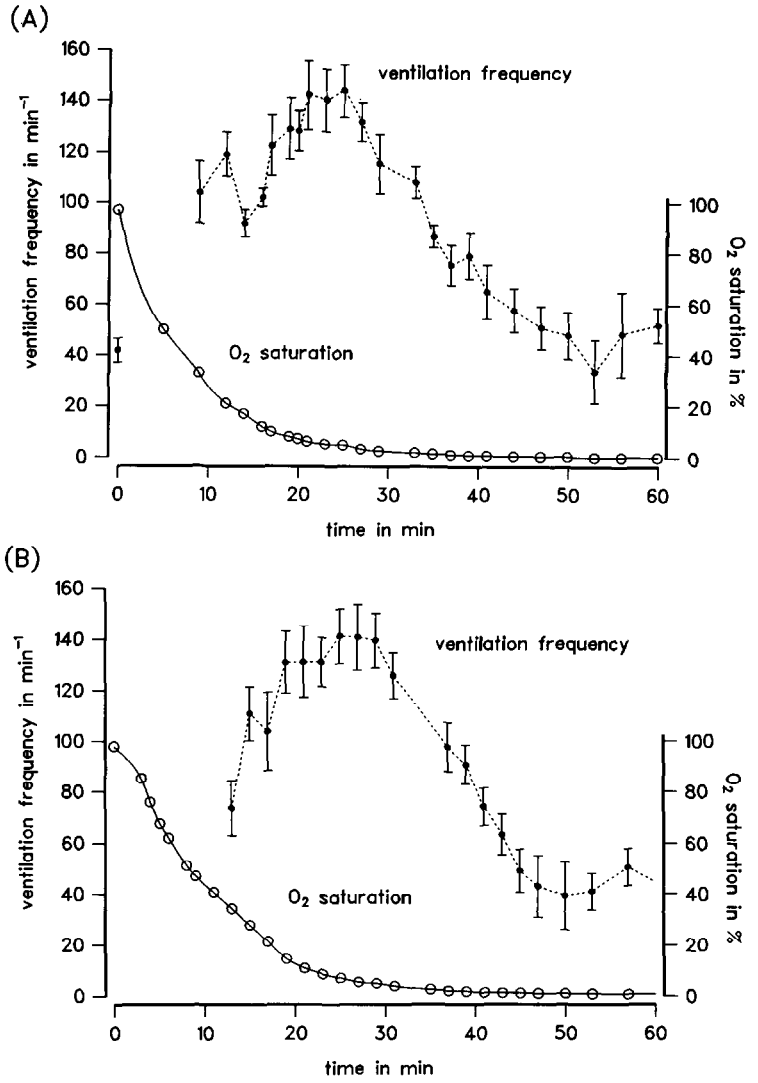

Fig. 1. Effects of hypoxia and hypoxia plus 2,4-dinitrophenol on ventilation frequency in goldfish. A group of 5 goldfish were kept in a Plexiglas tank and hypoxia/anoxia was produced as described in Section 2.2. Data are given as means ± SD ($n = 5$). (A) Effect of hypoxia/anoxia on the ventilation frequency of goldfish in the absence of 2,4-dinitrophenol. (B) Hypoxia/anoxia plus 2,4-dinitrophenol (6 mg l^{-1}). 2,4-Dinitrophenol had no significant effect on ventilation frequency during normoxia and hypoxia/anoxia.

The gas flow was then interrupted and the tank closed (airtight). Aliquots of water were drawn every hour through a small opening in the lid of the tank and analysed for ethanol as described.

3. Results

3.1. Behavioural effects of hypoxia and 2,4-dinitrophenol on Carassius and Brachydanio

Both zebrafish and goldfish have been widely used in ecotoxicological research. Zebrafish can easily be bred in the laboratory under well defined conditions. Its short life cycle and high rate of propagation make it an apt species for life cycle tests. On the other hand, zebrafish is not adapted to temperate climate; their natural habitats are small streams and ponds in India which have fairly constant temperature and O_2 concentration. *Brachydanio* is hence not an adequate "model fish" of temperate climates where fish have to cope with seasonal changes in temperature and oxygen concentration. Goldfish can tolerate marked changes in temperature as well as prolonged intervals of anoxia, especially at low temperature. This capability is crucial in the cold season when goldfish may be confined to the bottom of ice-covered ponds where O_2 is virtually absent.

