M.Sc. Thesis:

Routine Metabolic Rate and Critical Oxygen Levels of Cape Horse Mackerel Larvae and Juveniles in the Northern Benguela System

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Abstract: The Benguela Current Large Marine Ecosystem (BCLME) has its principal upwelling centre off the coast of Namibia, where hypoxic or even anoxic conditions occur in areas at depths as shallow as 50m. The BCLME features a high degree of variability over a broad spectrum of time and space scales, causing severe perturbations of the ecosystem with great impact on fish stocks of commercial interest. Larvae and juveniles of *Trachurus trachurus capensis* face a physiological challenge when exposed to hypoxic/anoxic waters and little is known regarding their tolerance.

This study aims to supply such data by measuring routine metabolic rate, citrate synthase and pyruvate kinase acitivty, oxygen critical partial pressure and by monitoring behavioral responses of this species. Specimens from 0.1g to 4.56g of fresh weight were tested in intermittent flow respiratory chambers. Results suggest the critical oxygen partial pressure to be between 4.8 and 3.9 mg(O_2)/l; routine metabolic rate follows a significant correlation with weight according to the allometric equation RMR= $1.2106W^{0.4872}$. Tests concerning the survival involved an exposure of 15 specimens up to 3 hours to oxygen concentration between 0.7 to 1.4 mg(O_2)/l; no losses were recorded, suggesting the possibility of this species to survive anoxic layers by means of tolerance and subsequent vertical migration. The catch records were compared with vertical oxygen profiles and no correlation could be found; we conclude that the vertical distribution of *T.trachurus capensis* is not primarily affected by the position of oxygen minimum zones.

Key words: Benguela, BCLME, *Trachurus trachurus capensis*, oxygen minimum zone, metabolic rate, hypoxia, RMR.

Introduction

The Benguela System

The Benguela Current Large Marine Ecosystem (BCLME) is located along the coast of south western Africa, ranging from east of the Cape of Good Hope in South Africa, to Angolan waters on the north, including the full extent of the Namibian coast. It is considered a Class I ecosystem based on SeaWiFS global primary productivity estimates, with more than 300 g C/m^2 yr, about six times higher than the North Sea ecosystem. It supports a large biomass of fish, crustaceans, sea birds and marine mammals and presents favorable conditions for a rich production of small pelagic fish such as herrings, sardines and anchovies (Boyer et al., 2000, Skogen, 1999).

The sources of the Benguela Current include Indian and South Atlantic subtropical thermocline water, saline low-oxygen tropical Atlantic water and cooler low-salinity subantarctic water. In the area between the continental shelf and Walvis Ridge it was found that 50% of the source water came from the central Atlantic, 25% came from the Indian Ocean, and 25% came from the Agulhas Current and the tropical Atlantic (Garzoli et al. 1996). The BCLME is considered one of the four major eastern-boundary upwelling systems, such as the Humboldt Current on the Peruvian coast, the California current and the Canary upwelling system. To a certain degree, the Eastern Boundary Current (EBC) ecosystems share similar features, such as cold bottom water from moderate depths, which are carried upwards by a combination of trade winds, Coriolis force and Ekman transport (Blanco, 2001). A unique feature of the Benguela upwelling system is its enclosure within warmer waters, on the north side by equatorial eastern Atlantic currents (Fig.1) and on the south by the Indian Ocean Agulhas Current (Shannon et al., 1999).

In contrast with the Pacific upwelling system, which has small seasonal and large inter-annual event, the Benguela system has lower inter-annual variability and major events are rare. However, the Benguela system shows a much higher level of unpredictability, with occasional events resulting in extreme perturbation of the ecosystem and mass mortalities of marine fauna, with great economical losses for commercial fisheries. These anomalous events include the intrusion of warmer, nutrient poor equatorial water across both the southern and northern boundaries. Such events are being commonly referred as Benguela

Ninos, despite the fact that the existence of a Benguela Nino is not universally accepted (Shannon and Nelson, 1996; Hamukuaya et al., 2001).

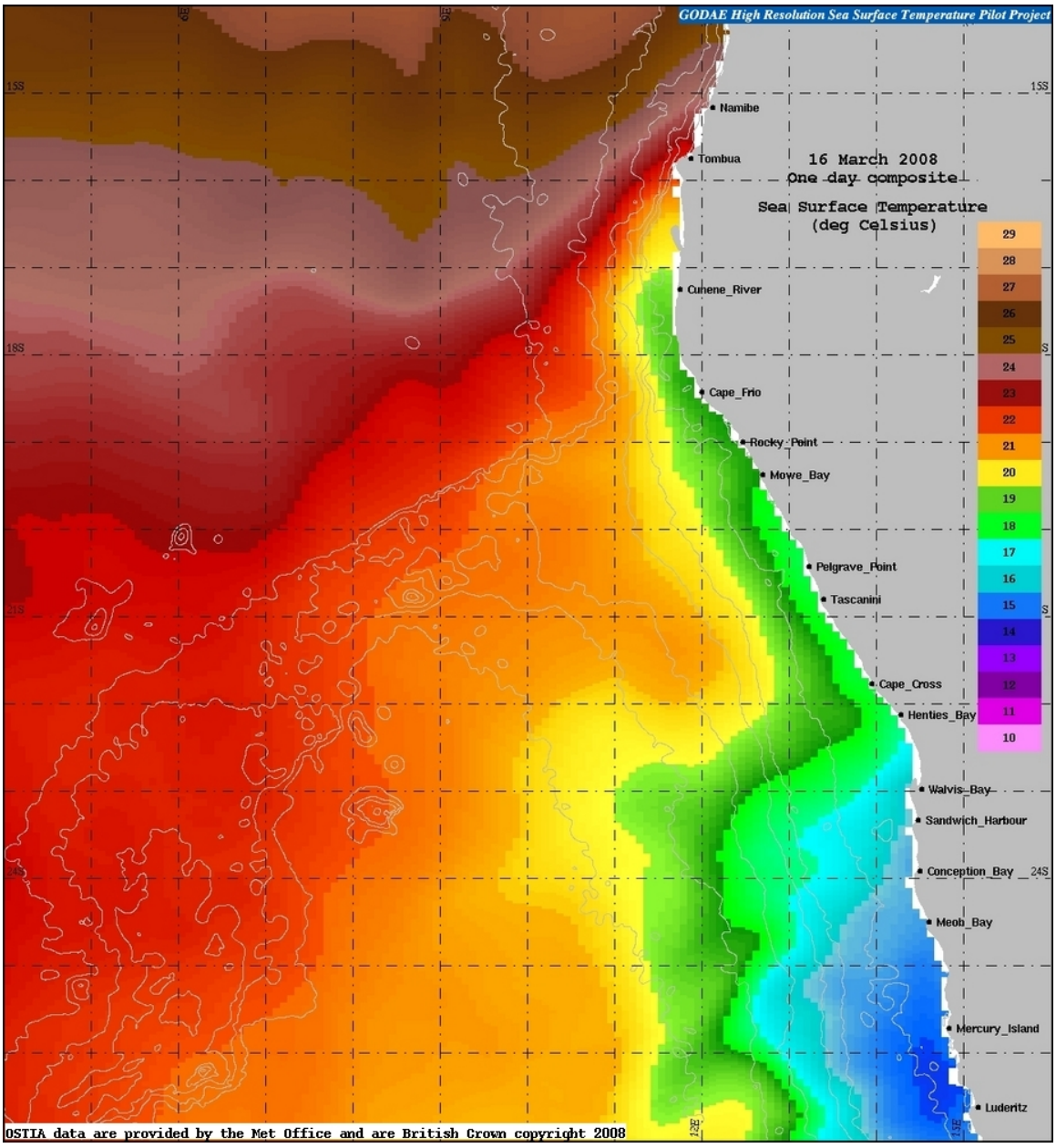

Fig 1: Sea surface temperatures recoded on 16-03-2008. Notice the coastal upwelling stretching northwards to the Cunene river mouth, past which a rapid shift towards tropical temperatures occurs.

The term "Benguela Nino" was coined by Shannon et al. (1986) to describe the large scale warming event, very similar to the Pacific El Nino that occurred along the southern Angolan and Namibian coast, roughly every ten years. The upwelling oceanic fronts of

the Benguela current are spatially highly affected by the large scale changes of intensity, direction and frequency of the wind field. The cooling and warming of large areas of the system alters the advection and stratification of water, which influence the concentration of dissolved oxygen in the water column. Few effects can be clearly attributed to a Benguela Nino in the BCLME. In 1995 a Benguela Nino followed a wide scale advection of low oxygen water into the northern Benguela current from the Angola Dome. This resulted in a temperature rise up to 8°C warmer than usual, which stretched for over 300km offshore from Cabinda to Central Namibia, causing fish mortalities, reduced recruitment and catches (Gammelsröd et al., 1998). Albeit the oxygen concentration in saturated water is reduced by increases in temperature and salinity, the magnitude of this effect is not likely to have much impact except under conditions of high demand, given the efficient uptake mechanism of most fish (Fry, 1971).

Widespread anoxia and hypoxia in the system, especially in the northern Benguela, can be a consequence of oxygen depletion by increased primary production and subsequent decay of organic matter (Helly and Levin, 2004). These events often occur upon a sustained and enhanced upwelling, which is responsible for the transport of bottom nutrient-rich waters towards the upper euphotic zone.

As described by Shannon (1985), nutrient-rich upwelled water boosts the growth of large-celled phytoplankton, which is beneficial for the zooplankton, principally copepods, feeding upon it. The resulting massive primary production triggers a rich secondary production in the upper pelagic layers, consisting mostly of sardines and anchovies. However, the abundant surface production leads to the creation of an extensive oxygen minimum zone (OMZ) located beneath the mixed layers (Fig.2). The oxygen minimum zone is, by definition, an area with less than 0.5mg O_2/l; within the OMZ severe hypoxic conditions might have oxygen concentrations below 0.2mg O_2/l (Levin, 2003; Helly and Levin, 2004). The OMZ off the coast of Namibia is generally located between 100m and 400m, although it can be found at shallower depths (Shannon et al., 1986).

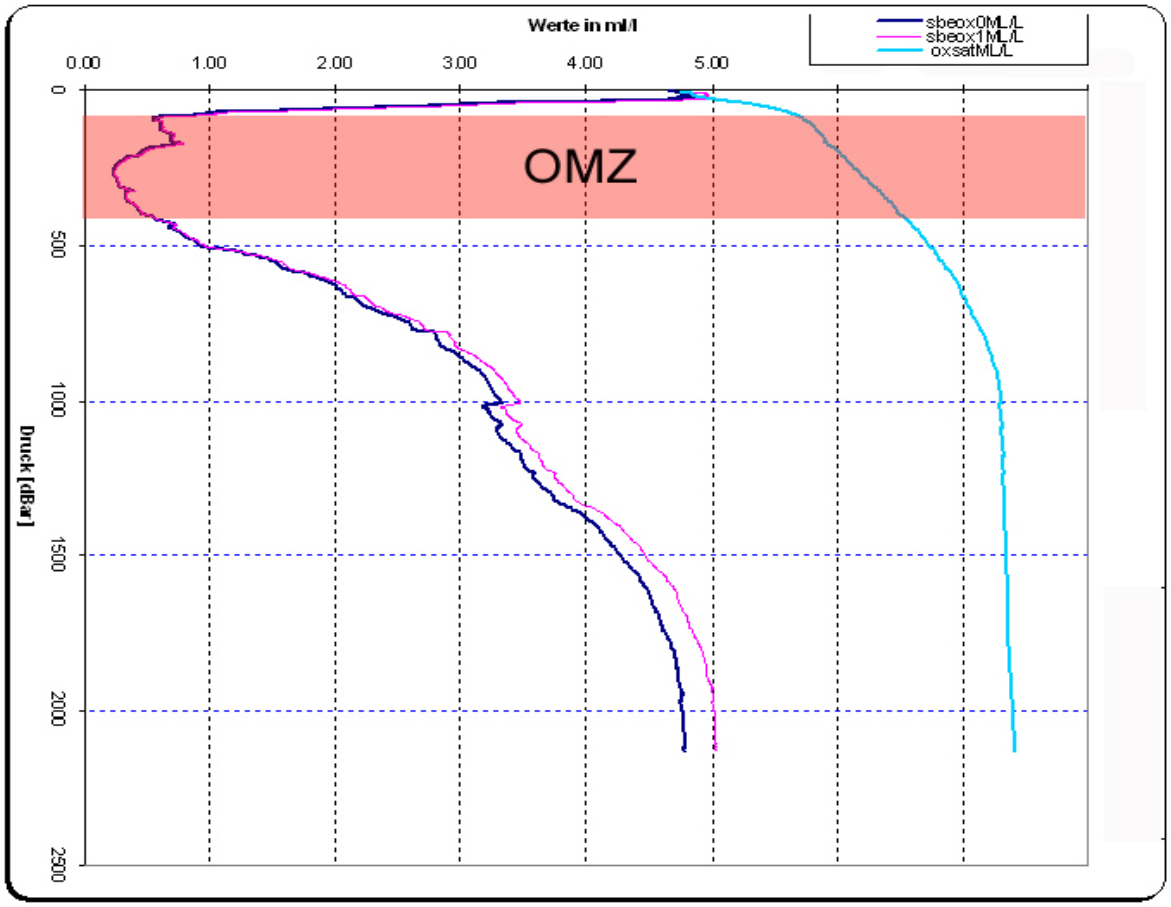

Fig 2: Vertical profile from a CTDO run performed beyond the continental slope. Note the sharp decline of oxygen concentration in the upper layer, suggesting the presence of strong upwelling that triggered an algal bloom. The photosynthetic activity ceases within the first tens of meter, the resulting depletion of oxygen from POM bacterial degradation causes an oxygen minimum zone 300m wide. On the surface a thick layer of microalgal aggregate was visible.

