Vol. 104: 109–117, 1994 | MARINE ECOLOGY PROGRESS SERIES Mar. Ecol. Prog. Ser. | Published January 27

Influence of long-term hypoxia exposure on the energy metabolism of *Solea solea*. I. Critical O_2 levels for aerobic and anaerobic metabolism

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ABSTRACT: Long-term hypoxia is a general phenomenon on the Italian Adriatic coastline, and is mainly caused by continuous eutrophication. The sensitivity of sole *Solea solea* to long-term hypoxia was investigated. Healthy *S. solea* obtained from trawls were kept at 19 °C in aquaria for at least 2 mo. The fish were exposed to hypoxia after a preacclimation period of 30 h at normoxia. Oxygen levels during normoxia were kept constant at 80% air saturation (16.6 kPa, 6.4 mg l^{-1} O_2); during hypoxia oxygen levels were set at 60, 40, 20, 12 or 6% air saturation (4.8, 3.2, 1.6, 1.0, 0.5 mg l^{-1}). During the experiment oxygen consumption was measured continuously. At the end of each experiment, blood samples were taken from anaesthetized specimens. Oxygen consumption patterns were statistically analyzed. A novel technique is described for the determination of the standard metabolic rate and the scope for activity of free-swimming animals. The resting metabolic rate and the scope for activity showed significant changes at reduced oxygen levels. Activity levels declined progressively starting at 40% air saturation. Resting levels remained constant between 80 and 20% air saturation, but fell below the standard metabolic rate at 12 and 6%. Blood lactate levels were increased at 12 and 6%, indicating anaerobic metabolism. Data show that 40% air saturation should be considered as a limiting level, while the incipient lethal level lies between 12 and 20%.

KEY WORDS: *Solea solea* · Hypoxia · Anaerobiosis · Oxygen consumption · Critical oxygen level

INTRODUCTION

Eutrophication of the Northern Adriatic Sea results in regular and long periods of hypoxia and occasional anoxia (Degobbis 1989, Annual Report of Emilia Romagna 1988). This implies that animal species living in this area, particularly those which are not able to leave, must be well adapted to these conditions. This certainly holds for sole *Solea solea*, a flatfish living on the sandbottom along the Italian Adriatic Coast. Previous findings have shown that the bottom of the York River Estuary (Virginia, USA) becomes severely hypoxic in summer, causing all demersal fish to migrate out of the danger zone. This migration appears to be related to both the level of hypoxia and the hypoxia tolerance of the different species, i.e. the most tolerant species leave last and return first (Pihl et al. 1991). The migratory capacity of flatfish is, however, limited, not only because of their negative buoyancy, but also because they are poor swimmers: aerobic swimming is restricted to maximally 10 min, as ascertained in swim tunnel trials (Priede & Holliday 1980). Thus, in most cases *S. solea* will not be able to leave the hypoxic area, especially when the O_2 level falls rapidly.

The hypoxia sensitivity of fishes is species dependent and ranges between 50 and 20 Torr (Itazawa 1971, Gee et al. 1978, Van den Thillart & Van Waarde 1985). At a certain [O_2] fish often show avoidance behaviour (Gee et al. 1978, Petersen & Petersen 1990), or move to the surface for different kinds of surface breathing (Kramer & McClure 1982). If the fish do not

migrate or switch to another breathing pattern, then their tolerance to hypoxia depends on 2 factors: maintenance of oxygen extraction and/or reduction of oxygen consumption. The extraction capacity increases predominantly by an increase of water flow over the gills, while net blood flow hardly changes in resting animals (Jones et al. 1970, Randall 1982). In active fish blood flow through the gills increases markedly (Jones & Randall 1978). The maximal extraction capacity is in most fish dependent upon the $[O_2]$ gradient over the gills, and therefore also upon the ambient $[O_2]$. Reduction of oxygen consumption is in most cases reached by a reduction in locomotor activity. The oxygen consumption rate ranges between standard metabolic rate (SMR), and the 5 to 15 times higher active metabolic rate (AMR). When oxygen extraction capacity is reduced due to a smaller O_2 gradient over the gills, the AMR level falls and so the scope for activity of the animal will fall too. At a much lower oxygen concentration the SMR will be affected. When AMR = SMR the scope for activity is zero, and the $[O_2]$-crit or incipient lethal level is reached. Further reduction of the $[O_2]$ will lower the oxygen consumption below the SMR. Assuming the animal does not try to escape, then there are 2 physiological reactions towards further reduction of the ambient $[O_2]$: (1) compensation of energy production by anaerobic processes such as depletion of phosphocreatine and accumulation of lactate; (2) depression of metabolic rate below SMR level, a response which often occurs with animals under adverse environmental conditions (Hochachka & Guppy 1987).