Zebrafish showed very little tolerance of hypoxia. Hypoxia caused increased motor activity and ventilation of great intensity which became prominent when the oxygen content was decreased to about 20% of the normoxic content. If hypoxia became more severe, zebrafish would transiently lose control of their motor activity, indicated by intervals of impaired equilibrium and brief bursts of activity (when O_2 was at about 6% of its normoxic content). Ventilation would then become erratic and faint. If, at this stage, air was not re-admitted within a few minutes, the zebrafish would not recover. Zebrafish hence proved unable to tolerate hypoxia and is therefore not a satisfactory "model" for studying metabolic effects of hypoxia in fish.

The situation is different with goldfish. Goldfish remain calm with decreasing O_2 concentration. The most prominent response was a transient increase in the frequency and intensity of ventilation (see Fig. 1A). Relatively soon the fish would not respond to knocking at the pane of the aquarium. With more severe hypoxia goldfish would at times drift in the water for a few seconds or show brief bursts of activity. With severe hypoxia or anoxia, ventilation became unsteady and might stop for up to 2 min. Goldfish recovered rapidly and completely from 8 h of hypoxia/anoxia. We have not tested longer intervals but goldfish have been reported to survive more than 20 h of hypoxia/anoxia at 20°C [3] and several days at 4°C [4,5].

Exposure of goldfish to 2,4-dinitrophenol (6 mg l^{-1} and 12 mg l^{-1}) did not cause significant changes in behaviour (motor activity) or ventilation frequency under normoxic conditions. Dinitrophenol in these concentrations also did not significantly affect the behavioural responses of goldfish to hypoxia and anoxia (see Fig. 1A,B). The LC_{50} (96 h) of 2,4-dinitrophenol in goldfish is 23 mg l^{-1} [6].

3.2. Heat production of fish during normoxia and hypoxia/anoxia: the effect of 2,4-dinitrophenol

The rate of heat production of both goldfish and zebrafish could easily be measured by means of microcalorimetry under normoxic conditions. The resulting curves characteristically show a basal value that is constant with time (see Fig. 2A), which we interpret to represent the standard metabolic rate of the individual fish, i.e. the metabolic rate nec-