The oxygen depletion is caused by large amounts of sinking particulate organic matter (POM), whose quantity exceeds the oxidizing capacity of aerobic bacteria. POM is the main source of energy for remineralising bacteria in the sea. However the way remineralisation occurs strictly depends upon the local chemical conditions, responsible for shaping the bacterial communities. Whenever oxygen is available, aerobic bacteria are the leading recyclers of organic matter as they feature the highest efficiency over other chemical processes. Oxygen therefore becomes the first element to be depleted in oceanic waters by means of microbial degradation. Approaching hypoxic condition and in presence of significant amounts of nitrate (NO_3^-), denitrifying bacteria take over aerobic

microbes (Knowles, 1982), which in return are outperformed by sulfate reducers once nitrate is depleted. This bacterial turnover normally occurs in all marine environments, especially in sediments, although in the water column this shift is less pronounced due to the higher instability of the environment; oxidation and denitrification may therefore occur simultaneously. Only in presence of enhanced upwelling the oxygen might be totally depleted. Indeed, in the northern Benguela low oxygen water from the Angola Basin is upwelled onto the shelf, adding to hypoxia caused by extensive microbial degradation of organic matter buried in the sediment. A phenomenon known as "secondary remineralisation" or "nutrient trapping" also increases the risk of oxygen depletion on the continental shelf. In this scenario cold, dense and nutrient rich water from offshore collects POM sinking from the upper layers while flowing above the continental shelf. This prevents POM burial and leads to an upwards redistribution of nutrients, increasing both the BOD and the risk of anoxia (Tyrell and Lucas, 2002). Throughout the Northern Benguela low concentrations of dissolved oxygen are normally present, however neither the mean oxygen level nor the amplitude of the fluctuations are clear. Moreover both the seasonal and annual process of oxygen replenishment remains unclear (Kristmannsson, 1994). It is therefore hard to predict the impact of widely ranging dissolved oxygen concentrations in the Benguela ecosystem.

Teleost Metabolism

The majority of fish can be regarded as oxygen regulators, as they posses the ability to maintain a constant oxygen consumption over a certain range of oxygen concentrations by adjusting the ventilation. This is achieved by increasing the stroke volume (Randall, 1982). By decreasing ambient oxygen saturation, a level is reached at which the standard metabolic rate cannot be guaranteed any longer by faster ventilation. This level is called critical partial pressure of oxygen (PO_2cr). Below this level, the oxygen consumption (VO_2) decreases along with the ambient oxygen partial pressure (PO_2), the fish is therefore no longer able to satisfy its metabolic needs and, based on its ability to adopt physiological adaptation, it might die after a certain amount of time (Randall, 1982).

The metabolism of fish can be regarded as standard, routine and active metabolic rate. The standard metabolic rate (SMR) corresponds to the minimum maintenance

metabolism of a resting fish in a post- absorptive state. The routine metabolic rate (RMR) refers to a more realistic metabolic activity of a fish that feeds, grows and undergoes reproduction. The active metabolic rate (AMR) is an index of metabolic requirements of a fish at swimming at the maximum speed (Fry, 1971).

Metabolic Scaling

The effects of body size are evident on all biological structures and processes from cellular metabolism to population dynamics (McMahon and Bonner, 1983). This dependence is generally indicated as a variable "Y" on the body mass "M" in the allometric scaling equation

$$Y = a * M^{b}$$

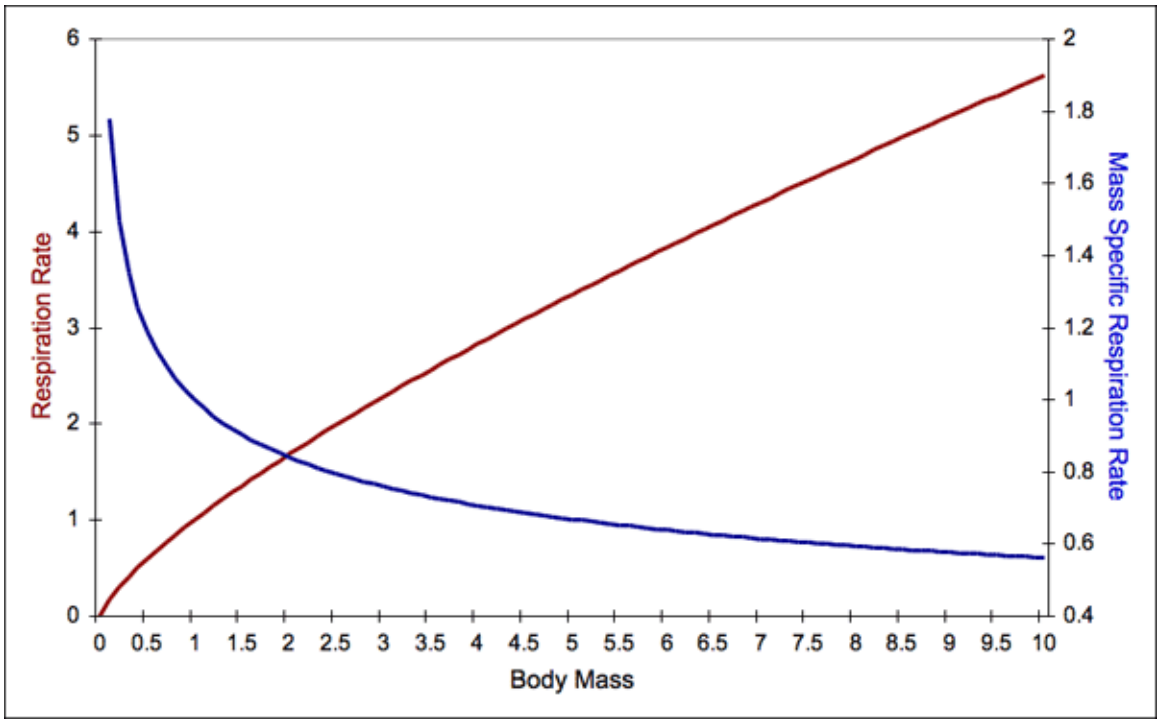

Fig 3: Generic empirical relation in ectotherm organisms, such as teleosts, between respiration rate and body mass (red line). The allometric equation shows an increase in respiration/body mass below 1:1. The exponent is generally between 0.5 and 1. This implies a logarithmic decrease of the mass specific respiration rate with mass (blue line).

where "*b*" is the scaling exponent and "*a*" a constant characteristic for the kind of organism. Most biological phenomena scale as three quarter powers of body mass (Schmidt and Nielsen, 1984), such as the metabolic rate of an entire organism that tends to scale as $M^{3/4}$. Most likely the explanation for this common index of fractioning is explained by the fact that multicellular organisms are sustained by the transport of elements by means of a complex network of vessels that branches to supply all parts of the organism (West et al., 1997).

The "scaling law" originates from the physical and geometrical implications of three principles: the delivery of elements occurs through a space-filling fractal-like branching scheme, the final branching is a size-invariant unit and the energetic requirements for its function follow the highest efficiency.

Body mass and temperature are the main external parameters that affect individual respiration in poikilothermic animals. If temperature is kept constant, the respiration (R) scales exponentially with the body mass (M) within species according to the allometric equation: $R = a * M^b$ (Fig.3). The logarithmic conversion of the data scale is used to linearize the allometric equation, allowing for easier correlation and interpretation (Peters, 1983).

Behavioral Responses to Hypoxia

The adaptation to environmental changes by means of behavioral and metabolic response is a general biological phenomenon. It appears that animals may show two opposite coping strategies upon exposure to stress conditions. They might either exhibit an increased locomotor activity or a passive strategy resulting in immobility and often reduced activity. Marine animals respond to hypoxia initially maintaining oxygen delivery by increasing the ventilation rate, the number of red blood cells and the binding capacity of hemoglobin (Randall, 1982). When this is not sufficient, they respond by conserving energy, which results in a further decrease of the metabolic rate and repression of protein synthesis (Hochachka, 1997). To survive prolonged exposure to hypoxia, the animal finally has to resort to anaerobic respiration (Randall et al, 1992).

The effects of hypoxia on fish growth, development and survival remain almost unknown. Diez and Davenport (1990) showed that embryos of dogfish suffered a rate of mortality of 100% if exposed to 20% PO_2, whereas the loss accounted for 0% when exposed to 50% PO_2. In coastal marine waters, hypoxia might shape species composition, community structure and decrease both species diversity and abundance (Pihl, 1994).

Among planktonic organisms most likely to suffer mortality from exposure to OMZ are fish larvae lacking fully developed motor capabilities (Breitburg, 1994). In consideration of the unpredictability of the position, strength and persistence of the upwelling oceanic fronts of the Benguela system, it is arguable that fish larvae and juveniles do not possess the ability to evade these hypoxic waters and therefore must adopt a series of physiological adaptation to increase their chances of survival. Many studies have already demonstrated the ability of adult fish to avoid or respond to low oxygen concentration (Kramer, 1987), but the behavioral response of larvae and juveniles is less understood, as the evading abilities vary among species. Post larval flounder (*Paralichthys lethostigma*) and large mouth bass (*Micropterus salmoides*) move vertically in response to low dissolved oxygen (Spoor, 1977), in contrast Atlantic salmon and brown trout are unable to avoid anoxic waters (Bishai, 1962). This suggests that species that normally thrive in environments periodically subjected to hypoxia are naturally disposed to seek the best conditions available, while species occurring in well oxygenated environments, such as streams and mixed pelagic layers, cannot cope with random events that lead to oxygen depletion.

Research relating hypoxia to growth and development of fish larvae is therefore essential to predict effects of hypoxic waters on natural fish populations. Moreover, the critical partial pressure of oxygen has been reported for several species of fish, but little is known regarding the ontogenic stages.

Metabolic Pathways and Biochemical Indices

Muscular tissue accounts for the majority of a fish body and different types of muscle fibers vary in proportion and location according to the ecological needs (Johnston, 1981). Muscle fibers are arranged in myotomes, in which "the type I" fiber or "red" muscle, accounting for up to 13% of body mass, usually runs along the lateral line in increasing amounts towards the tail end (Fig.4) (Dickson, 1995). This type of muscle powers sustainable aerobic swimming in most teleost fish. Type II or "white" fiber composes the bulk of the fish muscular structure and supplies fast powerful contractions from anaerobic ATP production. A much smaller portion of the myotome can additionally bear "type IIa" fiber, which feature fast oxidative muscular tissue (Johnston, 1981).

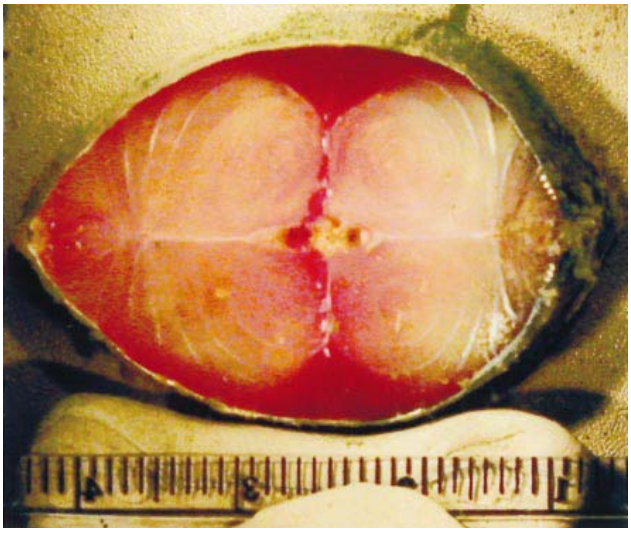

Fig 4: A cross-section through the body of a 233 mm fork length juvenile chub mackerel, *Scomber japonicus*. The red muscle is positioned along the midline on either side of the fish; the white muscle comprises most of the rest of the section (Gibb and Dickson, 2002).

The activity of key enzymes involved in these metabolic pathways is regulated according to the energetic demands of different situations.

Sustainable and burst swimming rely on different metabolic pathways to provide the ATP necessary for muscular contraction. White fiber relies on fast production of ATP by glycolysis using local storages of fuel and without being limited by oxygen availability and delivery rate. Sustained swimming relies on the much more energetically efficient aerobic production of ATP by means of complete oxidation of catabolites derived from glycolysis. This process takes longer and it is limited by the ability of the circulatory system to deliver oxygen and fuel to the cell (Weber and Haman, 1996). The oxygen availability and delivery, on the other side, can be limited by a series of factors, such as ventilation and perfusion rates, binding capacity of hemoglobin, level

of vascularization and myoglobin content in the muscle. The capacity to produce ATP can be further limited by the number of mitochondria per cell and last by the activity of the enzymes involved in the aerobic pathways.

Therefore the sequence of transformations along metabolic routes strictly depends on the enzyme efficiency. An evaluation of biochemical efficiency can be calculated by measuring enzymatic activity of key enzymes.

Citrate Synthase

Citrate synthase (CS) is a key component in the central metabolic pathway of aerobic organisms, the citric acid cycle (Fig. 5). In eukaryotic cells CS occurs almost exclusively in mitochondria, where it acts as a substrate for the citric acid cycle (Wiegand and Remington, 1986). Citrate synthase has been recognized as a reliable indicator of animal aerobic metabolism (Hochachka et al., 1970).

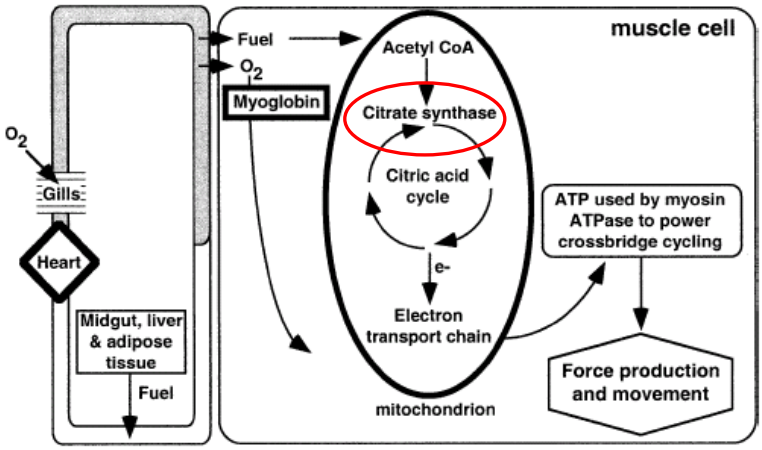

Fig 5: Schematic representation of biochemical pathways and fuel stores involved in aerobic ATP production in muscle fibers. The cell's efficiency in ATP production is greatly influenced by the rate of oxygen delivery by the outer organs. The heavy lining in the diagram indicates the subject as possible predictor of aerobic muscular activity. CS is highlighted by a red circle (Gibb and Dickson, 2002).

