HOMEOSTATIC AND MICROXIC REGULATION OF RESPIRATION IN TRANSITIONS TO ANAEROBIC METABOLISM

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I. Introduction

Classification of physiological responses into discrete patterns is a common scientific approach for describing and organizing results and evaluating new data sets in comparative studies. A classical example of such physiological classification is the distinction between respiratory regulation and conformity in the transition from normoxic to anoxic conditions (13; 24). However, a concise matching between such types of respiratory response to hypoxia and anoxic environments or mode of life is not generally possible. Homeostatic regulators and conformers alike are found among anoxia-intolerant (stenoxic) and tolerant (euryoxic) species. This intriguing predicament is due to the diversity of metabolic adaptations and the confinements of a typology which directs our attention mainly to one side - the normoxic reference - of the aerobic-anoxic spectrum.

This view was challenged recently by pointing out the adaptive significance of microxic regulation at low partial pressures of oxygen, p_{O_2} (8). The pattern of microxic regulation is characterized by a steep oxygen flux/pressure slope at very low oxygen, despite some degree of conformation at mild hypoxia. The steep slope exerts a dominating influence on the energetic state of the animal, in terms metabolic dynamics, energy saving, specifically carbohydrate sparing, ATP/substrate stoichiometry (5), and suppression of anaerobic emergency mechanisms (14). In the following discussion of microxic regulation some conceptual and methodological aspects are pointed out on the basis of selected examples showing a variety of patterns in the relation between oxygen flux and oxygen pressure. For a more extensive overview see Ref. 8.

On the subcellular level, generalized flux/pressure relations are frequently linear, such as relations between osmotic fluxes and osmotic pressure differences, diffusion fluxes of gases and partial pressure gradients, or chemical flux and chemical reaction pressure (7). Nonlinear flux/force relations of nonequilibrium thermodynamics (23) are linearized by transformation into flux/pressure relations in the far from equilibrium range (7). On the organismic level, however, nonlinear behavior is the rule rather than the exception. Organisms are complex, hierarchically organized systems which generate and maintain structures far from equilibrium on the basis of nonlinear feedback and feedforward control (23). Common linear regression procedures, and linear and static thinking in general, may conceal the diversity of physiological patterns which have evolved as adaptations to the variety and dynamics of natural environments.

II. Methodological Considerations

A. *Microxic and anoxic versus anaerobic conditions*

The microxic p_{O_2} range spans from $p_{O_2} > 0$ kPa to the rather arbitrary region of the microxic-hypoxic transition at c. 0.1 to 1 kPa (0.8 to 7.5 mmHg; 0.5 to 5% air saturation). This upper limit of the microxic range can be explained in terms of cellular physiology, as the range of p_c values in isolated cells, *e.g.* passive and active myocytes (31). The microxic region thus incorporates low oxygen conditions which are difficult to monitor by conventional methods (8). In some studies on animal anaerobiosis, microxic conditions are referred to as "anaerobic" when the exclusion of oxygen is attempted but not fully achieved (for a discussion see Ref. 8; 11). In contrast, the terms anoxic and microxic should be rigorously applied to conditions characterized by actual oxygen measurements, with reference to the sensitivity limit of the method for oxygen detection or to the tested limits of the respective oxygen removal technique.

