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CONTENTS

Microcalorimetric monitoring of biological activities. Ecological and toxicological studies in aquatic animals

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ABSTRACT

In many ecological, physiological and toxicological topics of research, the microcalorimetric method for monitoring biological activities provides not only a highly automatic and practical procedure but also an indispensable technique.

Several studies of animal energetics using ampoule and open-flow microcalorimeters are discussed. They show the importance of combining respirometric and calorimetric methods to separate total metabolic rates into the aerobic and anoxic components. Under environmental and physiological hypoxia, heat-dissipation rates are frequently higher than those indicated by respirometric or biochemical methods. This can be of practical importance in toxicological and pharmacological tests.

Direct calorimetry is applicable to bioenergetic studies and toxicological tests to the same extent as are respirometric (indirect calorimetric) methods. This paper concerns problems where direct calorimetry surpasses conventional respirometry and provides an indispensable technique for physiological and environmental studies.

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The simultaneous measurement of heat dissipation and oxygen consumption makes possible a distinction between aerobic and anoxic sources of metabolic energy (Herold, 1977; Hammen 1980; Shick, 1981; Gnaiger, 1983*a*, *c*; Pamatmat, 1983). The direct calorimetric method provides, in particular, the most general approach to the estimation of total biological energy conversion in oxygen-depleted microhabitats and anoxic aquatic environments (Gnaiger, 1979, 1980*a*; Pamatmat, 1980).

In the present paper, studies on aquatic animals will be summarized, whereas previous reports should be consulted for studies on bacteria and yeast (Belaich, 1980; Lamprecht, 1980), on soils, sediments and sewage systems (Ljungholm et al., 1979; Pamatmat et al.,

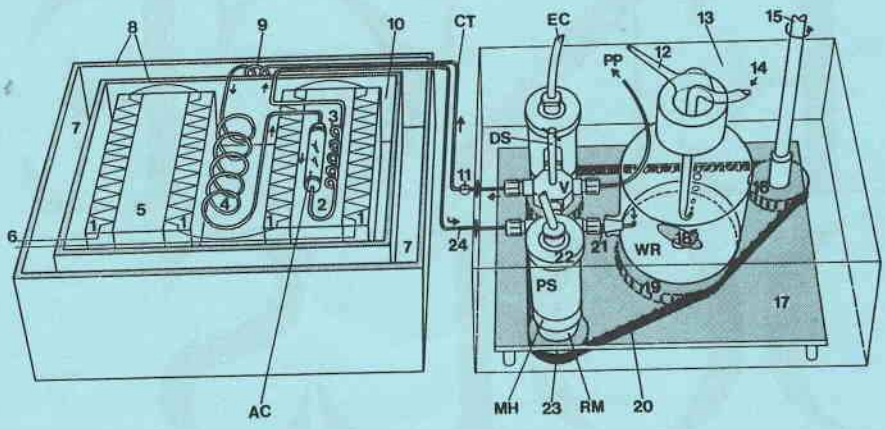


Fig. 1. The open-flow respirometer–calorimeter. LKB 2107 Flow-Sorption Microcalorimeter (left) connected to the Cyclobios Twin-Flow Microrespirometer (right) (from Gnaiger, 1983a).