Pyruvate Kinase

Pyruvate kinase (PK), key cytosolic enzyme in the glycolysis (Fig. 6), is responsible for the phosphorilation of ADP into ATP using phosho-enol-pyruvate as substrate. Like other enzymes of the glycolytic pathway, glycogen stores and lactate dehydrogenase, it supplies information about the potential for anaerobic work (Childress and Somero, 1979). Anaerobic metabolism does not suffer the shortcomings encountered in aerobic ATP production, although its energetic efficiency is low and sustainable only for short periods due to accumulation of catabolites such as lactate.

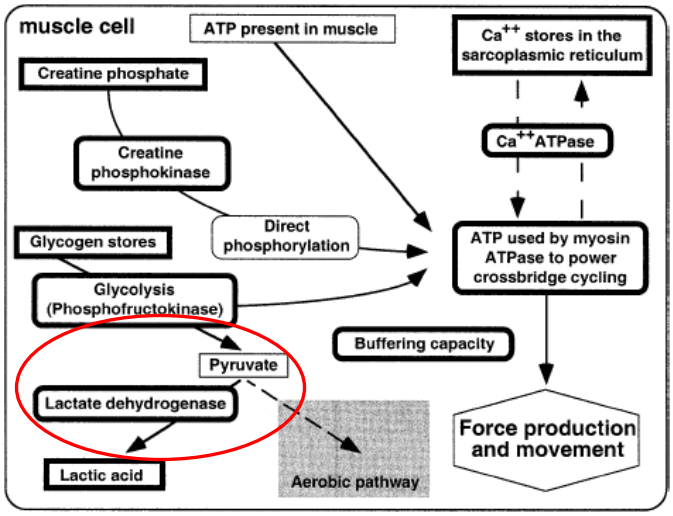

Fig. 6: Schematic representation of anaerobic production of ATP in muscular tissue and relative fuel stores. The heavy lining surrounding a variable indicates it as potential candidate for anaerobic performance. The red circle highlights the biochemical reactions triggered in vitro for PK activity essay purposes (Gibb and Dickson, 2002)

The activity of key enzymes involved in energy metabolism such as CS and PK is affected by endogenous factors like age (Marsch et al, 1999) and endogenous factors such as temperature (Vetter and Buchholz, 1997). Scaling studies have also shown that many actively swimming teleosts tend to increase their anaerobic potential with their body mass (Childress and Somero, 1990). This could be explained as a potential mechanism for maintaining a constant size-independent maximum burst speed. Larger animals, in fact, require proportionally more muscle power than smaller animals, as their mass increases faster than the cross sectional area of the muscle involved in locomotion (Wu, 1977).

Cape Horse Mackerel (*Trachurus trachurus capensis*)

The Cape horse mackerel (Fig. 7) (*Trachurus trachurus capensis*) is key component in the ecology of the Benguela system (Crawford et al., 1987), occupying a middle position in the food chain. Species of high commercial importance such as hake mostly fulfill their dietary needs by feeding upon Cape horse mackerel (Pillar and Wilkinson, 1995) and other higher predators such as Cape fur seals, tunas and most large pelagic species rely on this source of food (Barange et al., 2005; David, 1987). Studies on *Trachurus t. capensis* revealed the main diet consisting primarily of euphausids (krill), crustaceans and to a lesser extent fish. The diet varies according to the size of the specimen, being copepods the main food item during the early developmental stages (Jardas et al., 2004). Cape horse mackerel is assumed to be widely distributed throughout the Benguela system, although it is not clear whether environmental barriers such as the strong upwelling cell off Lüderitz prevent the migration of stocks from the Southern to the Northern Benguela and vice versa. The Northern limit for this species is roughly delimited by the intrusion of warmer water at the Angolan front, where the presence of Cape horse mackerel becomes scarce and discontinued. Larger individuals tend to move towards deeper water, whereas eggs and juveniles up to 14cm occupy pelagic waters at depths ranging from 20 to 100m. Adult specimens are known to undertake diel vertical migrations from mid water to the sea bed down to 200m. This migratory behavior seems to be absent or less evident in juveniles, especially when located in shallower waters (Barange et al., 1998). Horse mackerel in the Northern Benguela reproduce throughout the year, with a spawning peak in summer autumn (December – June) distributed between Walvis Bay and Cape Frio. Reading of otoliths from 1996 to 2004 surveys indicated the growth of *Trachurus t. capensis* to be relatively slow, with an achieved length of 4-6 cm during the first year and increments of 2-3 cm/year after the third year. The max life span has been documented by means of otolith reading to be 15 years, with a maximum length of 55cm.

Fig 7: Trachurus trachurus capensis. http://fishbase.de/images/thumbnails/gif/tn_Trcap_u1.gif

Aims

In this study the first aim is to provide information on the routine metabolic rate of juvenile Cape horse mackerel collected in the Benguela upwelling system, as such topic has not been approached yet.

Seeking the PO_2cr for this species is of outmost ecological significance, considering the random anoxic events that characterize the collection site. Measurements of key enzyme activity for aerobic and anaerobic metabolism such as CS and PK will further enrich our knowledge about the physiological adaptations of this species in extreme conditions. The outcome might reveal or exclude the existence of ecotypes among Cape horse mackerel populations in future comparative studies. In addition monitoring the behavioral responses of the specimens throughout the experiment will supply valuable information on the survival strategy of this species.

Questions:

1) What is the RMR rate of *Trachurus trachurus capensis* juveniles in the Northern Benguela system?

2) What are the effects of decreased PO_2 on RMR?

3) What is the PO_2cr?

4) What are the physiological and behavioral responses and/or adaptation of fish below the PO_2cr?

5) Is there a relation between ambient PO_2 and *T.trachurus capensis* distribution?

Hypothesis: the distribution of fish larvae/juveniles from the Benguela region is not affected by oxygen-depleted layers in upwelling areas off the coast of Namibia.

Material and methods

Sampling Site

Collection of live samples of *Trachurus trachurus capensis* took place on board the research vessel Maria S. Merian (RV Merian) along the Namibian- Angolan coast from 23°S to 15°S. This study comprised two expeditions (MSM07/02b – MSM07/03) from 09/03/08 to 19/03/08 and from 22/03/08 to 17/03/08, respectively. During the leg MSM07/02b RV Merian reached 21 stations, of which 3 transects of 5 stations each were

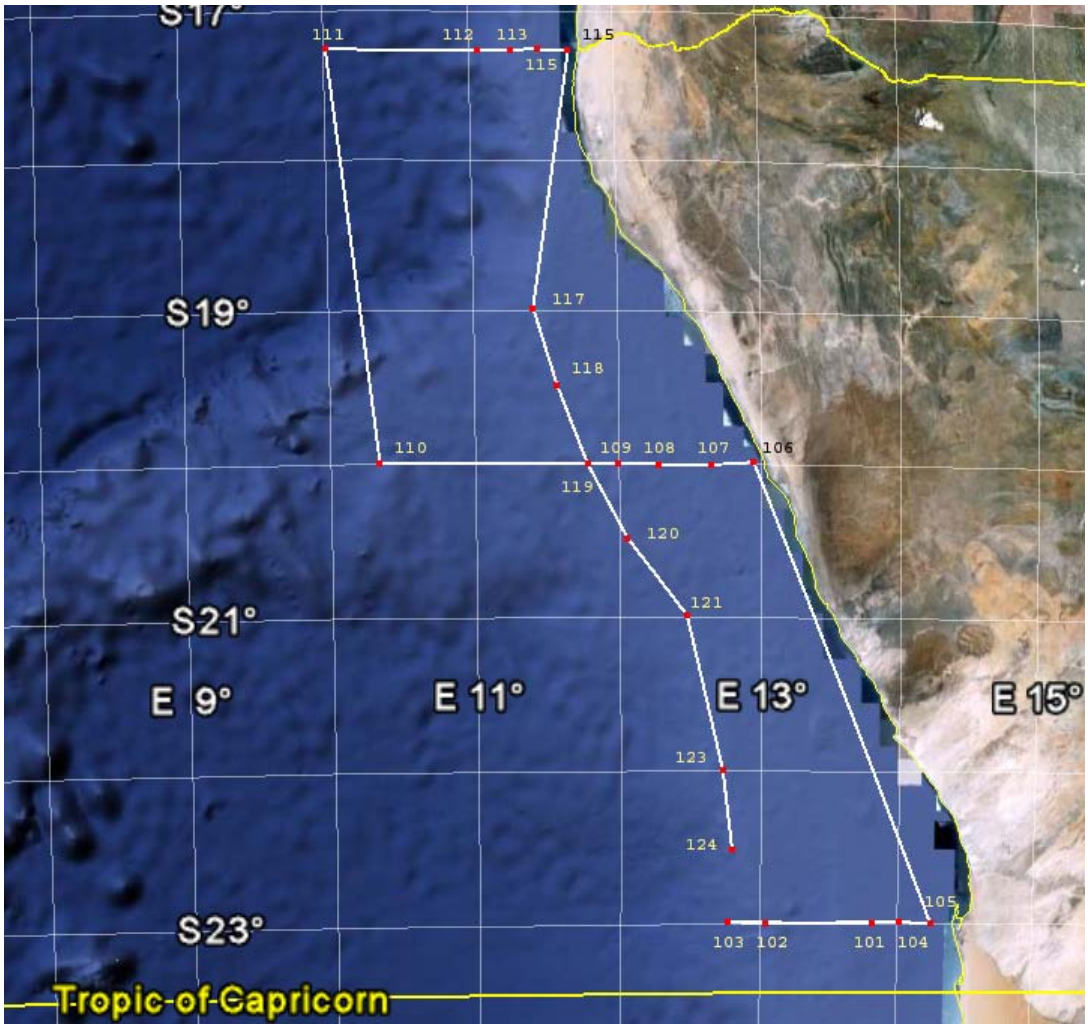

Fig 8: *Geographical position of the stations during leg MSM07/02b. The three transects of 5 stations each are visible at 23°S, 21°S and 17,5°S. Stations 117 to 124 follow the isobath at 450m.*

distributed perpendicularly to the coast at 17°S, 20°S and 23°S (Fig. 8), the remaining 6 stations ran along the coast following the isobathic line at 450m.

The leg MSM07/03 extended the sampling northern limit to 15°S, off the Angolan coast at Namibe harbor (Fig. 9). The longer duration of this leg allowed for a total of 41 stations, two of which enclosed a 24 hour sampling time (stations 228 and 236) for the recording of vertical migration of target species.

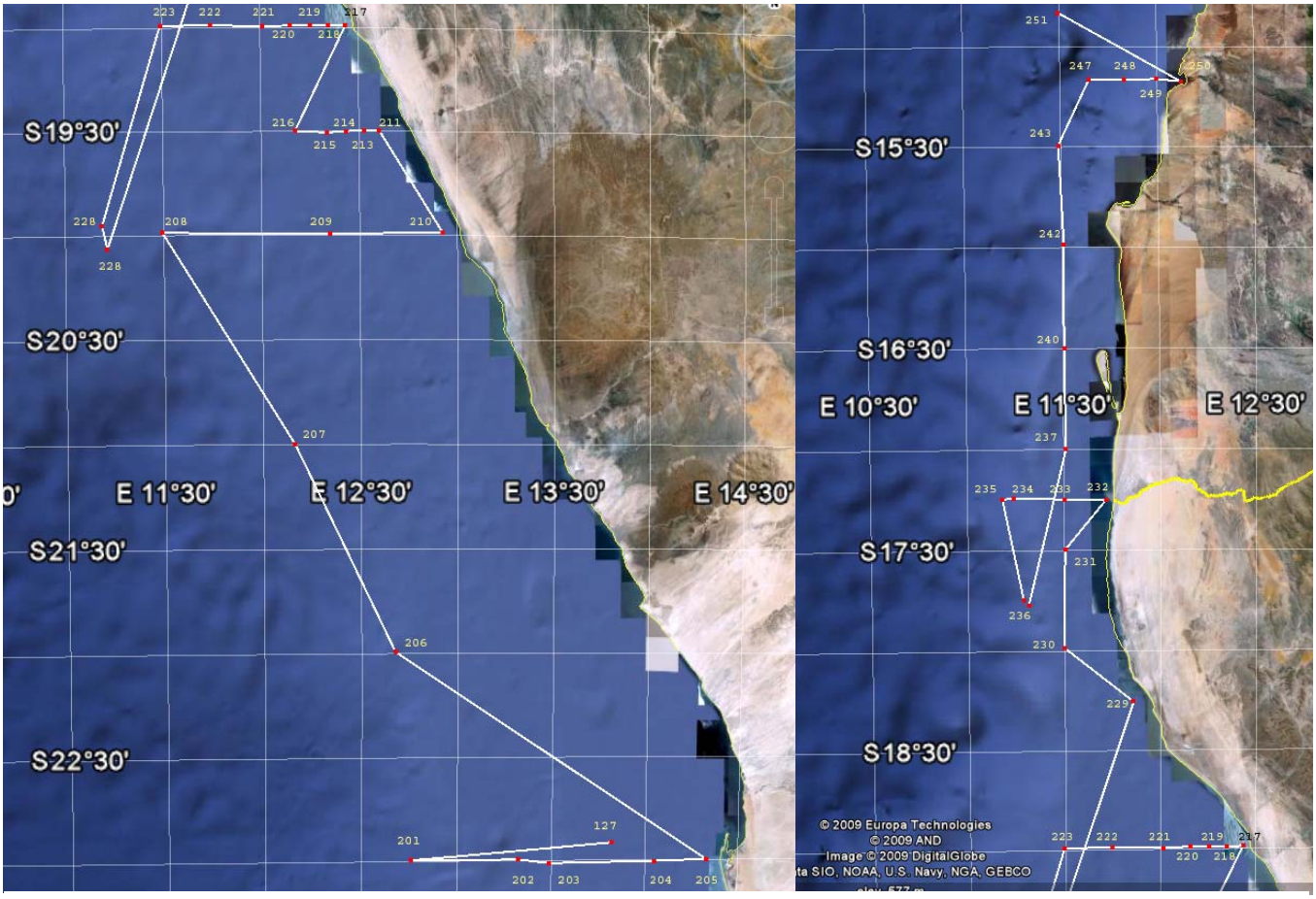

Fig 9: Geographical position of the stations during leg MS07/03. The route has been divided into two sections for fitting purposes. The left map refers to the first part of the expedition, in which station 228 represents the southern 24 hour sampling site. On the right side the remaining stations towards the harbor of Namibe. The second 24 hour station is marked by the number 236.