B. *Respirometry and calorimetry at low oxygen*

Respiratory and calorimetric measurements at low oxygen require extreme care to prevent the diffusion of oxygen, and highly sensitive instruments are required to measure the oxygen and heat fluxes which decline to a fraction of the fully aerobic fluxes (6; 8; 10). The phenomenon of aerobic shut-down (20) would imply that respiratory flux at p_{O_2} levels corresponding to <1% air saturation is insignificant. This paradigmatic and methodological background explains why rather little attention has been paid to respiratory adaptations at low oxygen in the transition to anaerobic metabolism.
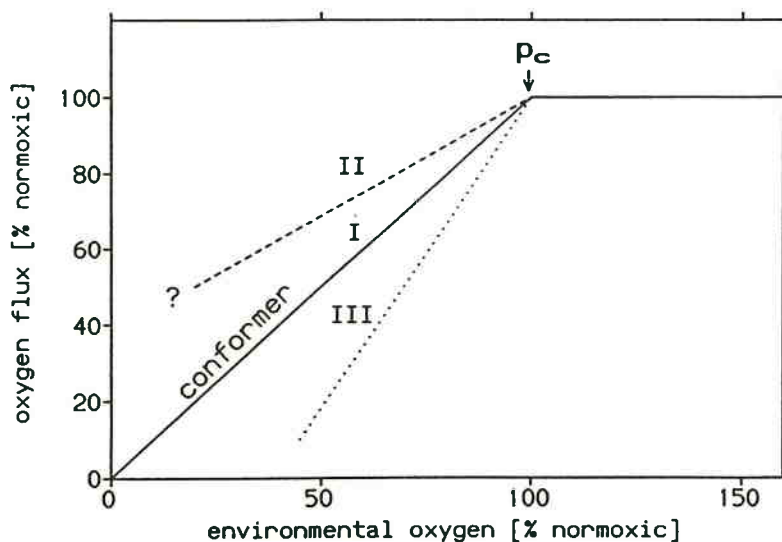
Figure 1. Three types of respiratory conforming patterns, according to Herreid (13). I - Perfect conformer. Oxygen flux, J_{O_2}, falls proportionally with environmental p_{O_2}. II - Conformer with some regulation. J_{O_2} falls slower than p_{O_2}. III - Conformer with decreasing O_2 conductance leading to a more rapid fall in J_{O_2} than in p_{O_2}. The respiratory pattern in the microxic range is not considered.

Oxygen diffusion into respirometers may cause a substantial artefact, underestimating oxygen flux, J_{O_2}, at low experimental p_{O_2}. Thereby the J_{O_2}/p_{O_2} curve is shifted to the right (Figure 1; from type II → I → III), concealing microxic regulation.

C. Open-flow and closed system respirometry

Steady state oxygen flux can be measured at controlled experimental p_{O_2} in open-flow or intermittently closed respirometers. This approach is superior to closed-chamber respirometry, since it allows acclimation of the animal to the experimental chamber, usually at normoxic perfusion conditions, and to each oxygen level. As a drawback, however, the oxygen regime in the perfusion chamber is not always well defined due to inflow-outflow p_{O_2} differences (6; 15). This is a particular problem at low oxygen in microxic regulators, since a well defined experimental oxygen level is required in the steep part of the oxygen flux/pressure relation. In addition, the number of data points along the oxygen gradient is necessarily limited to the number of selected steady state p_{O_2} levels.

A combination of open-flow and closed system approaches may be required to accurately quantify microxic regulation. The p_{O_2} regime in a closed respirometer is controlled by the oxygen uptake of the animal. The effect of duration of the experiment (changes in activity, prolonged starvation) is superimposed on the effect of p_{O_2} in the unidirectional oxygen regime of the closed respirometer. Importantly, high activity in the struggle to escape severe hypoxia must be separated from homeostatic regulatory responses such as high ventilation and circulation. If the effects of time and behavioral disturbances can be excluded, the closed respirometer chamber offers the advantage of continuously monitoring the first derivative of the oxygen signal, whence a continuous record of the oxygen flux/pressure relation is obtained. This requires high data sampling frequency and numerical treatment of the A/D converted records. In this case, models such as linear regression in an apparently linear region, or hyperbolic and quadratic polynomial functions, are not required for the description of the data. Interpolation or brute force mathematical fitting can be applied to delineate the flux/pressure relations for comparison.

III. Microxic Regulation

A. Oxygen flux/pressure relations viewed at increasing versus decreasing p_{O_2}

Even in comparative physiology, the traditional perspective on transitions to anoxia is dominated by an anthropomorphic or "anthropophysiologic" recognition which centers around the normoxic condition. We are aerobically poised and view the world from the preferred normoxic environment. Figure 1 shows relations of oxygen flux versus oxygen partial pressure (flux/pressure relations). Contrary to the practice to read English from left to right, a respiratory oxygen flux/pressure relation is inevitably studied from right to left: The curve is not seen to increase with p_{O_2} but to fall from normoxia to hypoxia, as it does in a closed respirometer. The decline of the flux/pressure relation toward the left is the traditional criterion for the conforming pattern. The plot may terminate at some arbitrary value without further attention as to the shape at low oxygen levels (Figure 1; *curve II*).