With respect to hypoxia very few experiments have been carried out on flatfish (Jørgensen & Mustafa 1980, Steffensen et al. 1982). There appears to be a difference in sensitivity between plaice and flounder, plaice being more tolerant than flounder. Flounder appears to be able to maintain a constant oxygen consumption rate over a wide range of oxygen levels (Steffensen et al. 1982). It switches to anaerobic metabolism at the relatively low oxygen level of 55 Torr (Jørgensen & Mustafa 1980). To our knowledge the sensitivity of sole to hypoxia has never been studied before. Bearing in mind the occurrence of long-term hypoxia in the Adriatic Sea at temperatures around 20 °C (summer), bottom-dwelling animals like sole must have special adaptations in order to survive. In this study we observed *Solea solea* over a 30 h preacclimation and a 12 h hypoxia period. Continuous oxygen consumption measurements revealed a large and frequent variability of routine activity. With statistical analysis of the data it was possible to establish the resting and active routine rate, both of which are very useful in describing the effect of hypoxia on the behaviour and physiology of this species.

MATERIALS AND METHODS

Fish. At the Marine Institute of the province Emilia Romagna at Cesenatico, Italy (Consorzio di Studi, Richerche ed Interventi sulle Risorse Marine), fresh seawater is pumped into a large storage tank of about 30 m^3 from an inlet point about 300 m offshore. After sedimentation and filtration the water is thermostatted and used to refresh the water in the tanks and aquaria where the animals are kept. The room is air-conditioned and was set for this study at 19 °C, the normal water temperature at the sea bottom (10 to 20 m) in September/October (Annual Report of Emilia Romagna 1988). Illumination was fixed at the daily cycle for September.

Solea solea were caught by (night) trawling along the coastline of Cesenatico (Adriatic Sea) by local fishermen, and kept alive in large plastic tanks on deck until they were carried to the lab at around 10:00 h. About 20 to 30 % of the fish died within a few days due to damage incurred by the trawling nets. The fish did not take food other than live worms, for which we used *Nereis* (sp.), a polychaete which is abundant along the shore. At first, the fish were kept in large glassfibre-polyester tanks, but it became apparent that they did not stay healthy for more than a month. It was assumed that the fish were stressed due to the absence of their normal substrate. Therefore we built a series of aquaria equipped with a (percolated) sand bottom (Van den Thillart & Dalla Via 1993); percolation is necessary in order to prevent the production of the poisonous gas H_2S due to rotting processes. About 8 fish weighing between 70 and 120 g were placed in a glass aquarium with a gravel and sand bottom of 40 × 100 cm surface and depth of 15 cm; the fish immediately buried themselves in the sand, and were barely visible except for the eyes, which protruded about 5 mm above the sand surface. The fish hardly moved under these conditions during the daytime. However, when feeding, they were very active and eager to catch the cut worms, which they caught when within 10 cm range. In these aquaria sole stayed healthy for many months. The fish were caught in May/June. About 70 specimens were kept in 8 aquaria equipped with sand bottoms. The experiments were carried out in September 1989.

Conditioning and oxygen consumption measurements. For conditioning and oxygen consumption measurements the respirometer setup described by Van den Thillart & Verbeek (1991) was used. The animal chambers of the respirometer had a volume of about 16 l. Water temperature was maintained at 20 ± 0.1 °C and kept at a constant $[O_2]$ by means of an oxygen controller (Kent Industrial Ltd, Stonehouse, UK). The latter opens a solenoid valve when the $[O_2]$

falls below a certain preset point; the flow of air-saturated water is set such that the $[O_2]$ stays within 2% of the set point. The oxygen consumption in the animal chamber is determined by the difference between the oxygen content of the inflowing ($[O_2]_i$) and outflowing ($[O_2]_o$) water and by the water flow. Since the $[O_2]_i$ is 100% air-saturated water, and $[O_2]_o$ is kept constant by the controller, the only variable is the water flow, which is measured continuously by a digital flowmeter (Rhodes, Romford, UK). The pulses from the flowmeter are registered by a datalogger (built by the electronic workshop, University of Leiden), and written on a removable EPROM cassette. The data were read afterwards into a MS-DOS computer via a RS232 input channel, and analysed using the software Quattro Pro 4.0 and StatGraphics 5.0.

Apart from the flowmeter readings, the oxygen consumption was also measured from the $[O_2]$ slopes after closure of the water inlet. Slope measurements were mainly used for blanks. Although all circulating water was constantly irradiated with UV-light in flowcells, a constant (low) blank respiration remained, probably due to micro-organisms attached to the walls and tubings. All oxygen consumption measurements were corrected for blanks measured at 80% before, and at the corresponding hypoxia level after each experiment. Blanks were never higher than 5% of total uptake.