1–Thermopile
2–Heat detector
3–Detector heat exchanger (gold CT)
4–Internal heat exchanger (gold CT)
5–Static reference detector
6–Thermovoltage transducer cables connecting to microvolt ammeter
7–Constant-temperature air bath of the calorimeter
8–Thermal insulation
9–External heat exchanger (gold CT)
10–Heat sink (aluminum block)
11–Three-way microvalve
12–Gas outlet
13–Constant-temperature water bath of the respirometer
14–Gas line from gas mixing pump to water reservoir
15–Drive shaft connected to synchronous motor
16–Gear wheel driving toothed rubber belt
17–Mounting plate
18–Magnetic stirring bar in water reservoir
19–Bracing wheel with impeller magnet
20–Rubber drive band
21–Stainless-steel CT
22–Retaining nut
23–Gear wheel driving stirring bar
24–Capillary connection from animal chamber to polarographic O_2 sensor
AC–Animal chamber (0.5 cm³)
CT–Capillary tube (gold) connecting respirometer to calorimeter
DS–Drive shaft operating the two four-way valves
EC–Electrode cables of polarographic O_2 sensor
MH–Magnet housing of stirring-chamber bottom assembly
PP–Outlet (Teflon tubing) to peristaltic pump
PS–Polarographic O_2 sensor sleeve
RM–Rotating magnet driving magnetic stirrer in PS
V– Four-way microvalve
WR–Water reservoir for p_{O_2} equilibration

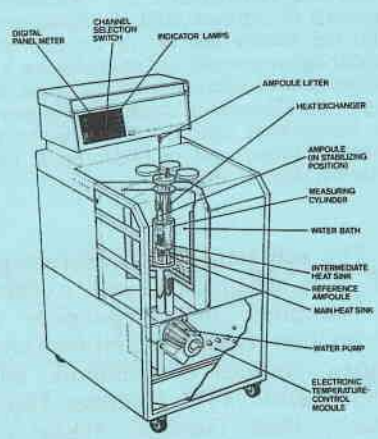
1981; Redl & Tiefenbrunner, 1981; Gustafsson & Gustafsson, 1983), and on tissues, blood and sperm cells, as well as medical applications (Spink & Wadsö, 1976; Wadsö, 1977; Andersson & Lovrien, 1979; Pätel et al., 1980; Woledge, 1980; Hammerstedt et al., 1983).

Microcalorimetric methods

The most versatile ecological applications of microcalorimetry are offered by heat-flow calorimeters with perfusion (open-flow) animal chambers (Fig. 1). The heat-conduction principle (Calvet & Prat, 1963; Wadsö, 1974) ensures practically isothermal conditions for experimental periods of unlimited length. Continuous perfusion, on the other hand, provides a means for maintaining or manipulating the organism's environment in investigations of physiological responses to ecological and toxicological variables (Gnaiger, 1979, 1981, 1983*a*; Famme et al., 1981). Depletion of oxygen may be avoided by perfusing the animal chamber. This is particularly important when studying aerobic (oxidative) processes in water. For processes in air, oxygen depletion is much less likely to occur, since air contains 20–50 times as much oxygen as does the same volume of equilibrated water (Forstner & Gnaiger, 1983). Closed, small-volume animal chambers (Fig. 2B) are therefore suited for measurements in air and for studies of anoxic metabolism.

Depending on the kind of organism under study, different methodological problems will be encountered. With increasing animal size the thermal capacity of the biomass increases, thermal diffusion paths in larger animal chambers are longer, and the ratio of heat-conducting surface to volume decreases. Consequently, the ratio of heat storage to heat flow increases with the mass of animal and chamber, which unavoidably detracts from the time resolution of the calorimetric system (cf. Fig. 3). Mathematical time-response corrections, therefore, have to be made (Suurkuusk & Wadsö, 1982). A complex dependence of the response time on the locomotory behavior of the animal may, however, be superimposed on these corrections (Gnaiger, 1983*a*). This problem has to be kept in mind when using large-volume animal chambers in heat-flow and adiabatic calorimeters.