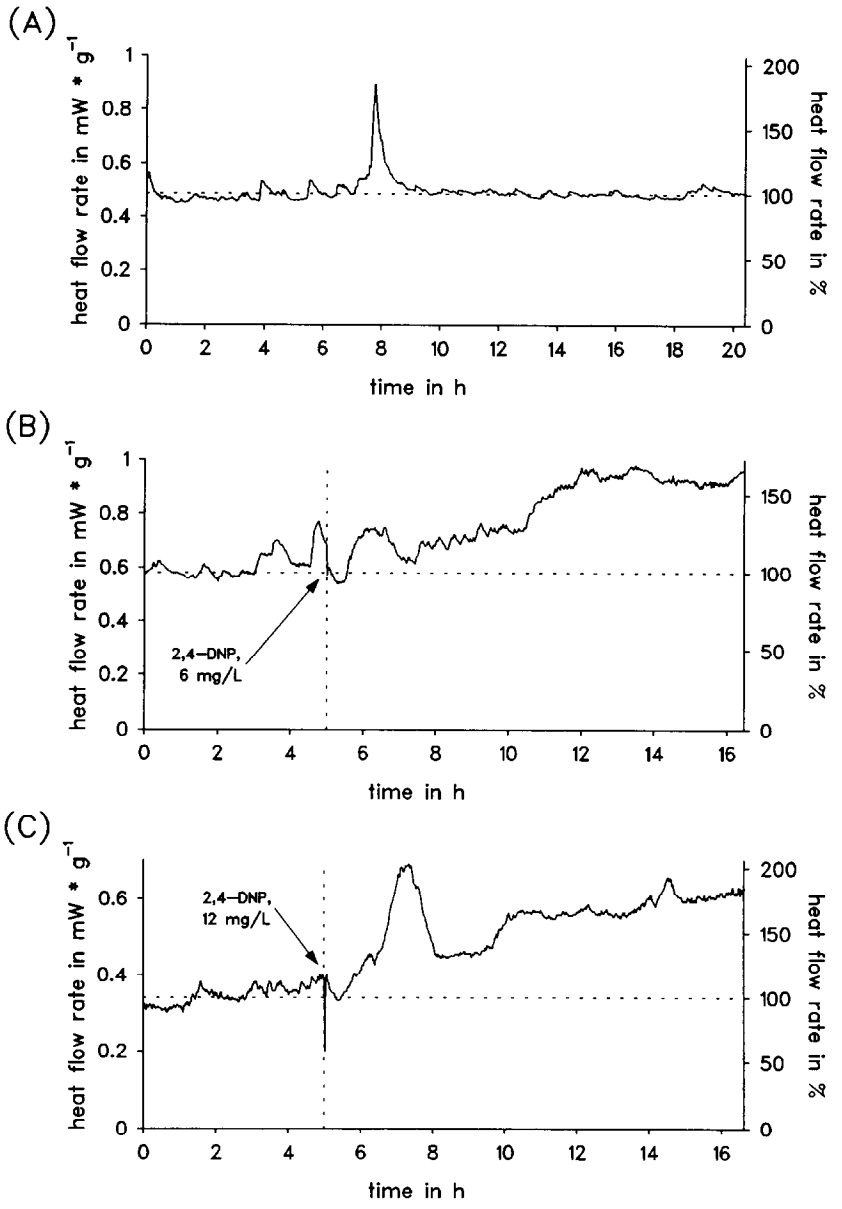

Fig. 2. Heat production of goldfish under normoxic conditions at 20°C, and the effect of 2,4-dinitrophenol. The broken line at 100% indicates the heat flow rate corresponding to the standard metabolic rate. Peaks of heat flow indicate episodes of motor activity of different duration and intensity. (A) Control: heat production of a goldfish (7.8 g body weight) in the absence of 2,4-dinitrophenol. (B), (C) Acute effect of 2,4-dinitrophenol on heat production of goldfish. The chemical was added as indicated by the arrows. (B) Dinitrophenol at 6 mg l^{-1}. (C) Dinitrophenol at 12 mg l^{-1}. At both concentrations 2,4-dinitrophenol causes the heat flow rate to increase above the standard rate.

essary to support the physiological processes of resting fish. Transient maxima (peaks) of heat flow were assigned to brief episodes of motor activity on the grounds of parallel experiments where fish were observed in purpose made transparent cells of the same size as the calorimeter cells.

Addition of 2,4-dinitrophenol to the calorimeter cells caused the heat production of goldfish to increase by 50% or more (Fig. 2B,C). The increased heat flow rate was maintained for at least 12 h; longer intervals were not studied.

Hypoxia/anoxia was produced by changing the gas flow from air to pure nitrogen. The decreasing oxygen content in the water had very different effects on heat flow rates in *Brachydanio* and *Carassius*. The effects mirrored the behavioural responses. Since we knew that *Brachydanio* would not survive prolonged severe hypoxia, we reduced the N_2 flow to 250 ml h^{-1} per cell and limited it to 50 min in order not to sacrifice the animals. During this time the oxygen content was reduced to about 6% of the normoxic value and the heat flow rate showed a tendency to increase with decreasing O_2 concentration (see Fig. 3).

In goldfish the switch from air to nitrogen provoked a transient increase in heat flow indicating a brief phase of muscle activity. After about 30 min, however, the heat flow rate fell precipitously to a value between 15% and 30% of the normoxic rate. A similar metabolic depression has been reported by van den Thillart and co-workers [7].