In order to determine the essential physical properties of sea water, at each station a probe measuring conductivity, temperature, depth and oxygen (CTDO) was deployed. This supplied a detailed vertical profiling of the above mentioned parameters, including the position and extension of the oxygen minimum zone (OMZ).

Catching Methods

The catching gear available on board RV Merian included vertical and oblique multinet, ring trawl and Multiple Opening and Closing Net with Environmental Sensing System (MOCNESS) (Fig.10). The mesh sizes ranged from 2000µm to 330µm.

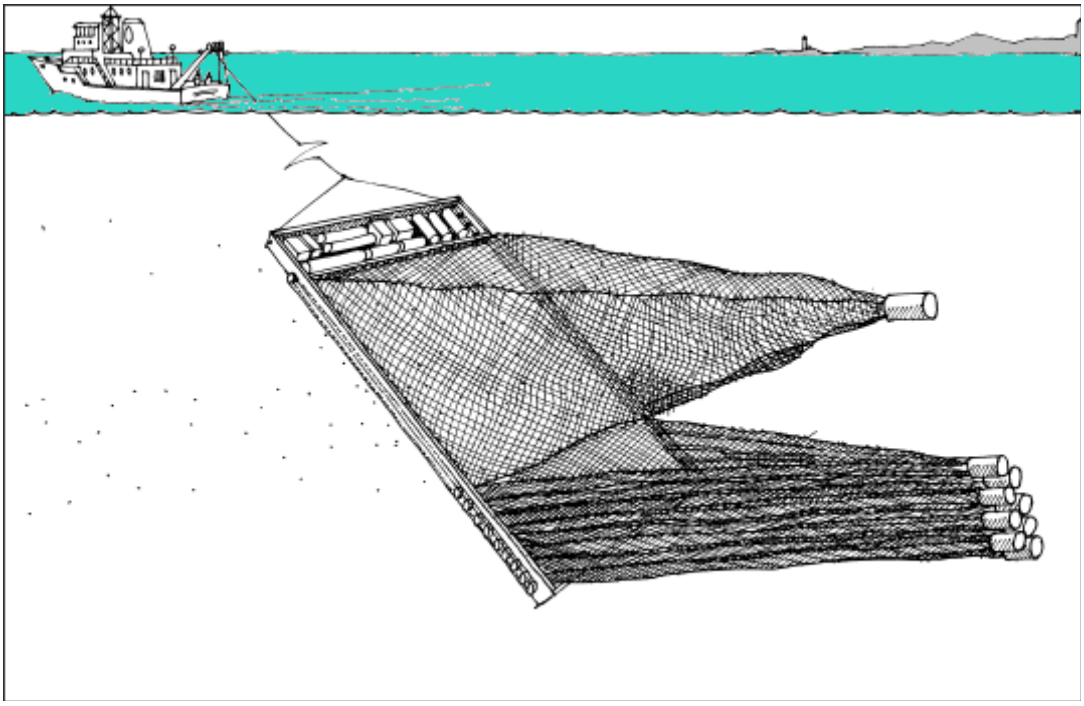

Fig 10:Drawing of deployed MOCNESS, note the numerous nets that can be set for opening and closing at given depths. *www.gma.org/onlocation/globcimg/mocness.gif*

Upon completion of the CTDO profiling, MOCNESS of either $10m^2$ or $2m^2$ were towed to investigate biodiversity. The length of these runs, up to several hours in the deepest areas, the pressure induced upon the fish by towing speed of 2 to 3 knots and the decompression sickness caused by quick hauling up, excluded the possibility to recover any live sample from the nets. However, due to the intrinsic feature of the MOCNESS, a quick examination of the contents allowed the planning of further shorter casts, aimed to the recovery of live specimens for physiological purposes. Juveniles of *T. trachurus* and other teleosts suffered from the presence of gelatinous zooplankton such as jellyfish, which tended to aggregate especially in the shallow layers. Jellyfish nematocyst would generally incapacitate and kill most of the catch once they entered the net. To minimize the risk of loosing valuable trawls, the MOCNESS was preferred to the Ring Trawl for its

ability of harvesting at chosen depths; in this case selectively avoid the “jellyfish zone”. The towing time was shortened to 10-15 minutes and the trawling speed reduced to 1.5 knots.

Timing was the key to ensure the survival of fish recovered from the nets. To speed up and facilitate on-deck handling and management of the equipment, the lightest $1m^2$ MOCNESS was used. A sea water pump flushed all organisms entangled in the net downwards the cod end while the MOCNESS was lifted on deck. The sampling container was quickly inspected and, in case of successful catch, the content was transferred into a larger container filled with 50l of sea water. Juveniles of *T. trachurus* were then transported with the greatest care to an aquarium, supplied with a biological filter and a cooling unit to maintain the optimal temperature of 16°C. Specimen that survived to this point would persist in a healthy state throughout the duration of the cruise. Fish were daily fed with live krill or copepod collected with vertical net tows and maintained in a healthy state throughout the duration of the cruise. Specimens were sorted by their size and allocated into separate compartments of the aquarium to avoid cannibalism. Three size classes were defined: class I had total length <30mm, class II comprised 30mm<fish<50mm and class III grouping all specimen >50mm. Fish were left undisturbed a minimum of 24 hours in the aquarium for acclimation. Interest in food items and quick association of human presence to food supply revealed the full recovery from the traumatic event of capture, allowing for the beginning of experiments in the respiratory chambers.

The number of *T. trachurus* recovered from the net tows was recorded for each station, including parameters such as depth, time and catching method.

Intermittent Flow Respiratory System

The equipment used in this study to assess both the RMR and the PO_2cr is a customized intermittent flow respiratory system, featuring selectable temperature, water-flow velocity and chamber volume. It consists of a semi-closed network of gas proof Tygon tubes (16mm), which links a Plexiglass chamber to an Ismatec pulseless-pump and a three-way valve (Fig. 11). Adaptors allow for the installation of temperature and oxygen probes, which are cabled to home-designed hardware for data collection prior the final elaboration through the software (DTMF 4.0). The oxygen probe was a standard portable oxy-meter, whose measuring principle is based on conductivity variations in a compartment separated by a semi-permeable membrane (WTW Oxi-340i). DTMF 4.0 was designed to work on a Microsoft platform, thus making the system compatible to most portable PC´s. The software displays both on-time and history overview data of oxygen and temperature levels, with the possibility of varying the sampling rate. In this study the number of variables between experiments was minimized to facilitate comparison of results, hence the sampling rate was maintained constant at 1/10 Hz. Additionally the software calculated the average oxygen concentration every six readings and recorded this value in the database. The narrow ranging in size of the specimens did not require any volumetric adjustment of the respiratory chamber. Therefore a round 232ml Plexiglass chamber was used for all experiments, which allowed sufficient room for the fish to position itself in the preferred angle against the water flow; this resulted in a fixed pump delivery rate too.

The strength of DTMF 4.0 relies on the possibility to remotely control a three-way valve that ensures the periodical opening and closing of the system whenever certain PO_2 is reached. This can be done by dialing both the upper and lower PO_2 limits. Once the lower limit is reached, the software automatically triggers the opening of the three-way valve, allowing oxygen saturated water to replenish the system. Upon reaching the upper oxygen limit, the valve is switched back to its initial position, thus closing the system and starting a new cycle. The system has two main advantages over manually controlled devices: it guarantees the survival of the specimen, preventing oxygen depletion in case of user's distraction, allowing the running of long experiments without constant supervision, and maintains a precise repetitiveness of the experiments.

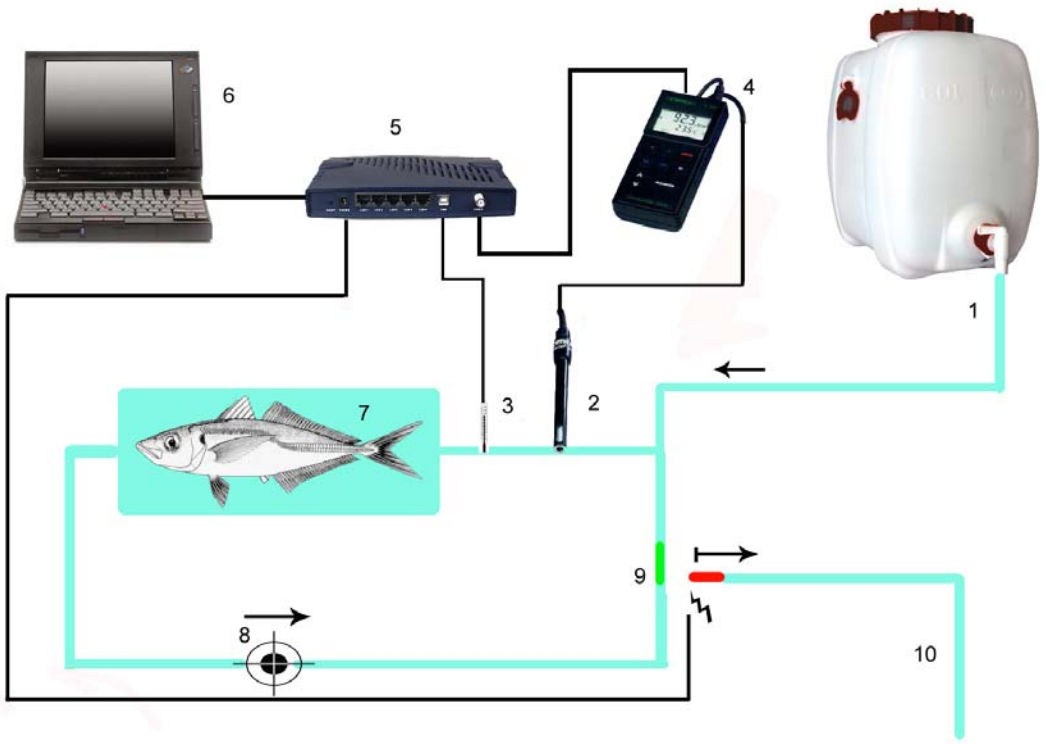

Fig 11: Respiratory system schematic representation. 1-Oxygenated water tank reservoir. 2-Oxygen probe. 3-Temperature probe. 4-Oxymeter. 5-Data collecting hardware. 6-Data processing software. 7-Respiratory chamber. 8- Ismatec pulseless pump. 9-Three way valve. 10- Output.

A 5l tank supplied the reservoir of oxygen saturated water. Sea water was previously pumped and mechanically sterilized through an Acropak 1000 (0.2µm) filter to minimize the oxygen uptake by bacterial respiration. The water reservoir was kept oxygen saturated by means of a standard aquarium aerator connected to a stone. A cooling unit was connected to two aluminum coils to provide a constant temperature of 16°C to both reservoir tank and respiratory system. To avoid temperature variations the whole system was installed into a black plastic container and submerged into cooled sea water. In addition a dark cover was placed onto the container to ensure the minimum optical disturbance to the fish. Prior to the sealing of the system, extreme care was taken to avoid the trapping of any air bubbles that would otherwise compromise the accuracy of the oxygen probe, which was calibrated prior the start of each experiment. The fish was left acclimatizing during the first 6 hours, for this purpose the lower oxygen limit was set at

80-70%. Once the oxygen consumption (VO_2 mg l^{-1} h^{-1}) stabilized, the lower oxygen limit was gradually lowered to seek the critical partial pressure of oxygen. The upper oxygen limit was kept at 90%, to investigate the fish behavior under sharp PO_2 variations and eventually to relieve the fish from hypoxia-induced stress. To secure reliable data, the down scaling of the PO_2 lower limit occurred every three cycles at 10% steps. Upon completion of the experiment, the system was kept opened for about one hour to allow a gradual recovery of the specimen, thus minimizing further stress from the consequent reallocation to the aquarium. In consideration of the fast metabolism of an organism during the developmental stage, the fish endured experiments for a maximum of 24-30 hours to avoid starvation, which could cause unwanted variation in the RMR.

Despite the use of sterilized sea water, the proliferation of bacteria into the system required the run of blank cycles, to exclude the bacterial respiration from the actual fish VO_2. Constant renewal of water and thorough cleaning with 70% ethanol of every component prevented both a bacterial film build up on the inner surfaces and the loss of accuracy of the probes.

Respirometer Data Processing

The software DTMF 4.0 collects the data in a .txt format file, sorting it in three columns each showing time, mean temperature and oxygen saturation [%]. The sampling interval is 1/10Hz, the data recorded every minute results from the mean of six measurements. The time is displayed in fractions of 1 day; hence the interval (t_x) of one minute equals to 0.00069 days. Time scale in minutes (t_x*1440) was chosen as standard for data processing. Temperature was recorded in Celsius degrees and did not require any conversion. A thermometer integrated to the oxygen-meter was monitored at regular intervals to double-check the readings with the separate temperature probe. Oxygen content in water was recorded as PO_2 [%], automatically calculated by the instrument for the local temperature and salinity. The WTW oxymeter was zeroed prior the start of each experiment. The .txt file was imported into a standard .xls format sheet, where cleaning of the raw data set took place. Instrumental errors were removed whenever negative PO_2 decreases were recorded ($\Delta PO_2<0$), so were the 5 minutes that followed the beginning of a new cycle, time required for an even diffusion of oxygen throughout the system. A graph was constructed by plotting the time of experiment on the "x" axis (expressed in minutes) against ΔPO_2 (Fig. 12).