Calorimetric measurements on aerobic and anoxic metabolism have been obtained with animal dry-weights ranging from 1.4 mg in a 0.5-cm³ chamber (Gnaiger, 1981, 1983*a*) to 60 g in a 1200-cm³ cham-



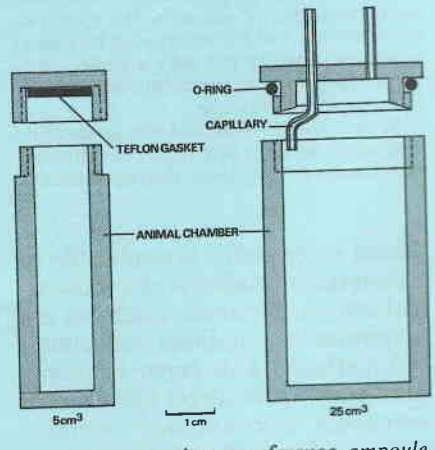


Fig. 2. The LKB 2277 BioActivity Monitor. A–The modular system (from Suurkuusk & Wadsö, 1982). B–Stainless-steel ampoules, 5 cm³ (i.d. 11 mm, inner height 53 mm) and 25 cm³ (i.d. 25 mm, inner height 53 mm). The 5-cm³ ampoule is used in a differential twin system against a reference ampoule (see Fig. 2A), whereas the larger ampoule comprises a single, nondifferential unit, which is not as free of noise as the twin system (cf. Fig. 3).

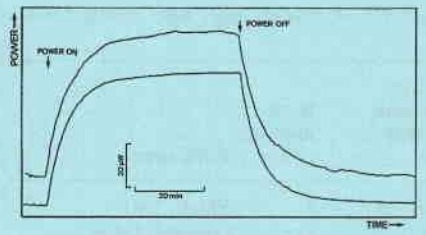


Fig. 3. Calibration run with the LKB 2277 BioActivity Monitor (prototype) with two 25-cm³ ampoules partially filled with water of 12 °C. The simultaneously recorded baselines were arbitrarily displaced relative to each other along the power axis in order to avoid overlapping of the traces. Vertical arrows indicate the switching on and off of the calibration power input of 100 µW. Data: recorder full-scale deflection: 250 mm ≃ 30 µV ≃ 301 µW; calibration steady-state deflection: 83 mm ≃ 9.96 µV; sensitivity: 0.100 µV/µW; calibration constant (scale deflection basis): 1.205 µW/mm.

The time-response curve did not follow a simple first-order exponential function; 95 % response was reached after 25 min, steady state after 50–60 min.

In comparison, the 5-cm³ ampoule was calibrated at a full-scale power range of 30 µW with a sensitivity of 0.33 µV/µW; 95 % response was reached after 6.8 min and steady state after 16–17 min.

ber (Jackson, 1968). The lower limit of animal and chamber size is set by the sensitivity and stability of the measuring system. With a stability of the order of ±0.2 to ±0.5 µW over 8 hours (LKB 2277 BioActivity Monitor Instruction Manual, LKB-Produkter AB, 1982, cf. Fig. 6), 0.1 mg would represent the minimum dry biomass required, whereas for most anoxic studies dry weights above 1 mg have to be used (cf. Table 1).

The perfusion flow-rates have to be adapted to the metabolic requirements. For every mW of heat dissipated aerobically, about 8 µmol O_2 are comsumed per hour (Gnaiger, 1983*b*). This would cause complete oxygen depletion at a flow of air-saturated water of 30 cm³/h at 20 °C. Perfusion flow-rates of 150 cm³/h (and higher with sea water) are required per mW to maintain 80 % air saturation in the animal's environment.

Ecological applications

Aerobic energy-balance studies

The ecologist's interest in heat measurements stems primarily from the widely recognized bioenergetic balance equation for heterotrophs, based on the first law of thermodynamics:

$$P = A + R + U. \quad (1)$$

Production, P, of biomass is the result of the net energy gain from assimilated food, A, and the loss of energy due to respiration, R, and excretion, U. Whereas biomass, food, and excreta may be converted to energy equivalents by bomb calorimetry, loss of energy by respiration may be measured directly by microcalorimetry as total rate of heat dissipation, d_tQ/dt [mW]. Alternatively, R may be estimated by respirometric methods (indirect calorimetry) as aerobic catabolic heat loss, d_kQ_{ox}/dt, with appropriately derived oxycaloric equivalents, $\Delta_kH_{O_2}$ [kJ per mol O_2] (for a critical discussion see Gnaiger, 1983*b*). For the application of indirect calorimetry, therefore, the catabolic heat-balance equation must be satisfied:

$$d_tQ/dt = d_kQ_{ox}/dt = dN_{O_2}/dt \times \Delta_kH_{O_2}, \quad (2)$$

where dN_{O_2}/dt is the respiratory rate [µmol O_2 per second]. Simultaneous direct and indirect calorimetry (for example in an equipment as that depicted in Fig. 1) provides the experimental test for Eqn. (2), which appears to be confirmed by recent studies on aquatic animals under balanced aerobic conditions (Pamatmat, 1978, 1983; Famme et al., 1981; Gnaiger, 1981, 1983*a*, see also Fig. 9).

Aerobic and anoxic metabolism

The above energy-balance method is restricted to aerobically respiring organisms. However, under hypoxic and anoxic conditions respirometric methods fail to determine the total catabolic rate, whence $|d_tQ/dt| > |d_kQ_{ox}/dt|$. Sessile marine animals living on the tidal shore may create a hypoxic microenvironment within their closed shells during air exposure (Bayne et al., 1976), and metabolism may be anoxic (de Zwaan, 1977) or fully aerobic even in air (Pamatmat, 1978, 1983; Shick, 1981). The common mussel *Mytilus edulis* shows constant, low levels of anoxic heat dissipation when exposed to air, but some individuals voluntarily break their isolation by gaping, which is accompanied by a sharp deflection in the power-time curve (Fig. 4A). This gaping behavior is particularly pronounced in the small-size class of *M. edulis* (Fig 4B). Metabolic rate in air therefore is a complex function of the relationship between size and respiratory rate (Bayne et al., 1982), of the relationship between size and anoxic rate (Pamatmat, 1980), and of the behaviorally controlled

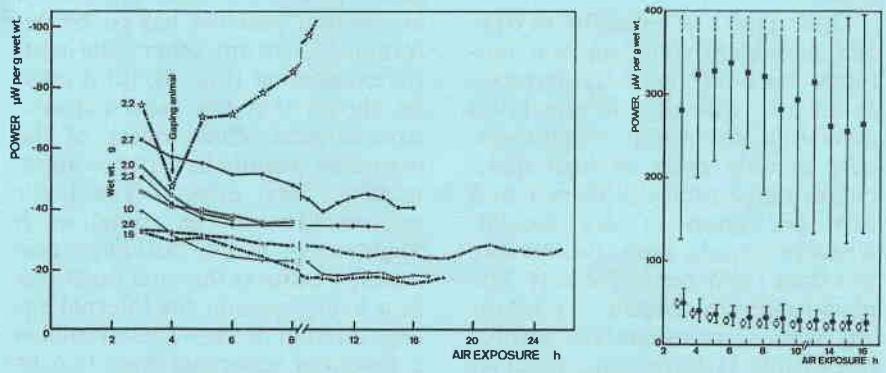


Fig. 4. Total specific rates of heat dissipation of the common mussel Mytilus edulis during exposure to air. A–Individual power–time curves of animals of 1.0–2.7 g wet weight, integrated over one-hour periods. One individual gaped after 4 h of air exposure. Another (weight 2.0 g, open squares) was placed in the calorimeter a second time after a recovery period of 3 h in water. When the shells were clamped completely tight, the anoxic heat dissipation reached a steady state at the same level as at 5–8 h in air. B–Average rates of animals of two size classes: 0.02–0.13 g wet weight (filled squares, 2–7 individuals per experiment), and 1.0–2.7 g wet weight (open circles: mussels acclimated to two 8-h periods of air exposure per day; filled circles: animals acclimated to subtidal conditions; gaping animals were excluded). Bars show one standard deviation.