A fundamentally different interpretation of curve II (Figure 1) is suggested by simply reading the graph from the intercept, the anoxic condition (alien to us humans) to normoxia (where we are from the beginning). The pattern of both the *homeostatic regulator* and the *microxic regulator* shows a steep increase of oxygen flux, J_{O_2} [relative units or nmol $O_2.s^{-1}.g^{-1}$], with p_{O_2} (Figure 2). This steep J_{O_2}/p_{O_2} slope in the microxic to hypoxic region distinguishes the microxic regulator from the true conformer. Within the p_{O_2} interval from zero oxygen to a partial pressure p_m, microxic regulation maintains a slope steeper than in the conformer, at $>5\%$ normoxic flux per kPa O_2. The slope levels off above the p_m to $<5\%$ per kPa. In the limit, the p_m merges with the critical p_{O_2}, p_c, in the pattern of a homeostatic regulator (Figure 2).
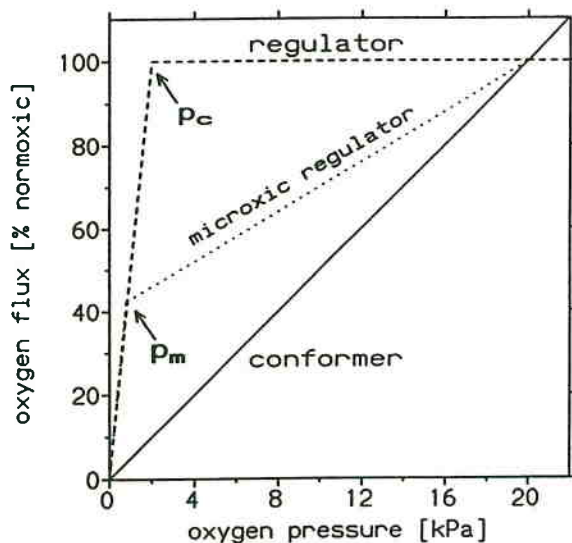
Figure 2. Three types of oxygen flux/pressure relations, contrasting regulation and conformation in the microxic range (up to 1 kPa). The difference between the homeostatic regulator and microxic regulator is restricted to the hypoxic range (1 to <20 kPa). 1 kPa = 0.133322 mmHg. Normoxia (100% air saturation) is at 20 kPa (150 mmHg). After Ref. 8.

B. Energetic significance of microxic regulation

Due to the steep slope of oxygen uptake in the microxic regulator, the aerobic fraction reaches typically 50% of total heat flux at only 1% air saturation (0.2 kPa or 1.5 mmHg O_2), whereas heat flux in the conformer is $>90\%$ anaerobic (8). This assumes that anaerobic heat flux is maintained at 10% of the normoxic level up to the p_m. Suppression of anaerobic mechanisms of energy transformation in proportion to the steep rise in aerobic respiration further enhances the energetic significance of microxic regulation. Thereby the limiting p_{O_2}, p_{lim}, is shifted to the left and fully aerobic metabolism is sustained over a wide range of hypoxia. The corresponding adaptive value can be quantified in biochemical and thermodynamic terms, particularly by the steep rise in the ATP/Glycogen stoichiometry observed in the microxic regulator but not in the conformer (8).

Several apparently anaerobic environments are in fact microxic or severely hypoxic, including microenvironments of the intertidal macrofauna, *e.g.* within the closed shell valves of mussels during air exposure (26). Therefore, it is important to study the physiological adaptations not only to anoxic but to microxic conditions. The small decline of oxygen flux with p_{O_2} in the hypoxic range, as opposed to full regulation, may indicate an energy saving strategy to reduce the costs of locomotion and avoid increased ventilation, irrigation and cardiac output.