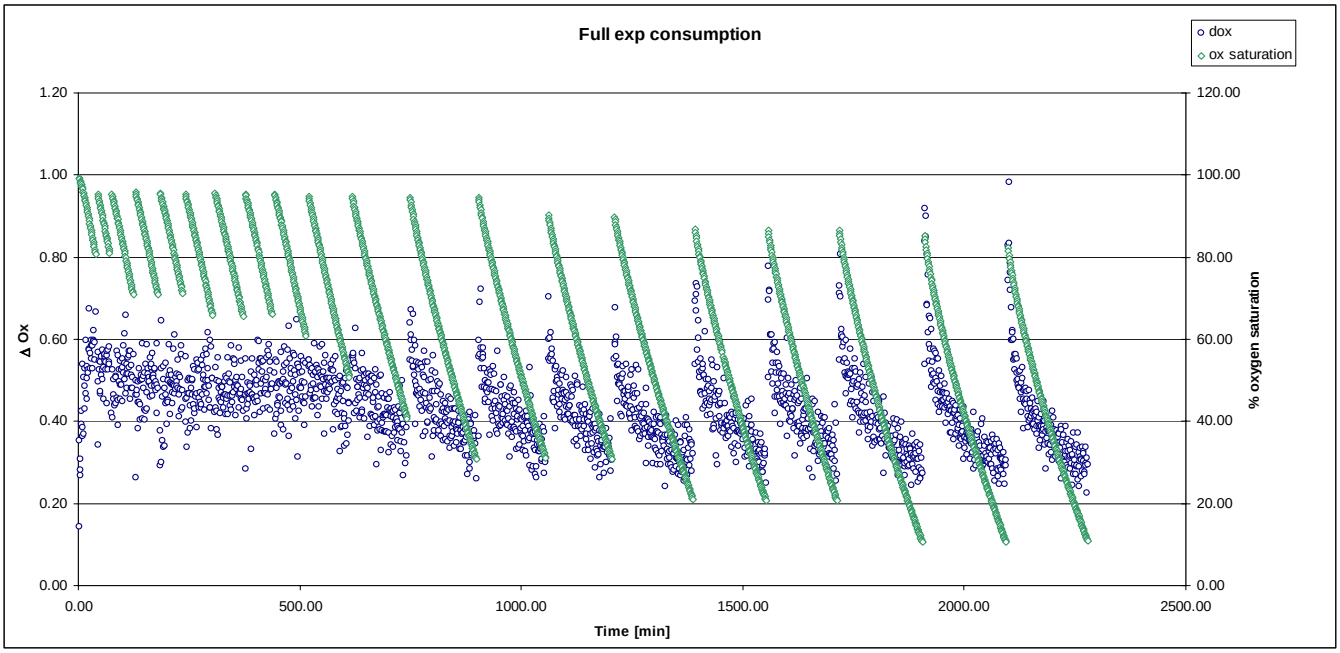

Fig. 12: Graphical representation of a typical respiratory experiment in an intermittent flow system. On the "x" axis time is shown in minutes, on the primary "y" axis the O_2 consumption is referred to as ΔPo_2 and visible in the graph as blue dots. The secondary "y" axis plots the PO_2 [%] and clearly indicates the cycle profile.

Oxygen Critical Partial Pressure

The PO_2cr was sought by monitoring the fish variations in oxygen uptake at time intervals of one minute (Fig. 12). By gradually lowering the lower PO_2 limit, the hypothetical PO_2cr is approached. Once this value is reached, VO_2 should decrease along with PO_2. This might not be clearly visible when PO_2cr has just been crossed. However by reducing the amplitude of the PO_2 downscaling, it was possible to determine the interval at which PO_2cr is reached. Additionally another valuable index that underlined the crossing of the PO_2cr came from the increased VO_2, once oxygen saturated water is restored in the system. Indeed, the oxygen debt caused by anaerobic metabolites built up, such as lactate, results in a drastic increase in VO_2 whenever the conditions allow for higher uptake. This phenomenon manifests itself in the graph as a sharp widening of the ΔPO_2 window and becomes more evident by either increasing the exposure beyond the PO_2cr level or by decreasing the lower PO_2 limit or both (Fig. 12).

The procedure involved repetitive cycles and gradual lowering of the PO_2 limit down to 10%, limit beyond which it was chosen not to proceed to avoid valuable specimen loss. The fish were returned to the aquarium, fed and allowed to recover for further analysis.

Routine Metabolic Rate (RMR)

The volume of water in the system (Vr), the weight of the specimen (W) and the variation of PO_2 (ΔPO_2) during the time period Δt permit the calculation of the RMR according to the formula:

$$RMR = Vr * \Delta PO_2 / \Delta t * W$$

The volume of water minus the volume occupied by the fish is reported in liters (l), oxygen content in water is converted into $[O_2]$ mg/l, Δt was expressed in hours (h) and W in kilos (kg). RMR is therefore defined as mg $[O_2]$ kg^{-1} h^{-1}.

Vr was measured by directly empting the system into a volumetric column. Calculation of ΔPO_2 was carried out by taking the average consumption prior reaching 60% oxygen saturation, beyond which a variation in VO_2 occurred, and converting it into O_2 [mg l^{-1} h^{-1}].

The weight specific oxygen consumption (RMR) and the absolute consumption of each individual (VO_2) were plotted against the body mass of the tested subject and statistically analyzed by Pearson´s test. A trend line was developed according to the general allometric equation $Y= aW^b$, being "W" the body mass and "b" the scaling coefficient.

Survival and Behavioral Responses Below PO_2cr

Eight fish of different size were chosen and separately brought to PO_2 of 20% (experiments 1 to 8) and left in those conditions for an amount of time ranging from 0 to 3 hours. The remaining 7 (experiment 9 to 15) fish underwent a similar experiment, where the lower PO_2 limit set at 10%. Varying the exposure time allowed the monitoring of behavioral changes under hypoxic conditions and the estimation of the survival ability. The number of gill beats per minute was also recorded throughout these experiments.

Keeping the ambient oxygen saturation within a narrow window range involved the manual override of the automated system. Brief openings of the valve restricted the increase of PO_2 up to 8%.

Enzymatic Essay

The tests planned for the calculation of the RMR had a three-fold objective: measuring the RMR, key enzymes activity of aerobic and anaerobic metabolism, and endurance in anoxic conditions, such as those encountered in OMZs.

All 15 live specimens were tested at PO_2 above the PO_2cr for measuring RMR, subsequently brought well below the PO_2cr to discover their ability to survive OMZs and finally frozen to perform enzymatic assays in lab facilities.

The preparation of raw tissue for measuring activity of CS and PK followed a common protocol. The fish were kept in a frozen state, with the aid of liquid nitrogen, throughout the dissecting procedures aimed to recover portions of plain muscular tissue, which was later on reduced into powder by means of a ceramic mortar and pester. The resulting powder was then weighed and collected into a 2ml tube. To avoid premature thawing of the tissue, an aliquot of extraction buffer had been previously frozen in the 2ml tube. The extraction was carried out on ice cold buffer of 75mM Tris/HCl (pH 7.6) and 1mM EDTA, with a ratio buffer/tissue 9:1. The tissue was then lysed in an ultrasonic bath at 0°C for 2 minutes. Cellular debris was then precipitated by centrifugation (20 min at 18000g). The supernatant was recovered, stored on ice and used within the next 3 hours for both CS and PK essays.

The enzymatic activity was measured in two separate spectrophotometers supplied with a cooling unit to maintain a constant temperature. This equipment allowed processing up to 12 samples at once, greatly reducing the risk of enzymatic degradation. Data sets of fresh body weight and enzymatic activity followed Person´s test for correlation validation.

Citrate Synthase (CS)

The optical density of a liquid (OD) is related to the absorbance (A) and by the optical path length (l) and is defined by the following equation:

$$OD = A / l = \varepsilon B * cB$$

The absorbance is a dimensionless number, the path length is fixed by the dimension of the spectophotometric cuvette. The molar extinction coefficient of the absorbing substance (ε B= 13.61 l $mmol^{-1}$ cm^{-1} for DTNB at 412nm) is specific for the compound studied at that particular wavelength. The absorbance (cB) increases with molar concentration of the substance. The rate of increase of the absorbance is the slope defined by dA/dt, which is in this case proportional to the enzyme activity.

Citrate synthase activity essay followed the protocol from Sidell et al. (1987). Photometry is applied to measure the enzymatic activity by adding thionitrobeinzioc acid (DTNB)

and triggering an irreversible reaction, whose product causes an increase in absorption at 412nm. The following reactions refer to the normal catalysis by CS of acetyl CoA-SH (1) and the same complex in presence of DTNB (2).

(1) $$\text{Acetyl-CoA + oxalacetate + } H_2O \rightarrow \text{citrate + CoA-SH}$$

(2) $$\text{Acetyl-CoA + oxalacetate + DTNB} \rightarrow \text{citrate + CoA-SH-DTNB}$$

The production of the complex Acetyl-CoA-SH-DTNB causes a linear increase of absorbance, proportional to the enzymatic activity. For comparability of data sets it is essential to maintain a constant temperature, which greatly influences the reaction speed. A constant temperature of 15°C was maintained throughout the measurements.
The following reagents can be stored at the specified temperature and reused up to a month:

100 mM Tris-HCl buffer pH 8.0: 1,58g/100ml, adjust to pH, store at 4°C,
20 mM acetyl-CoA: 0.025g/1.5ml, make aliquots and store at−20° C.

The remaining reagents cannot be used again once defrosted. It is advisable to prepare a stock solution to cover all experimental needs and distribute it in daily aliquots as even the slightest variation in reagents concentration is likely to increase the standard variation among replicates.

0.5 mM oxalacetate: 0.065g/25ml, single use, store at -20°C
0.25 mM DTNB: 0.052g/25ml, single use, add KOH to dissolve, store at -20°C

All solutions were mixed ice cold and allowed to reach the target temperature (15°C) in the cuvette rack, connected to a cooling unit. In 1ml plastic cuvettes 750µl 100mM Tris/HCl (pH 8.0), 50µl 5mM DTNB, 20µl 20mM Acetyl-CoA, 160µl H_2O + sample were added. The proper amount of sample was determined by a series of trials at different

concentrations until a linear response in absorbance was observed. The optimal volume was found to be 5µl of sample in 155µl H_2O. The spectrophotometer was zeroed using a random sample without the final reagent and engaged for one minute, prior the start of the catalysis, to test the ground absorbance of the remaining samples. The reaction was triggered by adding 20µl of 20mM Oxalacetate. Plastic sticks were used to mix all components into a homogenous solution. The reading time was set at 15 minutes, with a sampling rate of 15 seconds.

The rate of increased absorption (r_A) was automatically extrapolated by the software; the user's role is to manually exclude non linear variations from the slope.

The specific enzyme activity (v) is then defined as international units, IU (µM/min), by the following equation:

$$v = \frac{r_A}{l \cdot \varepsilon_B \cdot \nu_B} \cdot \frac{V_{cuvette}}{V_{sample} \cdot \rho}$$

- rA: (dA/dt) rate of absorbance change [min-1].
- l: optical path length (= 1 cm).
- εB: extinction coefficient of B (DTNB) at 412 nm and pH 8.1 (= 13.6 $mM^{-1}*cm^{-1}$).
- νB: stechiometric number of B (DTNB) in the reaction (= 1).
- $V_{cuvette}$: volume of solution in the cuvette (= 1000 µl).
- V_{sample} volume of samle added to cuvette.
- ρ: mass concentration or density of biological material in the sample (=0.8 for most fish)

One unit (U) of CS is defined as the amount of enzyme required to produce 1µM of citrate in one minute. The results were expressed as U/g of fresh weight. Two reactions were tested for each extraction. The concentration of the sample was deliberately doubled

in the second replicate, expecting an equal increase in enzymatic activity. A third replicate was considered in case high standard deviation was encountered. This mostly occurred as a result of a too high concentration of sample in the reaction, which interfered with the absorbance.

Pyruvate Kinase (PK)

Pyruvate kinase (PK) dephosphorylates phosphoenolpyruvate (PEP) into pyruvate using adenosindiphosphate (ADP) as the phosphate group acceptor, according to the reaction:

$$PEP + ADP \xrightarrow{\text{Pyruvate kinase}} Pyruvate + ATP$$

The pyruvate formed from PEP by PK is measured by the formation of NAD^+ in presence of lactate dehydrogenase (LDH):

$$Pyruvate + NADH \xrightarrow{\text{Lactic Dehydrogenase}} Lactate + NAD^+$$

NADH (extinction coefficient of 6.31 $mM^{-1} \cdot cm^{-1}$) is detected by the spectrophotometer at a wavelength of 340nm. The PK activity is proportional to the decrease in absorbance recorded once the reaction is triggered.

The sample preparation followed the same protocol used for CS extraction; both PK and CS essays occurred simultaneously in two separate spectrophotometers at the temperature of 15°C.

The reagents were added ice cold in the following order and consist of 500µl of 100mM Imidazol (pH 6.9), 200µl of 750mM KCl, 20µl of 500mM $MgSO_4$ x 7 H_2O, 20µl of 7.5mM NADH, 20µl of 50mM KNC, 50µl of 100mM ADP, 130µl of H_2O + sample and 5µl of LDH (550 U/mg). The reaction was triggered by 50µl of 50mM PEP.

100 mM Imidazole, adjust to pH 7.2 with HCl: 0.681g/100ml

100 mM Adenosine 5'-Diphosphate Solution (ADP): 0.251g/5ml

1000 mM Magnesium Sulfate Heptahydrate ($MgSO_4$): 1.23g/10ml

750 mM Potassium Chloride Solution (KCl): 2.8g/50ml

50 mM Phospho(enol)pyruvate Solution (PEP): 0.052g/5ml

7.5 mM b-Nicotinamide Adenine Dinucleotide (b-NADH): 0.053g/10ml

L-Lactic Dehydrogenase Enzyme Solution (LDH): 10mg/2ml

Reagents such as PEP, NADH and ADP must be prepared in aliquots, stored at -20°C and used fresh each time.

The transition from NADH to NAD^+ occurred within 1´30". The spectrophotometer was programmed to record the absorbance at 340nm, for 5 minutes, with a sampling rate of 15 seconds.

The calculation of the specific PK enzymatic activity followed the same equation used for CS essays. The optimal concentration of sample in the reactions was found to be between 10µl and 20µl. Again two replicates per sample were tested; a third followed in case of high standard deviation.

Results

Sampling

The successful recovery of 18 samples of *T. trachurus capensis* of weight ranging from 0.1g to 4.56g allowed for a total of 21 tests in the respiratory chamber, six of which endured between 18 and 37 consecutive hours for PO_2cr evaluation. The ratio of live samples versus the casualties in the net tows was as low as 1%. The loss of three fish that occurred during the respiratory experiment with new untested equipment rather discouraged the use of the latter. Records of the catches coupled with CTDO profiles allowed the investigation of the effects of low oxygen content on *T.trachurus capensis* distribution in the water column (see Fig. 13). Only 18 stations out of the 41 scheduled on the second leg MSM03/07 produced catches of Cape horse mackerel. Juveniles and larvae were never found either deeper than 200m or anywhere beyond the continental shelf.