The large variations in the small-size class (6 expts.) are due to large fluctuations in heat dissipation associated with gaping and aerobic metabolism, whereas the large-size class (6 or 7 expts.) metabolizes almost completely anoxically.

The results were obtained with the prototype LKB BioActivity Monitor and 5-cm³ (small-size class) and 25-cm³ (large-size class) ampoules. Partially adopted from Gnaiger (1983c).

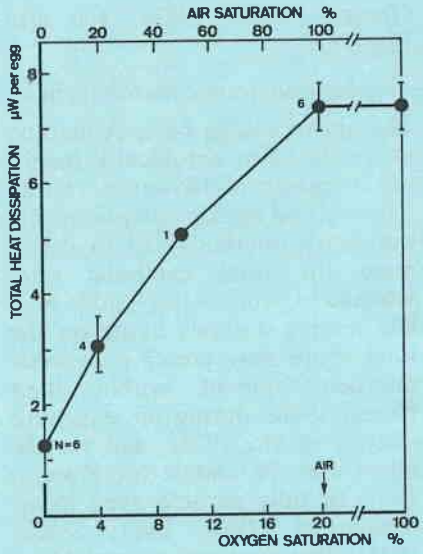


Fig. 5. Heat dissipation as a function of the oxygen saturation in eggs of Salvelinus alpinus at 8.5 °C. Mean ± S.D. of the number, N, of experiments. Each experiment was made with four eggs in the 0.5-cm³ glass chamber of the LKB 2107 Flow-Sorption Microcalorimeter. After Gnaiger (1979).

anoxic/aerobic ratio in total metabolism. The methodological variability offered by a modular system (Fig. 2) is particularly advantageous in studies of developmental stages of different animal sizes. I am not aware of any practical alternative to microcalorimetry in the study of such complex, ecologically significant metabolic patterns.

Due to the low solubility of oxygen in water, some aquatic biotopes become regularly hypoxic or anoxic, with drastic effects on metabolic processes. Weight-specific aerobic rates of heat dissipation range typically from 1 to 6 mW per gram of dry weight, whereas anoxic rates usually are less than 1 mW per g (Table 1). The planktonic copepod *Cyclops abyssorum* maintains its aerobic rate (Table 1) down to a critical air saturation of 5–10 % (Gnaiger, 1983*a*). It is an example of a nearly perfect oxygen regulator, whereas the eggs of the salmonid *Salvelinus alpinus* are linearly dependent on oxygen (Fig. 5). Below an intermediate, limiting p_{O_2}, anoxic catabolism supplements the diminishing aerobic rate. *S. alpinus* produces predominantly lactic acid (Gnaiger et al., 1981), whereas a complex pattern of multiple anoxic end products characterizes most

Table 1. Direct calorimetry (weight-specific power) of aerobic (ox) and anoxic (anox) metabolism in aquatic animals.

Animal		Temp. (°C)	Power ox/anox (mW/g dry wt)	Ratio anox/ox (%)	Reference
Anemone	*Actinia equina*	15	1.2/0.08	7	Shick, 1981
Annelid	*Lumbriculus variegatus*	12	2.6/1.1	43	Gnaiger, 1980*b*
		20	5.1/1.8	35	
Bivalves	*Mytilus edulis*	15	1.8/0.19	10	Shick et al., 1983
		20	7/0.9	13	Famme et al., 1981
	Cardium edule	20	0.7/0.29	41	Pamatmat, 1980
	Mulinia lateralis	15	3.4/3.2	97	Shumway et al., 1983
Crustacean	*Cyclops abyssorum*	6	6/1	17	Gnaiger, 1981, 1983*a*
Reptile	*Pseudemys scripta*	24	2.6/0.3	12	Jackson, 1968
Fish egg	*Salvelinus alpinus*	8.5	1.5/0.25	17	Gnaiger, 1979

euryoxic animals in the anoxic state (de Zwaan, 1977). Comparison of direct calorimetry and indirect biochemical assessment of anoxic enthalpy changes suggests, however, that the presently known biochemical pathways can account for less than 60 % of total anoxic heat dissipation (Gnaiger 1980*a*, *b*, 1983*c*; Shick et al., 1983). Microcalorimetric methods are therefore indispensable in investigations of metabolic capacities of anoxic animal life.