Switching the gas flow back to air resulted in a rapid increase in heat production to a value above the standard rate. This excess heat production was maintained for some time; it reflects the extra metabolism required for reverting to the normoxic state (paying back an "oxygen debt", for review see Ref. [8]). After 3 h of pure nitrogen the excess heat

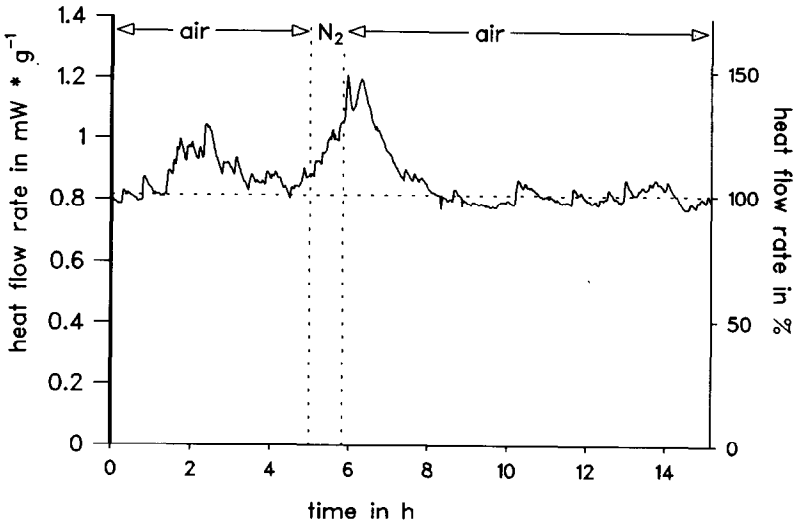

Fig. 3. Heat production of a male *Brachydanio rerio* (470 mg body weight) under normoxic and hypoxic conditions at 25°C. Hypoxia caused an increased heat flow, indicating increased ventilation and motor activity with decreasing oxygen content (for details see text).