Protocol. Sole were starved for 2 d before experimentation. Depending on the total weight, 2 or 3 fish were placed in each of the 2 animal chambers at about 14:00 h, and kept at a constant $[O_2]$ (80% air saturation; 16.6 kPa, 6.4 mg l^{-1} O_2) until the next day at 20:00 h. Then the $[O_2]$ was reduced by bubbling N_2 through an airstone, such that the decline of $[O_2]$ was about 50% h^{-1}. Thereafter the fish were held under hypoxia for 12 h until 9:00 h the next day. At the end of the experiment the fish were anaesthetized with 85 mg l^{-1} MS222 (3-aminobenzoic acid ethyl-ester, methanesulfonate salt; Sigma). After 10 min they were taken out and blood samples were taken from the caudal vein.

Before and after each experiment blank oxygen consumption measurements were made at the appropriate oxygen concentrations. The oxygen concentration of the water flowing in and out of the animal chambers was measured every day by Winkler O_2-titration, and these values were used to calibrate the oxygen consumption rates. The titration data showed an electrode drift of less than 2% d^{-1}.

Statistics. Data files from normoxic and hypoxic conditions were analyzed by Statgraphics 5.0. A frequency distribution of each data file was calculated, from which the oxgen consumption data at N = 0.05, 0.10, 0.50, 0.95 were extracted and analyzed by multifactorial analysis of variance (ANOVA). Differences with $p \leq 0.05$ were considered significant.

RESULTS

A total of 22 experiments were carried out at $[O_2]$ levels of 60, 40, 20, 12 and 6% air saturation (4.8, 3.2, 1.6, 1.0, 0.5 mg l^{-1}). In all experiments the oxygen consumption was monitored continuously over a period of about 42 h, of which the last 12 h were at a fixed hypoxia level, and the first 30 h at 80% air saturation. For each experiment 2 or 3 fish were used; in total 46 sole were used weighing 80 ± 21 g. Oxygen consumption was corrected for the weight differences according to Van den Thillart & Kesbeke (1978):

$$M_{100} = M \times 100^{0.8} / (W_1^{0.8} + W_2^{0.8} + W_3^{0.8})$$

where M_{100} = metabolic rate for a 100 g fish; M = measured metabolic rate; and W_1, W_2 & W_3 = respective weights of Fish 1, 2 & 3. The exponent 0.80 appears to be the same for different fish species and at different temperatures (Basu 1959, Beamish 1964, Yamamoto 1991, 1992), including 3 different flatfish species (Duthie 1982).

In Fig. 1 (A to E) typical experiments are presented for each condition. The oxygen consumption data are shown over a period of 42 h. The large variation in oxygen consumption over the first period (80% air saturation) represents changes in locomotion activity. Reduction of the $[O_2]$ from 80 to 40% air saturation had no visible effect. At 20% a reduction of the scope for activity is clearly noticeable, while at 12 and 6% the resting oxygen consumption rate is also significantly reduced.

Fish in the respirometer were generally quiescent; most of the time they were attached to the walls or the bottom of the respirometer, and did not swim regularly. The large changes in oxygen consumption may be related to the small movements as they attached themselves to the walls and bottom. In addition the fish showed extended periods of low oxygen consumption rates. The 4- to 5-fold change in O_2 consumption rate during the normoxic (80% air saturation) periods demonstrates the range of routine activity.

Data from all conditions were statistically analyzed. From the experiments shown in Fig. 1, the frequency distribution of the normoxic and hypoxic period was calculated and is shown in Fig. 2. It is clear that the range of O_2 consumption rates becomes smaller at decreasing $[O_2]$, while at 12 and 6% air saturation the hypoxia and normoxia distributions are completely separated. From the frequency distributions of 43 datafiles, the O_2 consumption values at N = 0.05, 0.50, and 0.95 were deduced, which can be considered as respectively resting, routine, and active levels. The difference between N = 0.05 and N = 0.95 should therefore be considered as the scope for activity. An exam-