Toxicological and pharmacological applications

In microcalorimetric toxicology the problem of establishing baseline levels is twofold: (1) the instrumental baseline has to be determined as in any other calorimetric experiment (Fig. 3); (2) it must be shown that the most extreme toxicological effect, death of the organism, results in zero (or undetectable) heat effects. The latter argument is not as trivial as it might seem to be. Although poisoning destructs the vital functions of a living system, the internal energy content of the system remains a potential source of heat two or three orders of magnitude above the power developed within an experimental period. An animal of 1 g ash-free dry weight and displaying a total heat-dissipation rate of 3 mW encounters a metabolic loss of mass of c. 0.5 mg/h or 0.05 %/h (1mW ≃ 0.15 mg organic biomass catabolized per hour; see Gnaiger, 1983*b*). After poisoning, the catabolic energy content of 1 g could theoretically sustain a normal heat-dissipation rate for about 2000 hours. In fact, however, the heat dissipation of fish eggs (and other organisms; Gnaiger, 1979) decreases to values close to zero after poisoning with a quaternary ammonium base (Fig. 6).

Aerobic standard tests and effects under ecological anoxia

Eutrophication of aquatic systems is an important cause of environmental hypoxia and anoxia, and is frequently combined with ecological pollution. The toxicological effects of pollutants on aquatic animals ought therefore to be routinely studied under relevant low oxygen conditions.

Although Streptomycin and Neomycin are known to be inactive against bacteria under anoxia (Dalla Via, 1983), they have a stimulating effect on the oligochaete *Lumbriculus variegatus*, even in the absence of oxygen (Gnaiger, 1980*a*). A lasting increase in heat dissipation is also seen in air-saturated water (Fig. 7A). On the other hand, the combination of Streptomycin and Penicillin caused a 30 % reduction in the aerobic rate of heat dissipation after a transitory increase, but had no effect on the anoxic rate (Fig. 7B). These antibiotics do, however, stimulate the rate as well as the pattern of heat dissipation of zooplankton (Gnaiger, 1981).

Direct and indirect calorimetry. Drug-induced physiological hypoxia

The eggs of *Salvelinus alpinus* are not affected by Streptomycin and Neomycin, which apparently do not penetrate the egg membrane

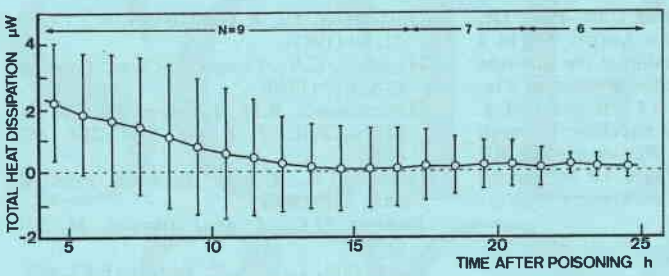


Fig. 6 (Above). Heat dissipation of four eggs of Salvelinus alpinus after poisoning with 0.5 % "Amoquar" (a quaternary ammonium base). Mean ± S.D. of the number of experiments, N, indicated. Before poisoning, the anoxic rate was 5µW. The baseline stability was better than ±2 µW at a perfusion rate of 3.3 cm³/h. After Gnaiger (1979).