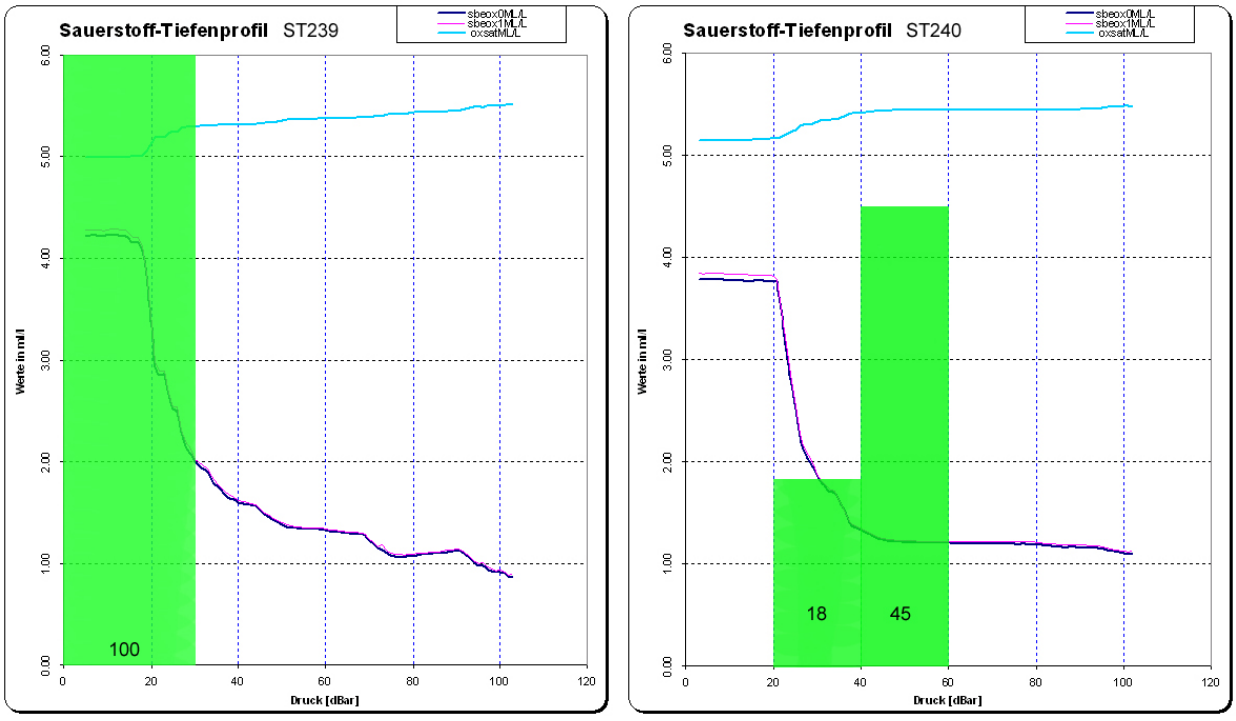

Fig. 13: Vertical profiles of oxygen concentration (ml/l) provided by CTDO at two sampling sites. The green bars represent the abundance of Cape horse mackerel in the water column at a given station (ST239, ST240). The number of individuals is reported on the green bars, while the width of the bar indicates the depth range at which they were caught. The MOCNESS proved itself valuable for detailed faunal surveys at given depth intervals.

The oxygen concentration at which Cape horse mackerel was caught ranged from the upper oxygen rich layers to the underlying OMZ down to 200m (Table 1). More details about the physical properties of water and local time at the sampling stations are available in appendix "A".

Table 1: List of stations with positive T. trachurus capensis catches. For each station the number of individuals, the depth interval and the local oxygen saturation are reported. The net tows were performed throughout the water column, see appendix "A" for the complete CTDO profiles.

Station	**209 (11:42)**	**213 (04:59)**	**214 (08:21)**	**214 (08:21)**	**215 (12:40)**	**216 (15:47)**	**216 (15:47)**	**218 (01:22)**	**218 (01:22)**	**219 (18:59)**
Individuals	10	14	17	4	1	5	3	3	1	6
Depth Range	140-200	40-80	0-80	0-20	18-50	0-35	0-200	20-50	0-20	0-15
PO2 [ml/l]	0.15-0.56	0.74-0.18	4.2-0.5	4.2-4.0	4.4-1.35	4.9-2.7	4.9-0.36	3.4-0.95	5.0-3.4	5.0-4.5

Station	**219 (03:56)**	**219 (03:56)**	**223 (21:43)**	**224**	**225**	**226**	**229 (22:22)**	**230 (03:55)**	**230 (03:55)**	**230 (03:55)**
Individuals	1	10	7	18	141	20	13	1	4	7
Depth Range	15-50	0-105	0-50	0-80	0-80	0-60	0-35	40-70	0-25	0-200
PO2 [ml/l]	4.5-0.78	5.0-0.42	2.9-2.3				4.0-1.19	2.52-0.7	4.4-3.9	4.4-0.54

Station	**231 (10:21)**	**231 (10:21)**	**237 (22:17)**	**238**	**238**	**238**	**238**	**239**	**240 (05:07)**	**240 (05:07)**
Individuals	10	16	30	3	30	2	3	100	18	45
Depth Range	10-25	25-60	25-40	0-25	25-40	40-60	0-95	0-30	20-40	40-60
PO2 [ml/l]	4.2-3.6	4.6-0.86	2.7-1.6	4.2-3.2	3.2-1.5	1.4-1.2	4.4-1.1	4.4-1.8	3.8-1.3	1.3-1.2

Oxygen Critical Partial Pressure

The six experiments carried out to target the PO_2cr involved specimens from 15mm to 70mm of length. Two experiments included the presence of two fish of equal size in the system, to avoid the loss of accuracy due to an inefficient ratio $mass_{fish}/volume_{system}$. The experiments endured from 18 to 37 hours. The experimental approach involved the downscaling of the lower oxygen limit by steps of 10%, whereas the upper oxygen limit remained between 80% and 100% (Fig. 12).

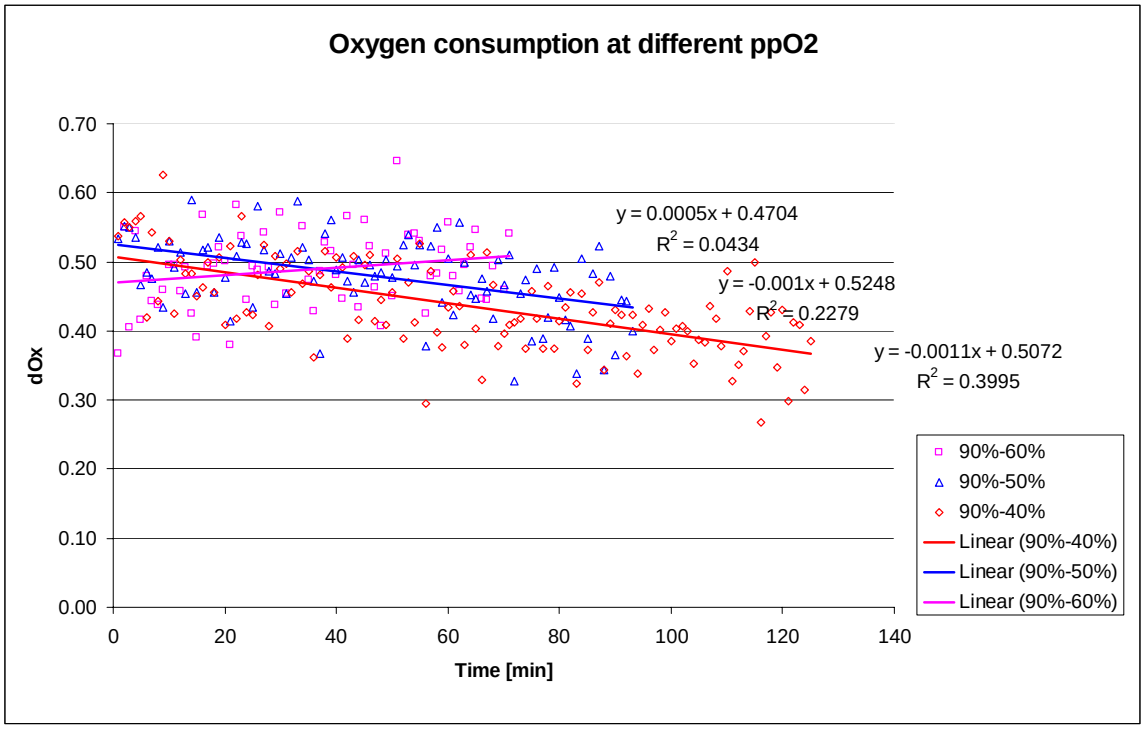

Fig. 14: Detection of the oxygen critical partial pressure by monitoring the linear pattern of oxygen consumption. The oxygen critical partial pressure is assumed to lie before the angular coefficient of the trend lines becomes negative; in this case the interval is 60%-50%.

The PO_2cr could be calculated with maximum accuracy of 10% (0.8mg/l O_2), being that the smallest interval detectable in our tests. The rate of oxygen depletion (ΔO_2) was calculated as variations of PO_2 [%] per minute. This was the primary index to detect the PO_2cr. Our expectations were to observe a gradual decrease of ΔO_2, once a certain PO_2 was crossed, followed by a sharp increase in oxygen demand once the system has been flushed with oxygen saturated water. This response was highlighted by the angular coefficient of a linear trend line describing the ΔO_2 pattern. The angular coefficient became negative approaching 50% PO_2, while it remained positive in the previous cycles down to 60% PO_2 (Fig. 14). Four experiments out of six indicated an oxygen critical partial pressure at PO_2 between 50% and 60%. One had to be considered unreliable, as the bacterial respiration overwhelmed the actual fish respiration, noticeably reducing the accuracy of the measurements. The largest sample (4.56g) did not show any appreciable changes in ΔO_2 until PO_2 reached 20%.

Routine Metabolic Rate

The routine metabolic rate could only be measured during the final phase of the study, as it involved the loss of the fish for weighing purposes and biochemical analysis. The VO_2 of 15 samples was measured before reaching the PO_2cr. Having deduced this value from previous test, we assumed RMR to be found at PO_2 higher than 60%, provided a constant ΔO_2 was observed prior reaching this value. The average bacterial respiration ($VO_{2\text{-bact}}$) was subtracted from the average total consumption ($VO_{2\text{-tot}}$). The resulting value ($VO_{2\text{-fish}}$) was then converted into O_2mg/l according to the conversion tables of O_2 solubility at 16°C, 35 PSU ($VO_{2\text{-tot}}$ * 7.97/100). The RMR is equal to the O_2 consumed in one hour (O_2 mg/ h) divided by the fish weight (kg). The RMR values, the average fish consumption per hour (routine oxygen consumption) and the body mass are reported on table 2.

The remaining 6 long term experiments, despite covering the complete range of PO_2 considered in this study, could not give any contribution in terms of either RMR or

average oxygen consumption, due to the impossibility of obtaining precise scale readings of fish weight under the continuous effects of ocean swell on board the research vessel.

Table 2: List of experiments and corresponding values of weight, RMR and average O_2 consumption.

Sample	Fresh Weight (g)	RMR (mgO2/kg h)	VO2 (mg O2/h)
1	0.74	798	1.23485698
2	0.65	444	0.60361619
3	0.1	1390	0.290879322
4	0.79	554	0.915195189
5	1.52	348	1.10731152
6	0.31	1126	0.730285134
7	0.56	963	1.127806313
8	1.55	458	1.485618631
9	0.6	938	1.177522513
10	1.13	530	1.253385411
11	0.72	854	1.286047284
12	0.79	694	1.147808553
13	2.9	290	1.758259595
14	3.3	310	2.142858894
15	4.56	267	2.547879832

Scaling of Metabolic Rate with Body Mass

Average oxygen consumption (VO_2) obtained (n=15) and body mass (W) were plotted against each other expecting to show an exponential pattern according to the equation $VO_2 = a*W^b$. The scaling equation was calculated by matching a linear regression to the data set, upon logarithmic transformation of both variables. Sample #2 was removed from the data set as outlier.

The allometric equation that most closely matched the data set is the following: $y= 1.2106x^{0.4872}$, featuring a $R^2=0.8819$ (Fig. 15). The correlation was found significant by Pearson´s test (r=0.93).

A similar test followed by plotting the RMR against the body mass. The mass specific respiration rate was expected to show an inverse logarithmic scale, resulting in the equation y= -333.55Ln +658.36, with $R^2 = 0.9154$ (see Fig. 16).

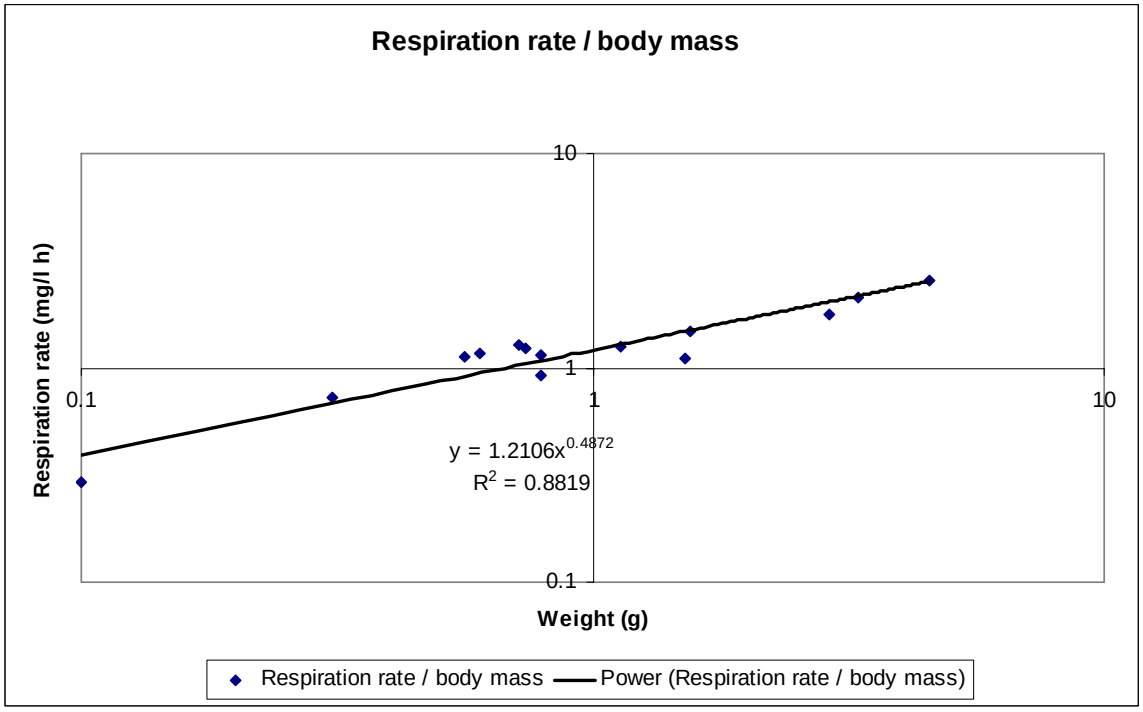

Fig 15: Fitting of exponential equation to the plotting of respiration over body mass. n=14 resulted in R^2=0.8819.