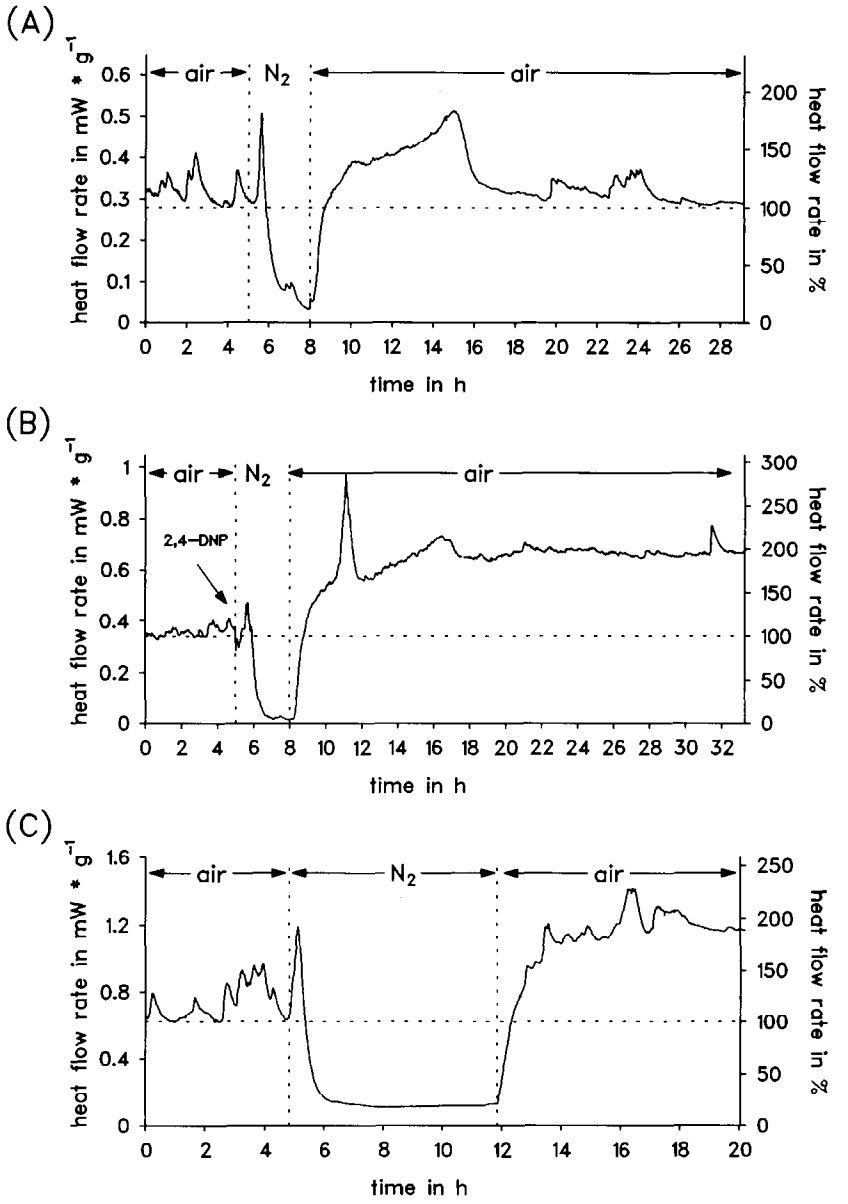

Fig. 4. Effects of hypoxia and hypoxia plus 2,4-dinitrophenol on heat production in goldfish at 20°C. (A) Hypoxia (pure nitrogen for 3 h). (B) The effects of combined exposure of goldfish to hypoxia (N_2 for 3 h) and 2,4-dinitrophenol (6 mg l^{-1}). (C) A similar experiment as in (A), but with prolonged hypoxia/anoxia (7 h of N_2). During anoxia no fluctuation in heat flow rate was observed indicating that the goldfish reduced motor activity and kept very quiet during anoxia.

flow reached a maximum of about 180% of the normoxic rate after 7 h of postanoxic recovery. During the following 5 h heat production returned to standard rate (see Fig. 4A). The amount of the "oxygen dept" and the time required for paying back depends on the duration of the anoxic interval. After 7 h of hypoxia/anoxia the elevated heat flow did not return to the standard level within 8 h of recovery (Fig. 4C).

The presence of 2,4-dinitrophenol (6 mg l^{-1}) did not change significantly the heat flow during hypoxia/anoxia (see Fig. 4A,B). The postanoxic heat flow, however, was markedly elevated above the standard rate and did not return to this rate in goldfish exposed to 2,4-dinitrophenol (see Fig. 4B).

3.3. Effect of 2,4-dinitrophenol on the rate of anaerobic ethanol excretion in goldfish

As with some other cyprinoid fish (crucian carp *Carassius carrasius* [9], bitterling *Rhodeus amarus* [10]) goldfish limit tissue acidification from anaerobic glycolysis by converting lactate to ethanol and CO_2, both of which can easily be excreted into the surrounding water. The synthesis and excretion of ethanol is a complex metabolic process; it requires the coordination of various metabolic pathways in different organs. We wanted to study whether this process was affected by exposure of goldfish to 2,4-dinitrophenol. As can be seen from Fig. 5 this was not the case at the concentration tested (6 mg 2,4-dinitrophenol per l added 24 h before initiating anoxia). Ethanol was produced during anaerobiosis at the same rate whether or not 2,4-dinitrophenol was present. After more than 5 h of anoxia there was a slight tendency to excrete more ethanol in the fish exposed to 2,4-dinitrophenol, but the difference was not statistically significant (Mann–Whitney *U*-test).

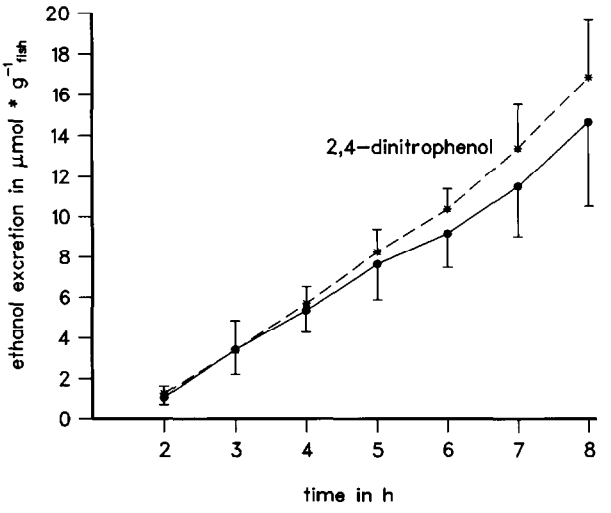

Fig. 5. Excretion of ethanol by goldfish during anoxia in the absence and presence of 2,4-dinitrophenol (6 mg l^{-1}).