C. Does microxic regulation distinct from conventional regulation exist?

There is extensive experimental evidence for the phenomenon of microxic regulation in lower vertebrates and many invertebrates (8). However, accurate oxygen flux measurements <2 kPa O_2 (c. 10% air saturation) are comparatively rare in animal physiology, since discussions on oxygen flux/pressure relations focused on the regulator-conformer dichotomy. A methodologically careful study on the respiration of common carp acclimated to an open-flow respiration chamber (15) suggests the importance of microxic regulation, based on the extrapolation of linear regression lines (Figure 3a). Microxic regulation is characterized by the decline of respiratory flux under progressive hypoxia, but the extrapolated intercept with the Y-axis is above zero (compare Figure 2). The oxygen flux/pressure relation of many invertebrates is well described by a polynomial function applied to normalized oxygen flux (expressed relative to the value observed at normoxia; Ref. 2; 20). In view of the polynomial fit (Fig. 3b; *dashed line*), the phenomenon of microxic regulation distinct from classical regulation disappears for the data at 20°C. The Y-intercept is not significantly different from zero.

For the data at 10°C the polynomial fit, however, intersects with the Y-axis at 38% of normoxic flux (Figure 3b; *full line*). This supports the conclusion on microxic regulation and the significance of a distinct p_m value at or below 4 kPa. Normalization of the oxygen flux is particularly instructive (Figure 3b). The patterns of the type II "conformer" (see Figure 1) at 10°C and of the homeostatic regulator at 20°C are distinct at mild hypoxia. Microxic regulation, however, effectively increases oxygen flux at severe hypoxia at least to the same extent compared to the pattern of the homeostatic regulator. A necessary requirement for the quantitative assessment of the p_m value is the measurement of oxygen flux at p_{O_2} levels well below the apparently linear hypoxic range.

Microxic regulation is an important adaptation to low environmental oxygen at low temperature (for a more general discussion of oxygen regulation and low temperature see Ref. 14 and 32). The common carp has a low anoxic tolerance compared to crucian carp, does not produce ethanol under anoxia (17), and additionally increases oxygen extraction at low p_{O_2} upon acclimation to hypoxia (19). The latter shifts the oxygen flux/pressure curve further to the left, yielding a steeper slope in the microxic range.
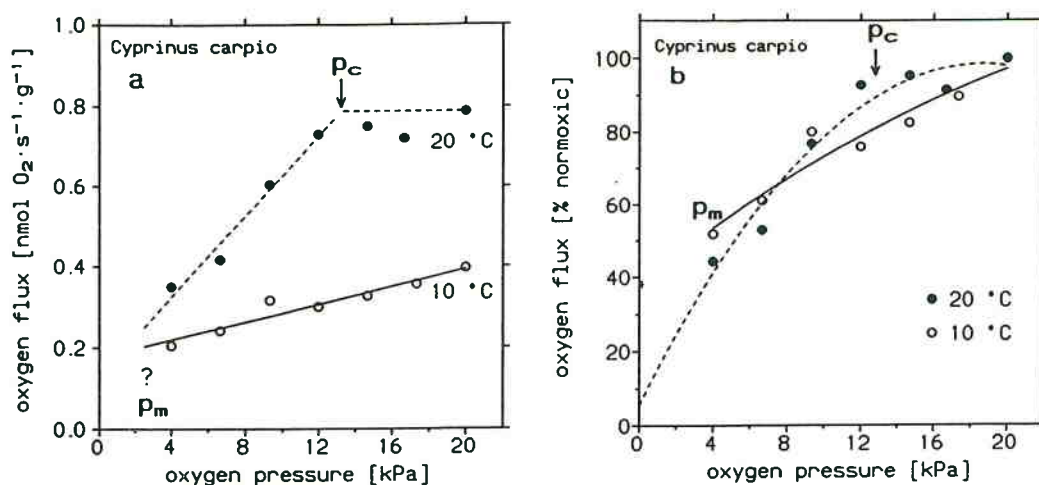
Figure 3. Oxygen flux [nmol $O_2.s^{-1}.g^{-1}$ fresh weight] as a function of environmental p_{O_2} in the carp, *Cyprinus carpio*, at two experimental (= acclimation) temperatures, measured in an open-flow respirometer. Data from Hughes *et al.* (15). 1 ml $O_2.h^{-1}$ = 12.393 nmol $O_2.s^{-1}$. *a)* Linear regression lines according to the original reference. The Y-intercepts at 44% and 16% of normoxic flux (10°C and 20°C, respectively) suggest microxic regulation. *b)* Normalized oxygen flux and polynomial fit (quadratic, second order). The continuous function does not indicate microxic regulation at 20°C (intercept not different from zero). However, it confirms microxic regulation at 10°C with a distinct $p_m \leq 4$ kPa (30 mmHg) in contrast to a conformer. The extrapolated intercept at 38% is significantly different from zero.