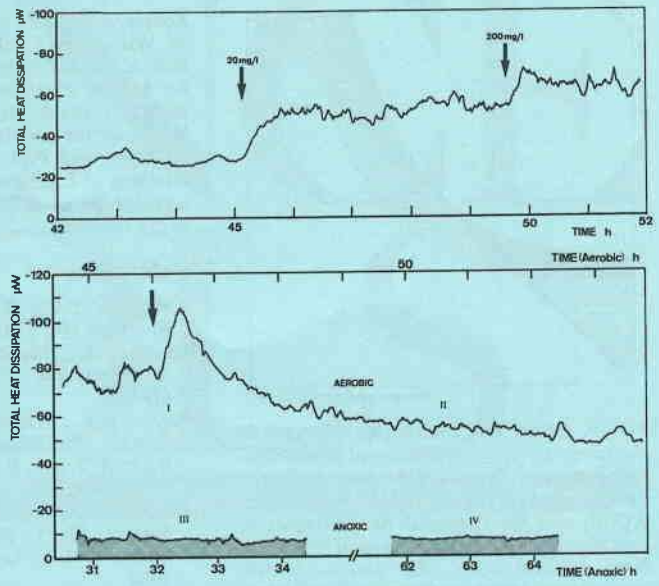


Fig. 7 (Right). Effects of sublethal doses of antibiotics on aerobic and anoxic heat dissipation of Lumbriculus variegatus at 20 °C. LKB 2107 Flow-Sorption Microcalorimeter. A–Increase in aerobic steady-state rate with increasing concentrations of Streptomycin sulfate and Neomycin sulfate (arrows; 20 mg/dm³ and 200 mg/dm³ of each, respectively). Total wet weight of ten individuals: 102.4 mg. After addition of the antibiotics, the anoxic rate increased from 0.27 to 0.34 µW per g wet wt. B–Decrease in aerobic steady-state rate (upper curve) after addition (arrow) of 500 mg Streptomycin sulfate and 1 000 mg Penicillin G per dm³. The anoxic rate (lower curve) remained unaffected by the addition of the antibiotics. Total weight of ten animals: 101.8 mg. Partially adopted from Gnaiger (1981).

(Gnaiger, 1981). After hatching, however, an immediate stress response to the addition of these antibiotics is observed as a nearly threefold increase in heat dissipation, whereas the oxygen consumption is only doubled (Fig. 8). This differential response in simultaneous respirometry and calorimetry is due to the activation of anoxic pathways sustaining the high metabolic rate (physiological hypoxia). The anoxic contribution evades detection when traditional respirometric techniques are applied alone.

Beezer & Chowdhry (1980) reviewed microcalorimetric studies of the action of drugs on microorganisms, and Dalla Via (1983) summarized the effects of antibiotics on the physiology of animals. Gnaiger (1981) discussed some biochemical implications of simultaneous direct and indirect calorimetry in analytical tests of drugs as related to the possible effect of uncoupling of the respiratory electron-transport system.

Conclusions

Aerobic and anoxic rates of heat dissipation are integratively detected by direct calorimetric methods. Simultaneous calorimetry and respirometry, on the other hand, furnish a quantitative distinction between these principal sources of catabolic energy. Whereas high metabolic rates, such as locomotory bursts or stress-induced peaks of activity, may be partially sustained by anoxic mechanisms (physiological hypoxia), low levels of heat dissipation are generally induced by environmental oxygen depletion. Under these conditions of hypoxia, anoxic pathways contribute to the total metabolic rate. Intermediate levels of hypoxia can cause even higher anoxic rates than those observed under strict environmental anoxia (Famme et al., 1981). Direct calorimetry represents the only nonspecific method for the detection of biological activity over the entire ecological and physiological range (Fig. 9).