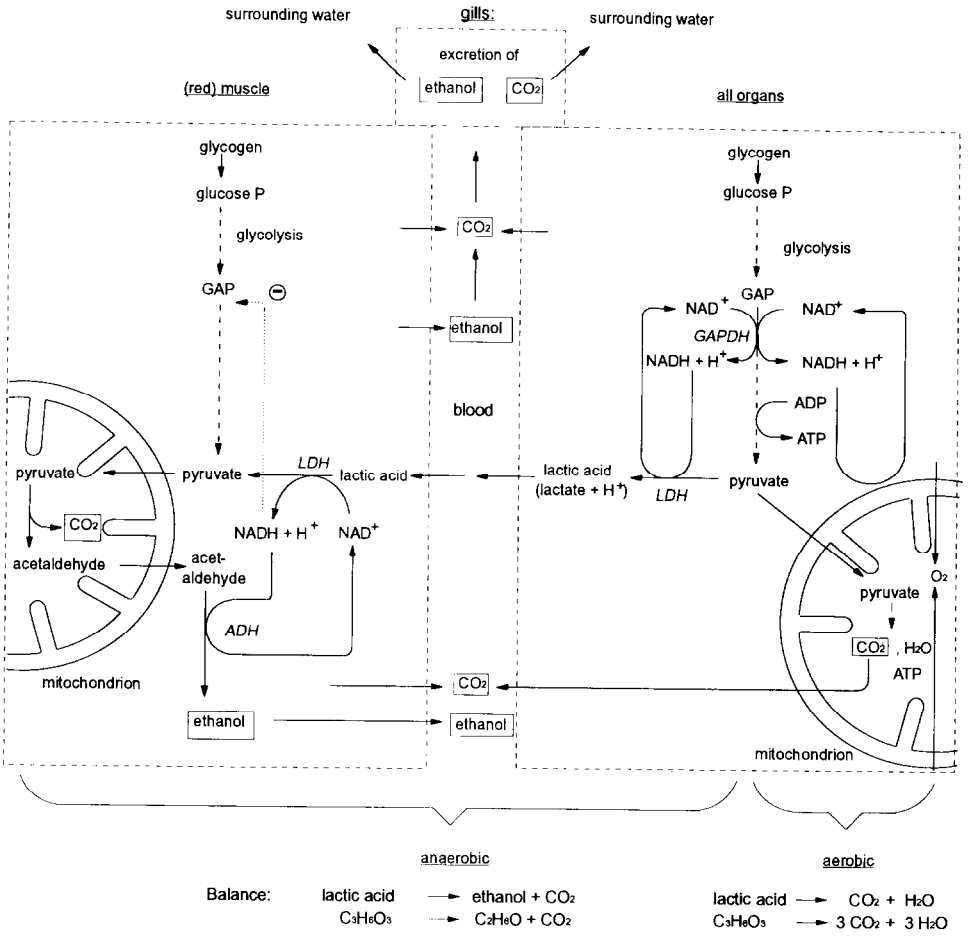

Fig. 6. Schematic representation of aerobic and anaerobic catabolism of glycogen in goldfish tissues (see [15,23,24]). Under aerobic conditions glycogen will be degraded to pyruvate and this can be fully oxidised to CO_2 and H_2O in the mitochondria as indicated on the right hand side of the scheme. Glycolytically produced NADH will be dehydrogenated to NAD^+ in the cytosol and transported via a shuttle mechanism into the mitochondria for full oxidation to H_2O. If oxygen is lacking, pyruvate and NADH cannot be oxidised this way. Instead, NAD^+ will be regenerated in a reaction catalysed by LDH. As a consequence carbohydrate (glycogen) is converted into lactic acid (lactate plus H^+). All fish organs are capable, although to different degrees, of both aerobic and anaerobic catabolism of glycogen. During prolonged anaerobiosis, however, the acidification of goldfish tissues will be limited by a special metabolic adaptation. Lactate will be taken up from the blood by muscle and reconverted to pyruvate by LDH (the resulting NADH will inhibit glycolysis in muscle). Pyruvate will be decarboxylated to acetaldehyde in the mitochondria by a catalytic activity of the pyruvate dehydrogenase enzyme complex. The acetaldehyde will be reduced to ethanol which can be excreted into the surrounding water. ADH, alcohol dehydrogenase; LDH, lactate dehydrogenase; GAP, glyceraldehyde 3-phosphate.