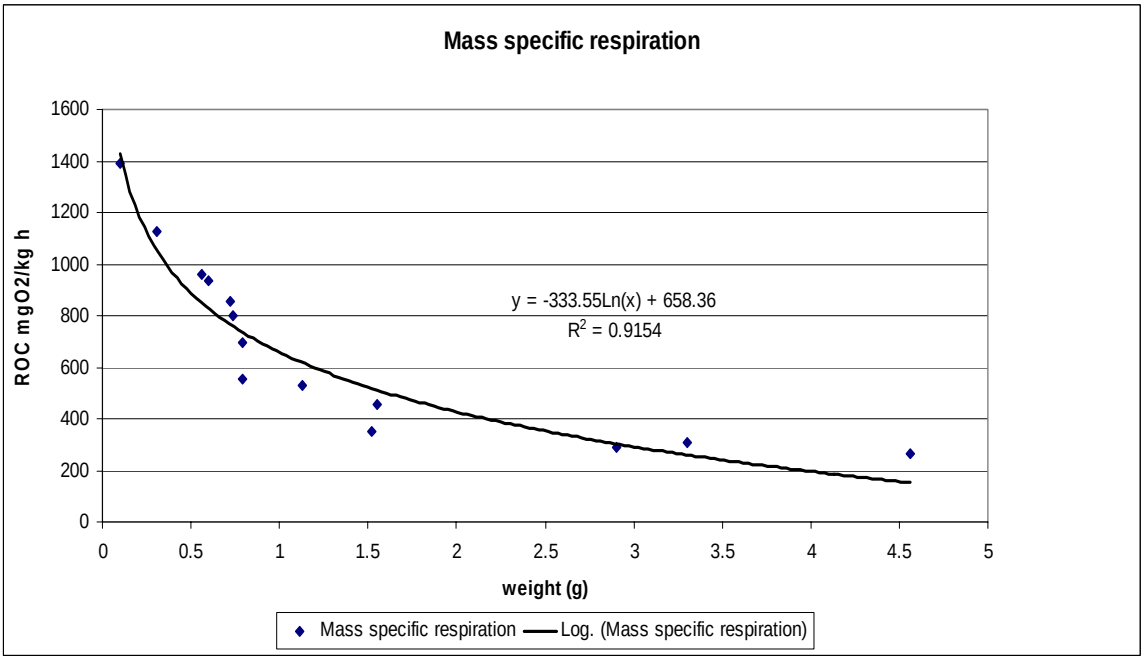

Fig 16: Mass specific respiration rate (RMR / body mass), featuring inverse logarithmic model. The correlation was found significant according to Pearson´s test.

Behavioral Responses

Fish activity was observed from the crossing of the PO_2cr to the end of the experiment. This species responded to progressive hypoxia by increasing the amplitude of gill ventilation down to about 40% PO_2, past which the phenomenon was accompanied by an increase in ventilation frequency (Fig. 17). The fish manifested hyperactivity approaching 20% PO_2 saturation, resulting in relentless changes of position throughout the respiratory chamber. This behavior persisted down to 10% PO_2 saturation, but ceased after 30 minutes. Testing the survival of this species to hypoxia was carried out by gradually prolonging the exposure to 20% and 10% PO_2. Each fish was therefore forced to endure from 30 to 180 minutes in hypoxic conditions. The VO_2 was reduced up to 50%. None of the specimens were lost due to asphyxiation.

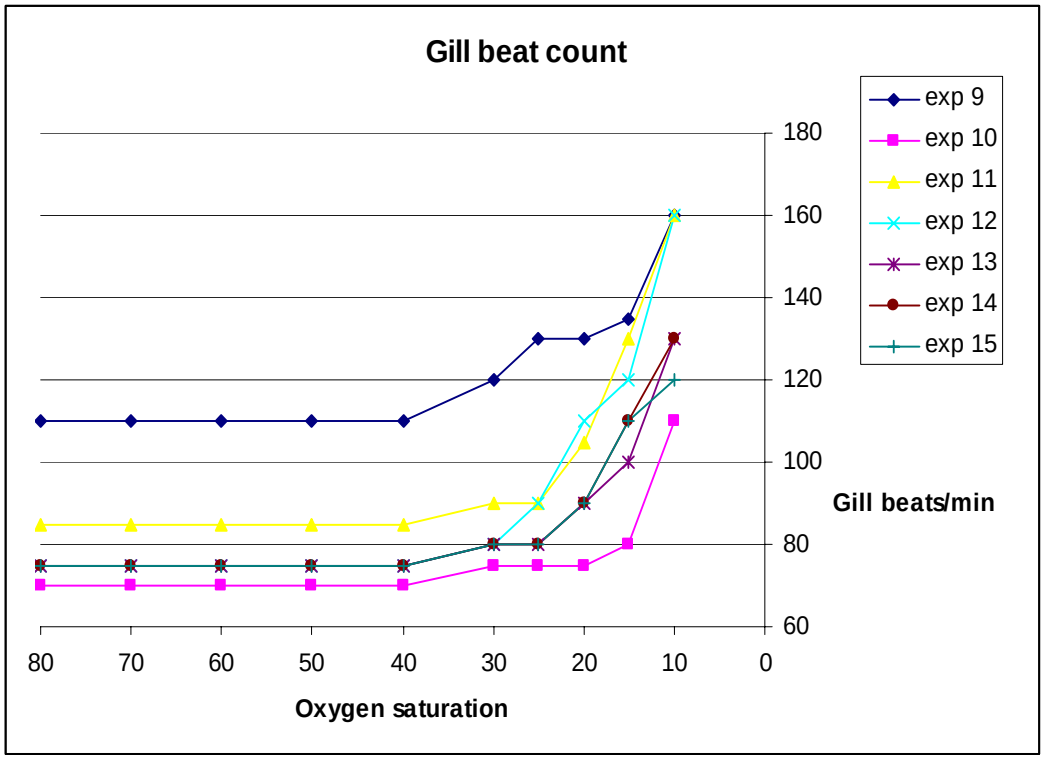

Fig. 17: Scheme of variations in ventilation frequency measured in gill beat per minute at PO_2 ranging from 80% to 10%. Note how specimens reacted by increasing their ventilation frequency past 40% PO_2. This graph refers to the set of data regarding the experiments 9 to 15 only.

The persistence at 10% PO_2 resulted in both a decreased motor activity of the fish and a reduced ventilation rate (Table 3).

Table 3: Changes in ventilation rates during prolonged exposure to 10% PO_2.

Time extension at 10% [min]								
		Gill beat/min						
	0	160	110	160	160	130	130	120
	30		100					
	60			150				
	90				140			
	120					140		
	150						100	
	180							100
Experiment		**9**	**10**	**11**	**12**	**13**	**14**	**15**

Upon opening of the valve and flushing of the system with oxygen rich water, the fish increased its oxygen uptake up to five times (Fig. 11), clearly showing the necessity to process the accumulated metabolites resulted from glycolysis. The VO_2 reached a stable level within 30 minutes.

Enzymatic Activities

Citrate synthase activity was measured in 15 samples, with at least two replicates per sample. In case of widely ranging or suspicious values a third replicate followed. For each sample the average activity was calculated and plotted against the body mass, the two variables were expressed into logarithmic scales to obtain a linear representation of the allometric equation $Y= aM^b$. The scaling coefficient "b" resulted to be positive (approximately 0.14), although the correlation of the two variables was not significant (Pearson test t=0.13). PK activity showed no correlation with body mass either (Fig. 18).

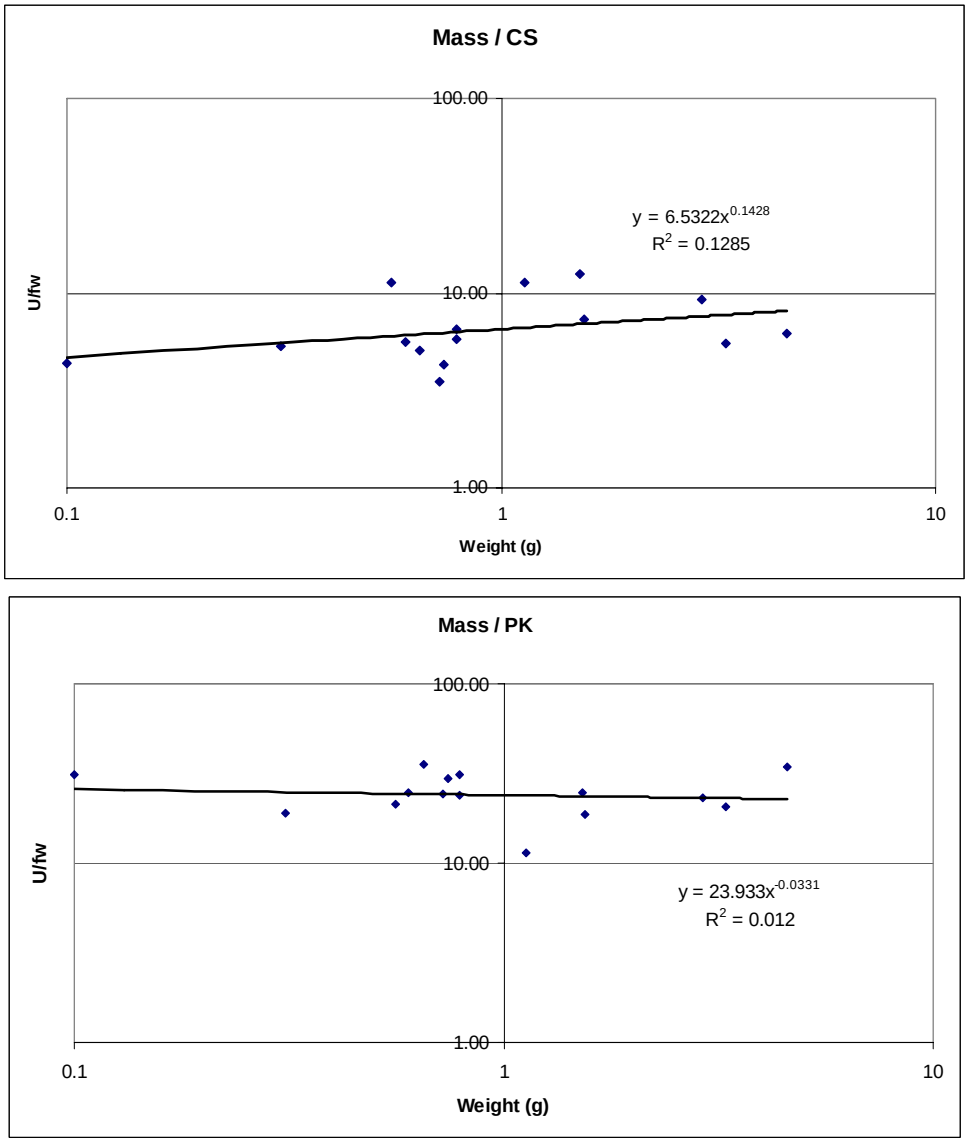

Fig. 18: Patterns of scaling of anaerobic (top) and aerobic (bottom) metabolic indices over the body mass. Both variables have been expressed into logarithmic form.

Discussion

The aim of the present study was to determine the routine metabolic rate (RMR) of Cape horse mackerel (*Trachurus trachurus capensis*) during the developmental stage and analyze both metabolic and behavioral responses in a broad range of ambient oxygen concentration. As shown in several studies, the most versatile tool to achieve this goal is the use of computerized intermittent flow respirometry (Steffens et al., 1994). Since horse mackerel is a pelagic species, which shows frequent spontaneous activity, the possibility of measuring the standard metabolic rate was excluded, as this would have involved too many approximations in a system in which the bacterial oxidation accounted for up to 50% of the total oxygen uptake and standard deviation in the ΔO_2. In addition the constant feeding accounted for an increase in metabolism caused by digestion, which could not be easily excluded. Feces were indeed expelled during the initial 8-10 hours. The volume of the chamber used in this study was less than 50% the volume of the whole system. This caused a significant elongation of the respiratory cycles, limiting the possibility of increasing the number of replicates due to the time restrictions imposed by the fish starvation and overall time availability. The fact that the chamber did not restrict the fish movements it is supposed to be beneficial for a faster acclimation. It is therefore suggested the chamber volume to be maintained and the pipeline system reduced to the minimum. The reduction of the inner surface would limit bacterial proliferation, allowing for more accurate measurements of the actual fish oxygen consumption. Other factors that negatively influenced the data achieved include the limitations imposed by the experiments being carried out on board a research vessel. This involved limited time, specimen availability and lack of equipment for biochemical assessment. Even the use of basic equipment, such a precision scale for fresh weight measurements, had to be ruled out due to the obvious implications of working on a platform affected by continuous variations in gravity.

The recovery of live samples immediately proved to be the most challenging part of this study. In spite of the availability of a large set of catching gear and stations, positioned across both latitudinal and depth range in the Northern Benguela, the development of procedures to minimize the losses by pressure and the lethal contact with nematocyst from jellyfish had a cost in terms of both time and stations close to 30%. The most

successful technique involved the inspection of other net casts, whose primary goal was to access abundance and biodiversity in the area. These trawls seldom produced live specimen, nevertheless they allowed for the meticulous planning of a dedicated short timed casts with a small $1m^2$ MOCNESS device. Despite all precautions, the mortality remained high and the costs of such a large research vessel excluded the chance of a second trial. Another limitation dictated by the tight time schedule on board denied the possibility of persisting on stations where large shoals of Cape horse mackerel were located; stations 225 and 239 produced 141 and 100 specimen respectively.