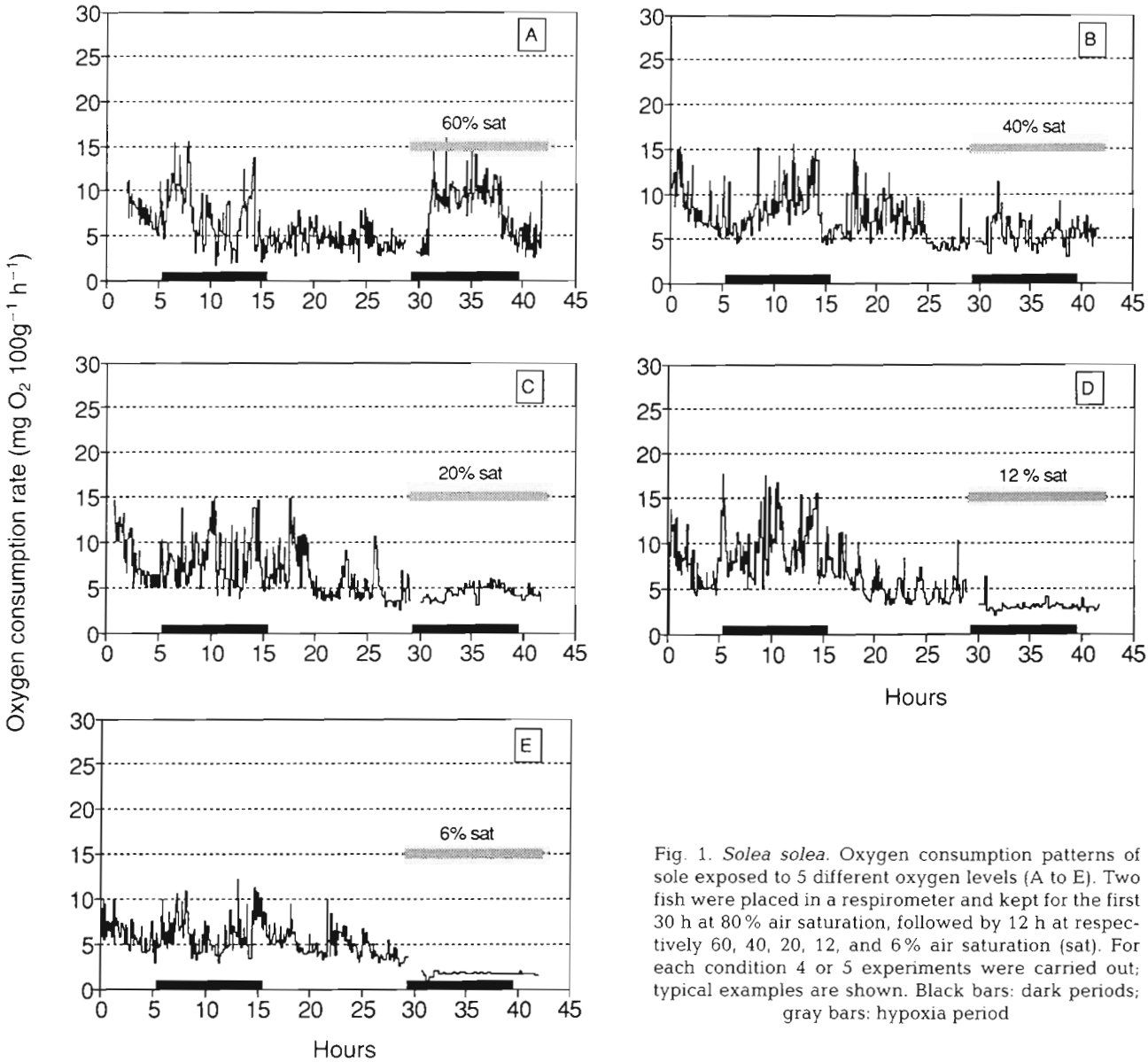

Fig. 1. *Solea solea*. Oxygen consumption patterns of sole exposed to 5 different oxygen levels (A to E). Two fish were placed in a respirometer and kept for the first 30 h at 80% air saturation, followed by 12 h at respectively 60, 40, 20, 12, and 6% air saturation (sat). For each condition 4 or 5 experiments were carried out; typical examples are shown. Black bars: dark periods; gray bars: hypoxia period

ple is presented in Fig. 3 in which the cumulative relative frequencies are presented, the values at N = 0.05, 0.50, and 0.95 being indicated. The frequency distribution of each condition shows how the fish spend their energy over the observed period. From this distribution we selected 3 important parameters: (1) the resting rate, defined at N = 0.05, (2) the median, defined at N = 0.50, and (3) the active rate, defined at N = 0.95. To prevent confusion with the well-known term active metabolic rate (the maximal aerobic capacity determined by forced swimming activity), we will call the active rate at N = 0.95 the active routine rate (ARR). A summary of the values of the 3 parameters from 43 datafiles is presented in Table 1, together with a multiple range statistical analysis (ANOVA). For N = 0.05 and 0.95 the data (± SE) are presented in Fig. 4.

Blood lactate levels are presented in Table 2. The values at 100, 60, 40, and 20% air saturation are not significantly different from each other, staying around 0.5 mM. The values at 12% (4.5 mM) and at 6% (19.8 mM) were found to be significantly different from each other, as well as from the data at 20, 40, 60, and 100% air saturation.