Diving water snakes studied by Abe and Mendes (Ref. 1) provide another impressive example for microxic regulation (Figure 4). Respiration falls continuously with progressive hypoxia, but the Y-intercepts of the quadratic polynomials are significantly above zero. This implies a very steep slope of 50% relative oxygen flux per kPa in the microxic range up to the $p_m \leq 1$ kPa (5% air saturation). This is a tenfold increased slope compared to a perfect conformer.

Estimation of the p_c in microxic regulators and oxyconformers requires measurement of oxygen flux in the hyperoxic range (Figure 1). In a calorimetric study of embryonic arctic charr it was shown that heat flux increased with p_{O_2} up to normoxia (21% oxygen saturation), but no further increase was observed up to 100% oxygen saturation (4). Apparently linear oxygen flux/pressure slopes under hypoxia lead to the classification of *Sipunculus nudus* as a conventional oxyconformer (22; Figure 5; *dotted lines*). The measurements in the closed chamber respirometer allow for the delineation of the data by a continuous function (polynomial, fourth order) which underscores the conclusions drawn from the linear regressions on the significance of a distinct p_m value (Figure 5). In fact, the flux/pressure relations of the **microxic regulator** *S. nudus* can be superimposed on the polynomial function of a **homeostatic regulator**, the common carp at 20°C,

up to the p_m values of 4 and 6 kPa (small and large individuals, respectively; Figure 5 and 3b; compare Figure 8).

A widespread occurrence of microxic regulation in intertidal tube dwelling worms is illustrated by the classic study on oxygen uptake in the polychaete *Mesochaetopterus taylori* (21; Figure 6). Oxygen pressure in the tube water declines to 2 to 4 kPa during 2 to 3 hours of tidal exposure (21). The p_m estimated on the basis of a polynomial fit is exactly at this lower limit of environmental p_{O_2} (Figure 6), indicating the defense of aerobic mechanisms of energy flux at low oxygen levels.
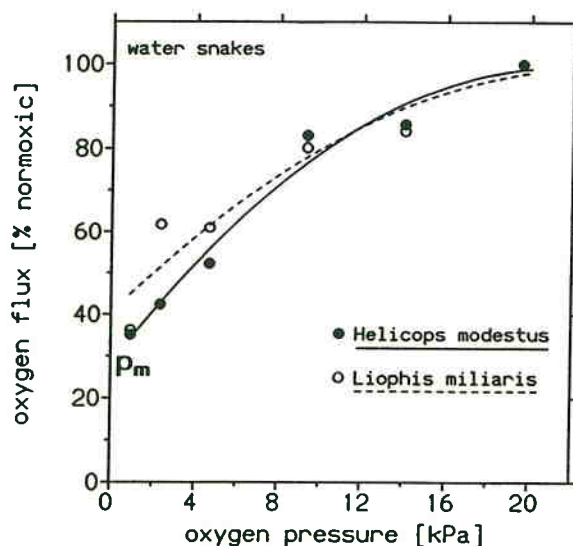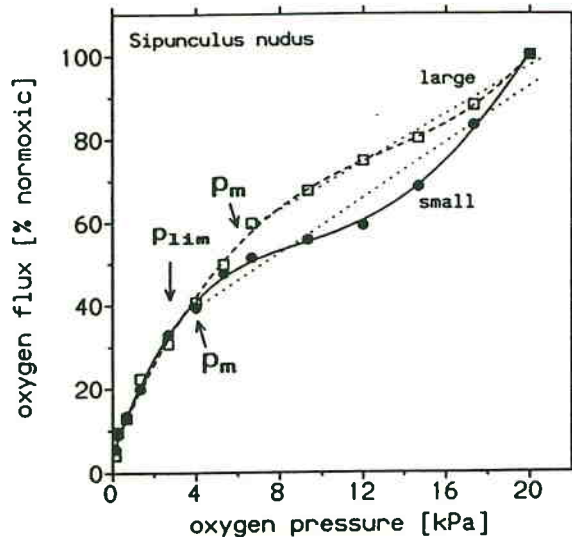