The investigation of these sites in the diurnal and nocturnal phase could have supplied significant contribution to the hypothesis that distribution of Cape horse mackerel follows other factors other than oxygen concentration. Daily vertical migration of *T. trachurus* has indeed been investigated in the Benguela region, the migrating pattern seems to vary across individual size, season and location (Barange et al, 1998; Barange et al, 2005), but a lot of speculation remains on this subject. Pillar and Barange (1988) and Axelsen (2004) initially proposed the main driving factor of vertical migrations to be a tactic to avoid predation, especially where they constituted the main diet of Cape hake. Later on they could not prove any correlation between vertical migration and either thermocline or feeding regime (Pillar and Barange, 1998; Barange and Pillar, 2005). In this study we hypothesized that the oxygen minimum zones, which occur frequently in the Northern Benguela, could shape the distribution and abundance of Cape horse mackerel. The extent to which the oxygen concentration influences either the vertical migration or the distribution of this species in the area seems to be negligible and not correlated. Comparison of the vertical CTDO profiles and the catches did not show any appreciable correlation or any clear pattern of diel vertical migration between diurnal and nocturnal hours. It might be argued that oxygen concentration still plays a role in shaping the distribution of this species, however it clearly cannot be considered a driving factor on its own. It is clear though that even the youngest juveniles and larvae possess a remarkable ability to survive near anoxic condition for up to three hours. The rate of mortality due to low oxygen availability remains unknown, it must be noted, though, that persisting at a PO_2 between 10% and 20% up to three hours accounted for null losses and, upon availability of oxygen levels above the PO_2cr, the VO_2 stabilized within 30 minutes after

intensive aeration. It might be argued that facing predatory pressure or the event of being surrounded by an extensive oxygen minimum zone, larvae and juvenile of Cape horse mackerel posses sufficient time to eventually persist in these condition for several hours, follow vertical migration of zooplankton and evade the danger of death by asphyxia or predation.

Routine metabolic rate

The metabolic rate of all ectotherms is strongly dependent on temperature and body mass (Peters, 1983). The fact that metabolism on most organisms displays a pattern described by the general allometric equation $Y= aW^b$ has been largely documented in past studies (Peters, 1983, Schmidt and Nielsen, 1984 and Childress and Somero, 1990). This metabolic scaling principle has mainly focused on terrestrial organisms, which group the majority of endotherms, leaving the individual body mass as the dominant variable responsible for metabolic regression. Nevertheless when including ectotherms, deeply affected by exogenous factors such as temperature, the metabolic scaling over the body mass remains faithful to this general trend (Schmidt and Nielsen, 1984). This relationship tends to be even more stable in aquatic organisms, where the high mass of water buffers the rate at which environmental temperature changes, reducing metabolic variations of organisms thriving in it. The measurements of routine metabolic rate of Cape horse mackerel showed a high correlation with body mass ($r = 0.93$). The scaling coefficient ($b= 0.48$) however does not match with the majority of the findings of metabolic scaling for teleosts, which display a scaling coefficient between 0.5 and 0.87 (Childress and Somero, 1990). Hermann and Enders (2000) performed a series of experiments on horse mackerel from the German Wadden Sea using a similar respiratory system. The routine metabolic rate was measured once the fish showed a linear VO_2, which generally resulted in a decreased oxygen consumption after the acclimation phase. Our experiments allowed an initial acclimatization of 6 hours leaving the system opened to continuous recirculation of oxygenated water. Surprisingly the VO_2 actually increased to a stabilized linear consumption during the first 30 minutes. This can be partially explained as the time required for a homogenous distribution of oxygen throughout the system. The results

obtained by Hermann and Enders (2000) indicate the routine metabolic rate of a 4.1 g specimen of *T .trachurus* from the Wadden Sea to be as low as 1.02 mg O_2 h^{-1}. Our findings suggest a standard metabolic rate of a fish of equal size to be about 2.36 mg O_2 h^{-1}, over twice the value found on specimen from the Wadden Sea. It must be pointed out that our tests were carried out at 16°C and not 13°C, this clearly contributed to a higher VO_2. Although temperature and body mass are reported to be the factors that mostly affect metabolism, other exogenous factors such as season and latitude also play a role in metabolic variation. Roberts (1964) found the oxygen consumption of sunfish (*Lepomis gibbosus*) to be unaffected by temperature variations of 7.5°C; it was found instead that the day length deeply affected the metabolism of this species. Nelson et al. (1994) conducted experiments of metabolic rate on different populations of cod, suggesting that even considering the same geographical range, differences in the respiratory physiology are induced by environmental differences.

The analysis of some blank cycles displayed a suspicious unstable VO_2. We can only assume that the condition of the WTW probe or its position was not optimized, some air bubbles could have remained trapped in one of the numerous links, interfering with the measurements. In fact a bypass connected the oxygen probe to the main system, featuring a restriction in the diameter of the tubing; this might have caused a shift in the actual VO_2.

The cumbersome set up of pipelines caused other problems with the heat dissipation through exposed parts of the system. The outer surface exposed to room temperature made the regulation of inner temperature problematic, especially when the difference of internal versus external temperatures becomes high. The use of insulating layers on the exposed surfaces proved to be ineffective. For all above listed reasons, it is suggested to reduce the volume of the tubing and eliminate the bypass that leads to the oxygen probe. Alternatively, the use of optodes in place of the WTW oxygen probe might prove beneficial for the analysis of small specimen, such as some of the smallest specimens used in this study.

Oxygen Critical Partial Pressure

The maximum accuracy with which the PO_2cr could be located was within a window of 0.79 mg/l O_2. The PO_2cr was therefore comprised between 3.9 mg/l and 4.7 mg/l O_2 at 16°C. This accounted for four of the six experiments that were carried out. However this value might be considered relative and not absolute during the developmental stage. The largest sample (W = 4.56g) seemed less affected by the oxygen depletion, as its VO_2 did not show any alteration until a concentration as low as 1.5 mg/l O_2 was reached. Even so, the oxygen debt manifested in a minimal increase in the routine oxygen consumption once the conditions allowed for metabolites oxidation. It is known that sustained swimming and burst swimming rely on different metabolic pathways to provide the ATP necessary to produce muscle contraction. To maintain size independent swimming speed, the larger animals invest in a higher amount of white muscle fiber. This results in a higher anaerobic capacity of larger animals, hence the possibility of more ATP produced by glycolysis. The anaerobic capacity results in a higher lactate production, which in return is accompanied by an increased CS activity in the white muscle. This is explained by the necessity to restore intracellular glycogen stores within white muscle (Dickson, 1995). The outcome in larger animals is likely to be a higher tolerance to anoxia. The role of size in hypoxia tolerance was shown in studies on coral reef fishes. An adult coral reef fish has a mass-specific oxygen consumption as low as 10% compared to what it was when it settled on the reef (Nilson et al, 2007). Arguably, because adults need less oxygen, achieving a lower PO_2cr should be less of a physiological challenge for larger animals rather than smaller ones. This was the case of a hypoxia tolerant cichlid of the Amazon river, in which the PO_2cr decreases from 50% of oxygen saturation to 30% with increasing the body mass (Sloman et al., 2006). On the other side, arguments persist on the fact that the scaling of respiratory factors, such as gill surface area and branching blood vessel, should actually make smaller individuals more hypoxia tolerant. These statements were also documented for other species (Rob and Abrahams, 2003).

Temperature variations are likely to affect the PO_2cr, as the oxygen solubility in water is reduced at higher temperature, while the metabolic rate increases along with environmental temperature in a poikilothermic animal. If the metabolic rate has to be maintained, the fish has to pump a higher volume of water through the gills with obvious

higher metabolic costs. The decreased efficiency of oxygen extraction from water further amplifies this phenomenon. Fernandes and Rantin (1989) found that *Oreochromis niloticus* had oxygen extraction efficiency up to 80% at 20°C, but this value decreased to 50% at 35°C. Temperatures higher than 16°C, the standard chosen in our experiments, are therefore expected to increase the PO_2cr of Cape horse mackerel and negatively influence the ability to survive in OMZ.

It might be argued therefore that the tolerance to hypoxia rather follows ecological needs, instead of scaling equations; in the case of Cape horse mackerel the study of a broader range of size classes and temperatures combined with a more accurate monitoring of the distribution could supply interesting findings on the anaerobic capabilities of this species and its ecological adaptations.

Behavioral Responses

Responses to lower oxygen saturation followed repetitive patterns. The specimens initially counter-acted the decreased efficiency of oxygen uptake by pumping a higher volume of water through the gill. This was interestingly carried out in a first moment by wider movement of the gill operculum and only later by increased ventilation frequency. Approaching an oxygen saturation of 20% (1.5 mg/l O_2) triggered hyperactivity, which is likely to be the first warning signal of unsustainable metabolite accumulation and physiological pH shift. The fish's persistent movements across the chamber highlight the ability of Cape horse mackerel to sense the danger of asphyxia and trigger a behavioral response that, despite the increase of energetic demand, will eventually lead to a safer area. Although numerous and contradictory hypothesis of vertical migration seem to be unable to locate the cause of this phenomenon, it cannot be excluded that this species is able to travel through the water column within hours and therefore evade the danger of anoxic waters. Interestingly after sustained persistence at low oxygen concentration, the hyperactivity ceased. This might indicate that either the glycolytic production of ATP has reached its limit or that the fish resorted to the final solution of reducing the metabolic rate to a minimum, or both.

Enzymatic Essays

For the purposes of our study we chose to measure key diagnostic enzymes activities to provide an estimate of the aerobic and anaerobic capacities of Cape horse mackerel. The index chosen for aerobic metabolism is citrate synthase, as it has shown to correlate with oxygen consumption rates in other studies on teleosts (Childress and Somero, 1979). CS activity is in fact representative of aerobic capacity and has been found to account for 90% of total respiration (Hochachka, 1970). As such we would have expected that CS activity would have scaled with the body mass to a similar rate as that found for oxygen consumption. Scaling relationship for CS activity in teleosts indicate an exponent of 0.75, although values from 0.5 to 0.87 have been documented (Childress and Somero, 1990). It is clear why an aerobic capacity indicator, such as CS, in the aerobic locomotor muscle would show a parallel pattern to the fish routine metabolic rate and routine oxygen consumption. However our findings do not show appreciable correlation with the values of routine metabolic rate, which display a significant fit to the allometric equation $Y= a*X^b$. The cause of this miscorrelation is most likely to be found neither in equipment malfunction nor in wrong execution of enzymatic essay protocols. The standard deviation between replicates was in most cases extremely low. However, carrying out a third replicate revealed an enzymatic activity varying up to two fold the outcome of previous findings. Nevertheless, the standard deviation of the third assessment was remarkably low. It is clear that the source of the problem has to be found within the extraction procedures, being this the only solution among the reagents that changed among the two essays. Any other issue related to the degradation of either the reagents or the enzymes contained in the tissue could be ruled out, as this would have most likely produced a decrease in enzymatic activity, rather than the increase that was observed in some cases.

The extracting procedures required a minimum of 40mg of muscular tissue to be recovered from each specimen. The dissection was carried out with the use of liquid nitrogen to preserve the integrity of the enzymes. This caused a series of shortcomings for the dissecting procedures. Although the abdominal cavity with the whole digestive apparatus, the head and the gills could be easily removed, the remaining portion of the fish still consisted of muscular tissue, but defined by different biochemical properties. The use of whole-animal versus single tissue homogenates has been discussed in other

studies (Berges and Ballantyne, 1991) and the outcome clearly shows that the homogenization of the whole animal appears to provide a reasonable index of total enzyme activity. This is true for enzymes with different substrates and cofactors requirements, as well as for those in different cellular compartments. Gibbs and Dickson (2003) carried out experiments on chub mackerel (*Scomber japonicus*) in which citrate synthase activity was measured in the red muscle, hearth and white muscle. The outcome clearly shows that the activity of CS varies between the three tissues; in particular the red muscle shows an activity ten times higher than in the white muscle. Pyruvate kinase was chosen as indicator of anaerobic potential, as key enzyme involved in the glycolysis. Scaling relationships for PK activity have been shown to vary with the tissue examined too (Childress and Somero, 1980). In a whole animal measurement, PK may be more closely related to the relative proportions of different tissues.

For the enzymes tested the partitioning of the animal into body sections appeared to provide an unreliable index of total enzymatic activity. Childress and Somero (1980) reported that PK activity scales with a slope greater than 1.0 in fish muscle, although it is found constant in other tissues, such as in the nervous system. In consideration of the difficulties encountered in dissecting frozen tissue of relatively small sized specimen used, it is advisable to rely on whole body homogenates for future enzymatic essays.

Conclusion

The reader might argue that the small population size (n=15) cannot supply sufficient data to draw conclusions on the physiological quests initially posed, thus increasing the chances of producing misleading interpretations. I respond that the aims have been fulfilled beyond expectations, not only for the quality of the data obtained, but especially in consideration of the high risk of failure involved. This study is not to be considered conclusive per se, but rather a more advanced starting point for future prospects in the Benguela system. So far, the tolerance of a middle trophic level species (Cape horse mackerel) to oxygen depleted waters has been revealed (3.9 mg/l – 4.7 mg/l O_2) along with the RMR at a temperature of 16°C. The sampling covered a relatively broad size range (0.1g – 4.56g) during some of the most delicate developmental stages; this allowed calculations of the metabolic scaling over the body mass ($y= 1.2106x^{0.4872}$) with significant correlation by Pearson´s test (r=0.93). The monitoring of behavioral responses to hypoxia suggests that this species actively seeks safety by means of vertical migration. The reason for the inconclusive PK and CS essays has been identified and can now be easily overcome.

Both data and technical experience have been gathered in this thesis and this expertise will serve future comparative studies. The cutting of costs and time requirements, the broadening of the data set and eventually the addition of other taxa are some aspects which are to be taken into consideration. Expedition costs and time restrictions for vessel time represented in fact some of the major limitations in this thesis. The technical and economical improvements therefore mostly focus on the catching methods and sample processing, which would benefit from the use of smaller, less expensive vessels coupled with an on-land based laboratory facility.

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