DISCUSSION

The oxygen consumption rate of fish varies under normoxic conditions between the standard metabolic rate (SMR) and the active metabolic rate (AMR), which is 5 to 15 times the SMR level. While the AMR can be measured by forcing the fish to swim at maximal

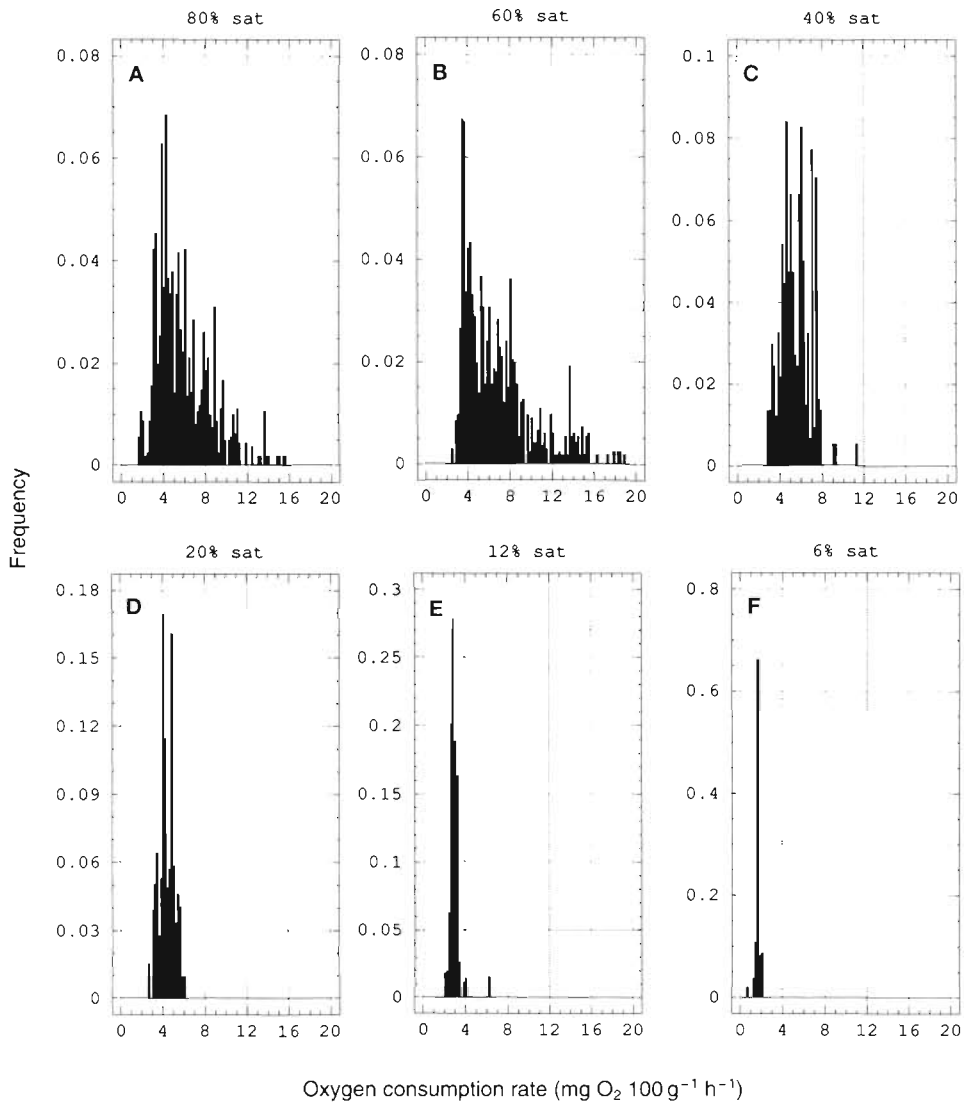

Fig. 2. Frequency histograms of the experiments shown in Fig. 1. The distributions of the oxygen consumption rates observed during the normoxic (80%) and the hypoxic period (60%) are from Fig. 1A. The distributions shown for 40, 20, 12, and 6% are taken from, respectively, Fig. 1B, 1C, 1D, and 1E. With decreasing $[O_2]$, the distribution band-width becomes narrower, while at 12 and 6% the oxygen consumption rates clearly fall below the standard metabolic rate