Figure 4. Normalized oxygen flux as a function of environmental p_{O_2} in two species of water snakes, *Helicops modestus* and *Liophis miliaris*, measured in an intermittently closed respirometer at 25°C. Data from Abe and Mendes (1). The intercepts of the polynomials (second order) with the Y-axis are significantly above zero (28 and 40%). A steep slope is indicated in the microxic range up to the p_m of c. 1 kPa (7 mmHg).

IV. Calorespirometry at Low Oxygen and the Limiting p_{O_2}

Respirometric studies cannot answer *per se* the energetically important question whether a reduction in metabolic oxygen flux represents quantitatively the reduction in metabolic energy flux, or if anaerobic mechanisms compensate for the decreased aerobic energy supply. Respirometry combined with calorimetry (calorespirometry) provides information on both aerobic and anaerobic aspects of energy metabolism (5, 9). The ratio of calorimetrically and respirometrically measured heat and oxygen flux (calorimetric/respirometric or CR ratio) is compared with the theoretically derived oxycaloric equivalent. The oxycaloric equivalent is the enthalpy change of the oxidative catabolic reactions per amount of oxygen respired, $\Delta_k H_{O_2}$, ranging from -430 to -480 kJ/mol O_2 (5; 9). CR ratios more exothermic than -480 kJ/mol indicate the simultaneous involvement of aerobic and anaerobic mechanisms of energy metabolism. In a variety of euryoxic and stenoxic animals, the CR ratios measured under normoxia indicate fully aerobic metabolism (11). In the transition from aerobic to anaerobic metabolism, there

is a limiting p_{O_2}, p_{lim}, below which CR ratios become more exothermic since anaerobic energy flux is switched on (8).

Figure 5. Oxygen flux as a function of environmental p_{O_2} in the intertidal worm *Sipunculus nudus*, measured in a closed-chamber respirometer at 15°C. Data from Pörtner *et al.* (22). The dotted lines show linear regressions as drawn in the original publication with slopes indicating significant microxic regulation, with different p_m values for large and small specimen. This view is corroborated by polynomial fitting (full and dashed lines) with intercepts not significantly different from zero. The limiting p_{O_2} (p_{lim}) was established by biochemical end product determination.

The toad *Bufo bufo* becomes hypoxic during 2 h of submergence at 20°C, as judged from a reduced heart rate and a fall in oxygen flux to 20% of its normoxic value, but the heat flux drops to an identical level, indicating no or no detectable contribution by anaerobic metabolism, although blood lactate levels show a slight tendency to increase (18). By comparison, the frog *Rana temporaria* shows the same level of metabolic depression to a heat flux of 20% of the normoxic level after 2 h of exposure to anaerobic conditions, with a similar decrease of the heart rate (29; compare Ref. 16).