The physiological and biochemical interpretation of biological heat dissipation requires detailed thermochemical analyses (Gnaiger, 1980*c*) and stimulates in-

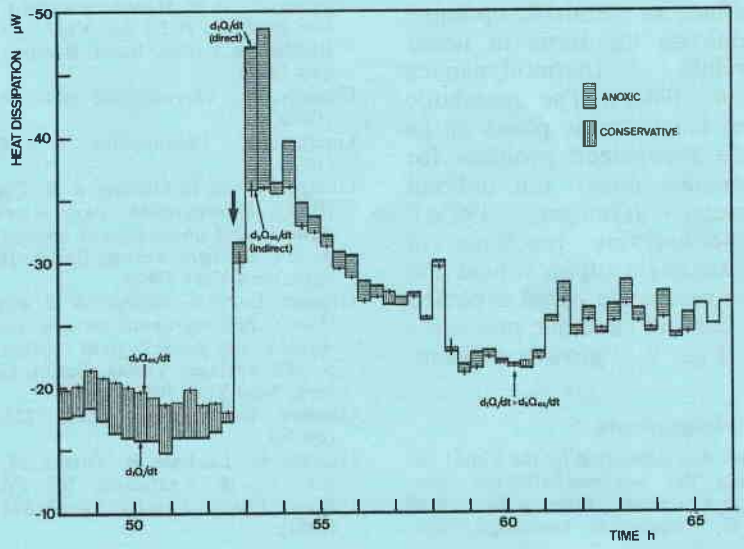


Fig. 8. Direct and indirect calorimetry of the stress response of Salvelinus alpinus after hatching (5.1 mg dry wt., 8 °C, 48% air saturation) after addition of 200 mg/dm³ Streptomycin sulfate and 200 mg/dm³ Neomycin sulfate (arrow). The experimental heat equivalent of oxygen consumption as determined under normoxic conditions, –451 kJ/mol O_2, was used to convert oxygen consumption into catabolic heat dissipation, d_kQ_{ox}/dt (Eqn. (2)), and instrumental results were corrected for delays (Gnaiger, 1983a). The vertical bars indicate the most probable range of oxygen uptake, accounting for the varying time constant of the respirometer. The total rate of heat dissipation, d_tQ/dt, indicates a high anoxic contribution to the stress reaction.

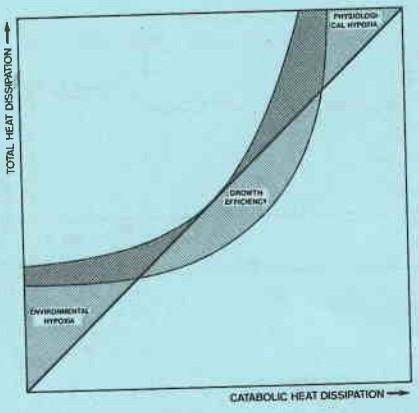


Fig. 9. Direct and indirect calorimetry in the range of environmental and physiological hypoxia. The straight line indicates agreement between total heat dissipation, d_tQ/dt, and the catabolic heat dissipation calculated on the basis of oxygen consumption, d_kQ_{ox}/dt (Eqn. (2)). Due to anoxic mechanisms under environmental and physiological hypoxia, low and high levels of d_tQ/dt, respectively, are higher than d_kQ_{ox}/dt. Anabolism ("growth efficiency") may suppress the total heat dissipation below the expected value of d_kQ_{ox}/dt. Based on experiments with fish larvae, Gnaiger (1983a).

terdisciplinary research (Gnaiger et al., 1981; Shick et al., 1983). Physiological and environmental hypoxia are two domains of biological activity (Fig. 9) where microcalorimetric monitoring will play an increasingly important role. The divergent biochemical traits giving rise to these anoxic sources of heat were conceptionally defined as metabolic optimum functions on the basis of linear irreversible thermodynamics (Gnaiger, 1983*c*). The metabolic state of biosynthesis poses an as yet less recognized problem for simultaneous direct and indirect calorimetry (Gnaiger, 1983*a*): Energy-conserving reactions of anabolism might suppress heat dissipation below the signal expected on the basis of catabolic processes alone (Fig. 9, "growth efficiency").

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