speed, the SMR cannot be enforced; in contrast the energy consumption level of the fish will increase and stay elevated for a long time every time the fish is handled. Although the oxygen consumption may increase due to higher muscle tone without external activity, there is a correlation between oxygen consumption and activity, which can be used to extrapolate to zero activity (Beamish & Mookherjii 1964). When individuals are not stressed, the reading at zero activity should give the SMR. The extrapolation method has been used as far as we know only by Beamish & Mookherjii (1964). Activity readings have been carried out by other authors (e.g. Spoor 1946, Heitman & Siegmund 1989, Forstner & Wieser 1990); the results indicate that the fish fall back to zero activity from time to time when observed for several days under low stress conditions. The lowest oxygen consumption rates are found at zero activity. In most papers this approach is not followed, and both preconditioning and observation period are usually too short to know that the fish are at low stress and at zero activity. When fish are put in a respirometer, it is likely that they are not at their SMR level for most of the time. When these fish are exposed to hypoxia, they will respond with an avoidance reaction (Petersen & Petersen 1990) or a reduction of external activity. In the latter case the response will show a conformer type response. In the literature it is suggested that this response is due to a reduction of the SMR. The toadfish, which is regarded as a classical example of a conformer (Prosser 1973), has been revealed however by Ultsch et al. (1981) to be a regulator. This could be proved only after sufficiently long observations. In a similar way Ott et al. (1980) showed that the critical oxygen ($[O_2]$-crit) level for carp and trout is much lower than described by most authors. The $[O_2]$-crit is the same value as the incipient lethal

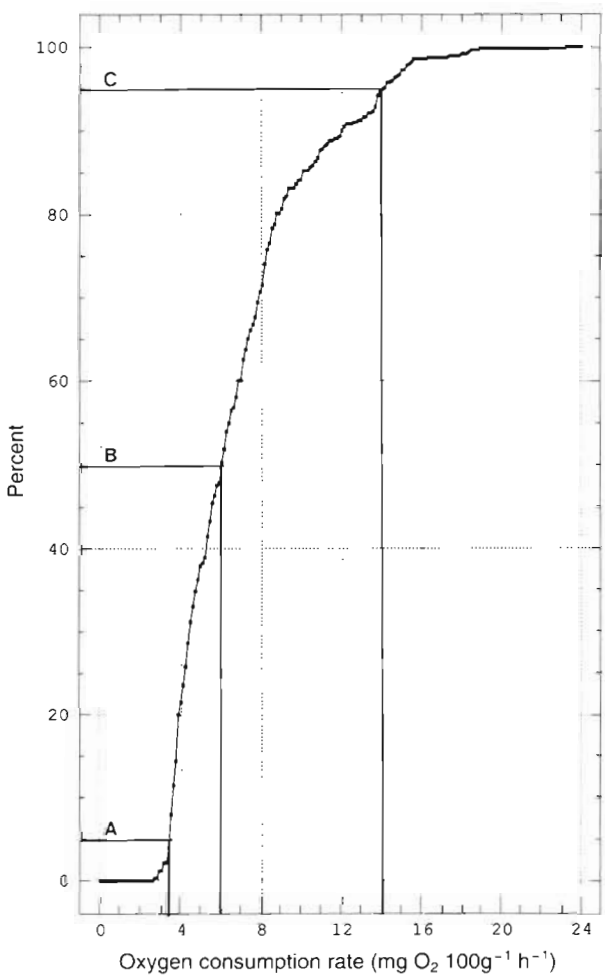

Fig. 3. Cumulative relative frequencies of the hypoxia period displayed in Fig. 1A. The oxygen consumption rates at the frequencies N = 0.05 (A) and at N = 0.95 (C) were taken as respectively the resting and active routine rates. The median at N = 0.50 (B) is also indicated to show the uneven distribution pattern

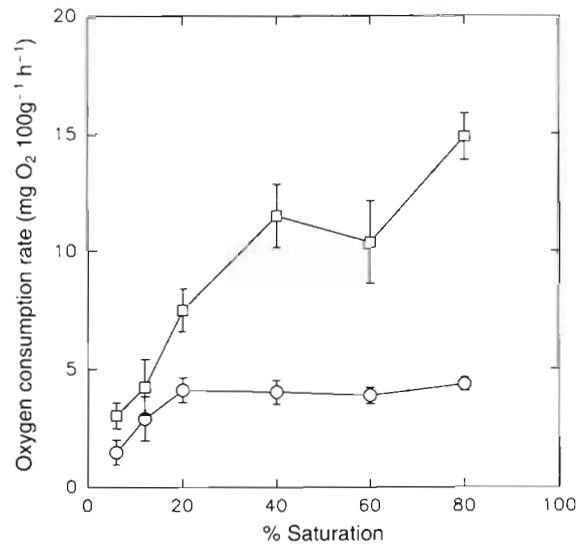

Fig. 4. *Solea solea*. Oxygen consumption rates of sole at different ambient oxygen levels. The resting (○) and active (□) oxygen consumption rates are derived from the frequency distribution of the measured rates over a 12 h observation period, as shown in Fig. 3. Each point represents the mean ± SE of 4 or 5 independent experiments (for statistics see Table 1)

level (Fry 1947, 1971), and is the $[O_2]$ where the scope for activity is zero, or where the AMR is reduced to the SMR level. In general the lethal level for fish will be higher than the $[O_2]$-crit, because fish will use their muscles from time to time. Thus a small scope for activity is necessary for the fish to stay alive for a few days under hypoxic conditions. This is clearly shown in Fig. 4; the resting metabolic rate falls below the SMR at 12 and 6%, however the active routine rate (ARR) is still at the SMR level at 12%. If we extrapolated the SMR line to the ARR line, then the $[O_2]$-crit would be 12% instead of 20%. Thus, although the fish are able to extract oxygen at the SMR level, they do not in fact do so, perhaps because the cost of ventilation is too high.