Calorespirometric investigations of the freshwater oligochaete *Lumbriculus variegatus* (8; 11) revealed a metabolic depression under anoxia to 15% of the normoxic heat flux, indicative of anoxic ATP turnover of c. 30% of the aerobic level (5, 11). *L. variegatus* is a homeostatic oxyregulator, with a hyperbolic function yielding the best fit (Figure 7; *open symbols, full line*). Oxygen flux is at 30% of the normoxic level at a p_{O_2} as low as 0.5 kPa (2% air saturation). Only below this p_{lim} in the microxic range, the CR ratios deviate significantly from the aerobic value (Figure 7). At low oxygen the oxygen flux/pressure slope in the homeostatic regulator *L. variegatus* is steeper than in the microxic regulator *S. nudus*. Correspondingly, the p_{lim} in the sipunculid worm is shifted to the right, at c. 3 kPa as shown by biochemical end product analyses (Figure 5; 22; see also 11). This difference is correlated with the more severely microxic and

anoxic environmental conditions found in the habitat of *L. variegatus*, but the size dependence of the p_c, p_m and p_{lim} levels has to be considered in any comparison. The small mussel and oyster larvae are mostly homeostatic regulators with p_c values down to 2 kPa, and p_{lim} levels at 1 kPa, as measured by calorespirometry (28; 30). There is a tendency to change the pattern with increasing size from a homeostatic regulator to a microxic regulator (28). Gill tissue of the bivalve *Mytilus edulis* responds to hypoxia by a gradual reduction of mitochondrial cytochromes, respiration and heat dissipation (3). CR ratios under hypoxia are not significantly different from those at normoxia and are in the range of the theoretical oxycaloric equivalent. Therefore, the p_{lim} is far below the p_c both in gills stimulated to high ciliary activity and in the controls (3).
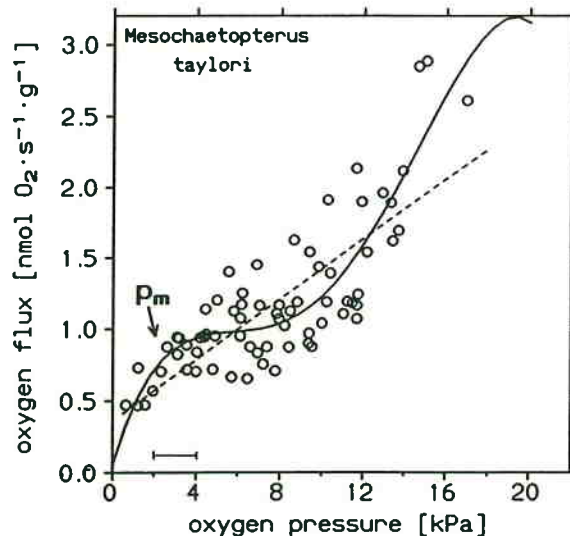

Figure 6. Oxygen flux [nmol.s^{-1}.g^{-1} dry weight] as a function of p_{O_2} in the intertidal polychaete *Mesochaetopterus taylori*, measured in a closed-chamber respirometer at 10°C. Data from Petersen and Johansen (21). The linear regression over all data and the polynomial function (fourth order, intercept not different from zero) indicate microxic regulation, with a p_m of 2.2 kPa. The bar shows the minimum p_{O_2} range measured in the tubes during intertidal exposure.

In contrast to the aerobically balanced metabolism of animals, tissues and harvested cells under normoxic and a wide range of hypoxic states, many cultured cells are frequently below the limiting p_{O_2} under standard aerobic culture conditions, incurring simultaneous aerobic and anaerobic metabolism (9).

V. A Comparative View on Microxic Regulation

In Figure 8 oxygen flux/pressure plots are superimposed, for a quantitative comparison of the examples discussed in the preceding sections. Under microxic conditions, the oligochaetes and water snakes have the same response, although the water snakes are not homeostatic regulators under hypoxia. The polychaete and sipunculid worms show the same increase in respiration up to 2 kPa O_2, although the p_m for the