4. Discussion

The LC_{50} test is an established method to assess the acute toxicity of chemicals. Because these tests require large numbers of animals and relatively high concentrations of chemicals many attempts have been made to reduce or replace LC_{50} tests in ecotoxicological research.

Wide use has been made of cultured cells in order to reduce animal experimentation, but this approach has major limitations because: (1) isolated cells are not representative of the whole organism, yet it will be the whole organism that is exposed to the pollutant; (2) even if all cell types of an animal were tested (and provided their susceptibility to chemicals remained unchanged during cell culture), tests on cells would not take into account the intricate interdependence and cooperation of cells and tissues on which the physiological function of multicellular animals are based. It is, therefore, not surprising that chemicals that are very toxic in intact organisms may have little effect on cells in culture [11,11a].

We wanted to assess the effects of pollutants on intact fish without harming the animals. We have therefore used concentrations well below the LC_{50} for relatively short periods of exposure and we have chosen criteria that reflect the complex physiological integration of intact fish, such as motor activity, ventilation frequency, rate of heat production, response to hypoxia and anaerobic ethanol excretion. All these criteria are based on coordinated functions of various organs. Heat flow rate proved to be the most sensitive of these criteria, and in this light it is surprising that microcalorimetry has hardly been used in ecotoxicological research on fish [12]. We could not observe significant changes in motor activity and ventilation frequency at concentrations of 2,4-dinitrophenol that brought about a marked increase in heat production. The effect was observed during normoxia as well as during postanoxic recovery, but not during anoxia.

The latter observation indicates that 2,4-dinitrophenol interferes with aerobic metabolism but has little if any effect on anaerobic metabolism which would be in line with the presumed mechanism of 2,4-dinitrophenol as an uncoupler of oxidative phosphorylation [13,14]. This view is further supported by the fact that 2,4-dinitrophenol had no significant effect on the anaerobic excretion of ethanol which is produced only during anaerobiosis.

The formation and excretion of ethanol is an adaptation to environmental hypoxia (for reviews see Refs. [15,16]). It requires the coordinated action of various organs and pathways as outlined in Fig. 6. By measuring ethanol excretion, anaerobic metabolism of goldfish can easily be quantified without using invasive or destructive methods. Whether pollutants other than 2,4-dinitrophenol would have an effect on the rate of excretion of ethanol is an interesting question that needs further study.

Inducing anaerobiosis does obviously not harm goldfish because these animals are well adapted to anoxia which they may encounter regularly in their natural habitats. Vertebrates tolerant of anoxia remain quiet when oxygen is in short supply (see Fig. 4C); they decrease their metabolic rate and reduce the function of organs including the brain (for reviews see Refs. [8,16–19]). Breathing nitrogen instead of air would not cause pain in man either, but unlike in animals tolerant to anoxia it would bring about a rapid loss of consciousness and a total breakdown of physiological functions [20,21].

Microcalorimetry has been shown to be a sensitive method for demonstrating acute effects of chemicals on fish. It could provide a valuable tool in ecotoxicological research. The method is not limited to fish but can be used with all kind of organisms including invertebrates, plants and microorganisms. It could also be used to assess the effects of a combination of chemicals, a likely situation in polluted habitats. In this respect the low specificity of the method is an advantage rather than an disadvantage. Microcalorimetry will detect small changes in heat production (i.e. in metabolism) irrespective of the underlying mechanisms but it cannot give information as to the reactions involved. Answering this question would require additional more specific methods.

The ultimate goal of ecotoxicological research is to provide reliable assessments of possible risks from pollutants in ecosystems. It is unlikely that this goal will ever be reached because even "simple ecosystems" are extremely complex, comprising many species of organisms and their developmental stages which are exposed to fluctuating abiotic factors. Moreover, in addition to acute metabolic effects chemicals can affect genetic material, the rate of reproduction, etc. [22]. As a consequence compromises with respect to the extent and strictness of ecotoxicological testing will be unavoidable but compromises in releasing chemicals into the environment should be restricted to a minimum because they will always carry the risk of unforeseen damage to ecosystems.

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