Continuous oxygen consumption measurements of fish under hypoxic conditions are rare (Van den Thillart & Verbeek 1991); almost all measurements are derived from the $[O_2]$ decline after closing the animal chamber, or from short-term measurements with a flow-through system. The problem with oxygen consumption measurements under hypoxic conditions is that we are dealing with several variables simultaneously. (1) It takes time for an animal to adapt to a new situation; respiration and circulation have to be tuned to the new oxygen level, as they do for each level of activity (Jones & Randall 1978). (2) The activity level of an animal varies between 2 extremes: the resting metabolic rate and the maximal aerobic rate. So in order to know the 'real' resting level under a certain hypoxic condition, it appears necessary to observe the animal continuously over a long enough period. The lowest levels would then approach the resting levels. Without activity reading, extrapolation would still be difficult. With the use of frequency analysis, however, we are able to estimate the resting level quite accurately. The distribution patterns at $[O_2] \geq 40\%$ are skewed, with a very steep increase at the low rates. Thus, the error made in determination of the SMR is small. We have chosen the 5% level, but at 10% the oxygen consumption rate is virtually the same.

Demersal fish like sole have a negative buoyancy, and are rather poor swimmers. Flatfish (flounder, plaice and lemon sole) can be forced to swim in a tilted swim tunnel, although most experimental fish are not cooperative and swim no longer than 10 min (Priede &

Table 1 *Solea solea*. Effect of long-term hypoxia on oxygen consumption patterns

% Saturation		V_{O_2} (mg O_2 $100g^{-1}$ h^{-1})							
		Resting		Median		Active		Range	
	n	Mean	SE	Mean	SE	Mean	SE	Mean	SE
6	4	1.49	0.63	2.14	0.95	3.04	1.84	0.92	1.12
12	4	2.91	0.63	3.52	0.95	4.20	1.84	1.07	1.12
20	5	4.11	0.56	5.84	0.85	7.49	1.64	2.52	1.00
40	4	4.02	0.63	6.98	0.95	11.51	1.84	5.82	1.12
60	5	3.88	0.56	7.71	0.85	12.38	1.64	6.77	1.00
80	21	4.36	0.27	7.86	0.42	15.22	0.80	8.50	0.49

Multiple range analysis

	Resting	Median	Active	Range
6 vs 12	n	n	n	n
20	s	s	n	n
40	s	s	s	s
60	s	s	s	s
80	s	s	s	s
12 vs 20	n	n	n	n
40	n	s	s	s
60	n	s	s	s
80	s	s	s	s
20 vs 40	n	n	n	s
60	n	s	s	s
80	n	n	s	s
40 vs 60	n	n	n	n
80	n	n	n	s
60 vs 80	n	n	n	n

s: statistically significant at $p \leq 0.05$; n: not significantly different

Holliday 1980, Duthie 1982). The factorial scope (AMR/SMR) obtained from these swim tunnel experiments is around 4. In the experimental setup described in this paper we were able to monitor the oxygen consumption of the fish continuously under free-swimming conditions. Statistical analysis of the data revealed the resting routine rates (RRR) and the active routine rates (ARR). Although it can be expected that ARR/RRR < AMR/SMR, the factorial scope observed in 22 experiments at 80% air saturation was 3.6 ± 0.8 (range 2.5 to 5.2), which is not much lower than the values obtained with forced activity (Priede & Holliday 1980, Duthie 1982).

The activity peaks of *Solea solea* during the experiments were all very short, lasting not much longer than a few minutes (Fig. 1), which fits the general behavioural pattern of flatfish. That this type of fish is not able to perform long aerobic activity follows from the lactate measurements of Duthie (1982): at all swimming speeds lactate levels were increased. The blood lactate levels in our experiments stayed at around 0.5 mM between 100 and 20% air saturation, so we

Table 2. *Solea solea*. Effect of long-term hypoxia on blood lactate levels

% Saturation	Lactate (µM)			**Multiple range analysis**					
	n	Mean	SE	6	12	20	40	60	100
6	6	19.84	1.16	–	s	s	s	s	s
12	7	4.46	1.07	–	–	s	s	s	s
20	10	0.70	0.89	–	–	–	n	n	n
40	10	0.47	0.89	–	–	–	–	n	n
60	10	0.51	1.00	–	–	–	–	–	n
100	8	0.26	0.85	–	–	–	–	–	–

s: statistically significant at $p \leq 0.05$; n: not significantly different

may conclude that routine activity peaks do not result in a significant anaerobic load.