polychaete appears to be lower than in *S. nudus*. The largest differences in these patterns occur in the intermediate hypoxic range, since 1) the examples were chosen to show a wide variety of patterns, and 2) normalization of oxygen flux eliminates all differences at the normoxic level. For a complete description of the oxygen dependence, the measurements should be extended into the hyperoxic range, which is most relevant for animals with symbiotic algae (25).
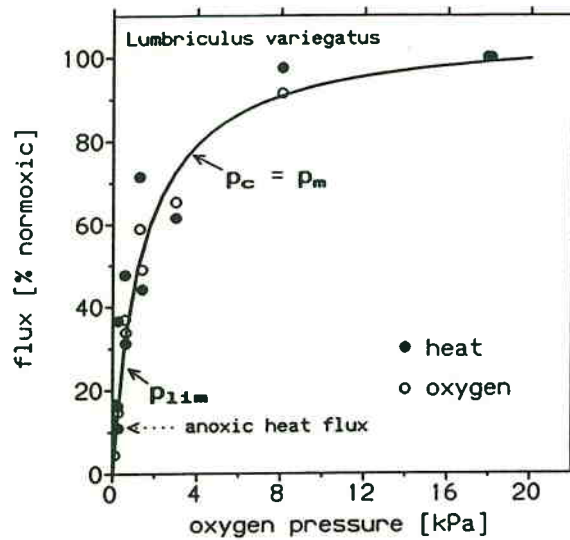
Figure 7. Normalized heat flux and oxygen flux as a function of environmental p_{O_2} in the freshwater oligochaete *Lumbriculus variegatus*. The full line shows the hyperbolic fit to the oxygen data. Polynomial functions yielded abnormal results, probably due to the large measurement intervals in the range of mild hypoxia. Below the limiting p_{O_2} (p_{lim}) of ca. 0.5 kPa the calorimetric/respirometric ratios are more exothermic than -500 kJ/mol O_2 and indicate a significant anaerobic energy flux which declines under anoxia to 11 % of normoxic heat flux. For a hyperbolic function, the definition of a p_c (merging with p_m) is arbitrary. Steady state measurements in the CYCLOBIOS Twin-Flow calorespirometer at 20°C. The normoxic heat flux and oxygen flux were -3.8 mW.g^{-1} and 8.3 nmol O_2.s^{-1}.g^{-1} dry weight. Therefore, the normoxic calorimetric/respirometric ratio was -0.46 mJ/nmol or -460 kJ/mol O_2, indicating fully aerobic metabolism. See Ref. 8 and 11 for experimental details.

The variety of physiological patterns in the transition to anaerobic metabolism is obscured by a strictly typological view. For comparison of the oxygen dependence, the parameters derived from mathematical models (20; 27) are less informative than the set of curves themselves.
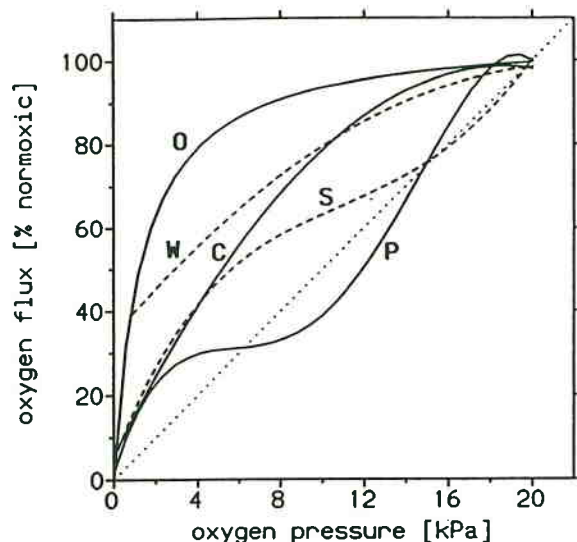
Figure 8. Normalized oxygen flux/pressure relations, for a comparison of respiratory patterns in homeostatic regulators (O - freshwater oligochaete; C - common carp, 20°C) and microxic regulators (W - water snakes; S - sipunculid worms, large and small combined; P - polychaete). The dotted line is the line of correspondence in a theoretical oxyconformer (hyperbolic and polynomial fits from Figures 3 to 7).

Complex patterns should not any longer be confined into categories such as oxyconformity and oxyregulation, nor should we place too much weight on some typical values such as p_c or p_m, since these have different significance depending on the actual environmental oxygen regime. The patterns are best *seen* in normalized flux/pressure diagrams, and can thus be related to the relevant environmental oxygen regime.

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