The SMR depends on several factors, such as temperature, satiation, starvation, life cycle, weight, and season. To correct for metabolic weight, the allometric relation $M = aW^b$ is used. The value for b approximates 0.80 for carp, goldfish, and tilapia over a wide range of weights and temperatures (Basu 1959, Beamish 1964, Yamamoto 1991, 1992). This value has also been observed for 3 different species of flatfish (Duthie 1982). For comparison purposes, all data were recalculated to mg O_2 $100g^{-1}$ h^{-1}, for a metabolic weight of 100 g. Steffensen et al. (1982) give 6.2 and 8.8 mg O_2 $100g^{-1}$ h^{-1} as SMR values of, respectively, flounder and plaice at 10°C, and Duthie (1982) gives 5.5, 5.5, and 6.9 as the SMR values for, respectively, flounder, dabs, and lemon sole at 15°C. These authors took care to keep the fish as quiet as possible; however their observations were much shorter than ours, which is probably the reason why we found a lower SMR of 4.1 mg O_2 $100g^{-1}$ h^{-1}, even at a higher temperature (19°C).

Although sole are sedentary and hardly swim when observed directly, the oxygen-consumption curves show an irregular pattern with a 5-fold range between lowest and highest values (4 to 20 mg O_2 $100g^{-1}$ h^{-1}). Progressive reduction of the scope for activity occurred when the $[O_2]$ was reduced to lower values (Fig. 4, Table 1). A different pattern was observed with the resting rates. Between air saturation levels of 80 and 20% the resting rates stayed around 4.1 mg O_2 $100g^{-1}$ h^{-1}. At 12%, and even more at 6%, the oxygen consumption rate falls clearly below the standard rate, indicating that the critical level for aerobic metabolism lies between 20 and 12% air saturation. Blood lactate levels measured after a 12 h exposure to hypoxia showed increased concentrations: 4.5 mM at 12%, and 19.8 mM at 6%, but no effect at 20% air saturation (Table 2). This indicates that between 20 and 12% air saturation there is also a critical oxygen level for the activation of anaerobic metabolism, and that anaerobic metabolism is activated in order to compensate for the loss of aerobic energy production. To know how far the fish are able to compensate, it is necessary to measure metabolites such as lactate, ATP and phosphocreatine in the whole fish, which will be the subject of a subsequent paper. Animals that can endure extreme environmental conditions like hypoxia and anoxia are often able to depress their metabolic rate below the SMR level (Hochachka & Guppy 1987). This happens not only as a response to anoxia such as in goldfish (Van den Thillart et al. 1989, Van Waversveld et al. 1989), but also under hypoxia, such as with the bivalve *Scapharca inaequivalvis* (Van den Thillart et al. 1992). The moderate lactate accumulation during 12 h hypoxia suggests that such a mechanism is also operative in *Solea solea*.

Apart from the incipient lethal level, Fry (1947, 1971) introduced the incipient limiting level, which is the $[O_2]$ below which the maximal oxygen consumption rate becomes depressed. For determination of the relation between $[O_2]$ and active metabolic rate most authors used a swim tunnel. Because flatfish like sole do not swim for long, active metabolic rate can best be deduced from spontaneous activity, since the fish will stay aerobic this way. In this paper we have shown that the frequency distribution of the oxygen consumption rates of free-swimming *Solea solea* can be used to determine the resting and the active routine rates (ARR). From Fig. 4 it is clear that the ARR declines progressively with lower $[O_2]$ levels. The first significant value is at 40% air saturation (Table 1), therefore we must conclude that the limiting O_2 level for *S. solea* lies between 40 and 60% air saturation.

Acknowledgements. The experiments were carried out at the marine research institute of the province Emilia Romagna and the University of Bologna: 'Consorzio di Studi, Richerche ed Interventi sulle Risorse Marine', Viale Vespucci 2, I-47042 Cesenatico (FO), Italy. The project was funded by the Commission of the European Communities 4th Environment R&D programme under contract #EV4V-0122-NL. We thank Dr O. Cattani for valuable advice and assistance and Prof. Dr W. Wieser for critically reading the manuscript.

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This article was presented by W. Wieser, Innsbruck, Austria

Manuscript first received: October 4, 1993
Revised version accepted: October 22, 1993