Thesis for the Master's degree in Molecular Biosciences
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Sulphide and Anoxia Tolerance in a Namibian Fish

How to Exploit a Hostile Environment

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Abstract

The Benguela upwelling in Namibia and South Africa experienced an ecosystem collapse after overfishing in the 1960s and 1970s, and as several industrially important species became scarce, others grew abundant. One of these was *Sufflogobius bibarbatus*, also known as the pelagic goby. The Benguela ocean floor has many areas with low levels of oxygen combined with high concentrations of H_2S. These potentially lethal conditions are avoided by most species, but the pelagic goby spend daytime in this inhospitable mud. In April 2008 a the research vessel "G.O. Sars" left port in Namibia, with a goal of investigating exactly how and why the goby prefer to seek shelter in such areas.

Cytochrome *c* oxidase (COX) is the fourth complex in the electron transport chain of mitochondria. It uses oxygen as the terminal electron acceptor during oxidative phosphorylation. Without oxygen oxidative phosphorylation stops and ATP production has to rely on anaerobic glycolysis. Accumulation of the end-product lactate is potentially deadly, and must be avoided. H_2S binds to COX and inhibits the interaction with oxygen, thus stopping oxidative phosphorylation, making the organism functionally anoxic. In this thesis I have used respirometry to investigate the hypoxia-tolerance and H_2S-tolerence of the pelagic goby. Finally I have done real-time RT-PCR experiments to examine the expression of COX subunits I-III during exposure to anoxia and/or, H_2S.

My findings indicate that the pelagic goby is exceptionally good at taking up oxygen in hypoxia, being able to maintain resting oxygen consumption down to a water oxygen level of 5.3 % of air saturation. It does not appear to have any special mechanism for tolerating H_2S, asides from the fact that it can survive exposures to anoxia for hours. During anoxia it accumulates lactate and builds up on oxygen debt. In nature, this oxygen debt is most likely paid off during the nocturnal migration from the bottom to well-oxygenated pelagic waters, and could be a main reason for the diurnal migration pattern of the pelagic goby. The expression of COX subunits I-III does not appear to be affected by either anoxia or H_2S exposure.

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1. Introduction

During the 1960s to 1980s, there was a major ecosystem collapse in the Namibian Benguela upwelling as a result of overfishing. Dominating fish species in the ecosystem had until then been sardines (*Sardinops sagax)* and anchovies (*Engraulis encrasicolis*), but after the collapse several new species grew abundant in the absence of the predatory fish, among them a pelagic goby species (*Sufflogobius bibarbatus*). The reasons behind the pelagic goby's success have not been clear.

Lack of oxygen, or anoxia, is deadly for most vertebrates. In spite of this there are a few species that can survive very long exposures to anoxia ([$O_2 \leq 0.5$ % of air saturation), including some fish and some species of freshwater turtle. They survive on anaerobic metabolism and their metabolism is depressed to save energy.

Studies done of the bottom of the Benguela current show that there are large hypoxic pockets on the bottom where no vertebrate life can be found, except for *Sufflogobius bibarbatus*. The pockets also contain high concentration of H_2S (Emeis et al., 2004). H_2S is generated by bacteria in aquatic ecosystems and it reacts with oxygen to produce hypoxic or even anoxic areas. H_2S has also a direct inhibitory effect on respiration, making the animal functionally anoxic. Thus, for most species exposure to high H_2S levels rapidly lead to death.

In April 2008 the Norwegian research-vessel "G. O. Sars" was the scene for an expedition studying the Benguela ecosystem in Namibia. This thesis is in part a result of studies done on this excursion, and in addition the thesis attempts to explain the physiological adaptations that make the goby so well suited to living in these challenging conditions.

1.1 The Benguela Upwelling System

The Benguela Current is the name of a coldwater current moving north from the coast of Namibia and South Africa. It is created by a meeting of the warm Indian ocean, subtropical Atlantic water and cold sub Antarctic water, and it was one of the four most productive ecosystems in the world (Cushing, 1971). Several studies of this area have divided it into two major parts, the Northern Benguela and the Southern Benguela, with

the dividing area at the Orange river mouth at the boarder between Namibia and South Africa (Shannon and Jarre-Teichmann, 1999; Shannon et al., 2003). By being a very nutrient rich this area has historically been able to support many species of fish like sardines and anchovies.

Namibia and South-Africa has been populated for thousands of years, and fish have always been an important part of the diet for anyone living close to the sea. With the large increase of catch size that came with developing technology in the 1950s and 1960s, overfishing of anchovies and sardines caused the Benguela system to go through an wasp-waist ecoshift where the removal of some species has lead to changes in population for other species as well (figure. 1) (Cury and Shannon, 2004).

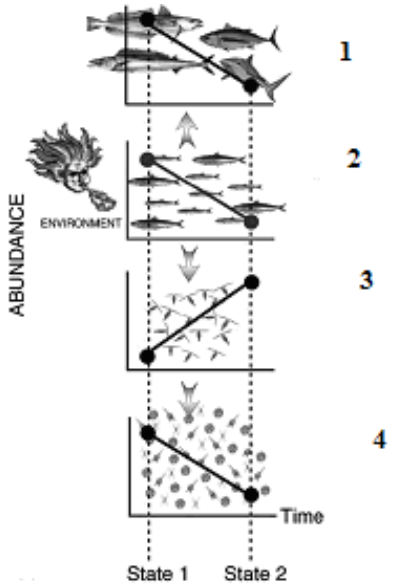

Figure 1. Wasp-waist ecoshift: As one species is reduced in abundance it leads to shifts in other layers of the food chain. Here is shown a reduction in layer 2, leading to a decrease in abundance of layer 1 as well due to lack of food. Layer 3 experience an increase because of the reduced amount of predators. This in turn causes the 4th layer to be reduced. See text for further explanation. Figure adapted from Cury and Shannon (2004).

Overfishing of sardines and anchovies (layer 2 in figure 1) have greatly reduced the abundance of these species, which in turn is reducing the amount of larger fish (layer 1) that prey on them. In the absence of predators other species that would serve as a food-source for these species have become abundant (layer 3), in turn reducing the abundance of their food (layer 4). This has lead to a general shift in abundance of larger predatory fish (layer 1 and 2) to an increase in plankton-eating pelagic fish and smaller invertebrates (layer 3). The ecoshift allowed *Sufflogobius bibarbatus* to be one of the species replacing the sardine, and the goby has become a very abundant species in the area, along with other

less industrially important species like several species of jellyfish (*Chrysaora hysoscella* and *Aequorea forskalea*) (Griffiths et al., 2005; Lynam et al., 2006). The pelagic goby is of great importance to the ecosystem as it is one of the main sources of food for the predatory fish, birds and seals in the area (Shannon and Jarre-Teichmann, 1999). Studies of *Sufflogobius bibarbatus* are few, but it is very important to understand how it fits into the ecosystem and how these changes in a few decades allowed the goby to become a dominant species.

1.2 *Sufflogobius bibarbatus*

The species *Sufflogobius bibarbatus* is part of the Gobiidae-family, consisting of more than 2000 species (figure 2). These small fish are found in numerous places like shallow pools of both fresh- and saltwater, and coral reefs, and some are even highly popular aquarium fish. *S. bibarbatus*, or the pelagic goby, is a small fish found on the coast of Namibia and South Africa (Hewitson and Cruickshank, 1993). Early studies on the goby reported that its main source of food was phytoplankton (Crawford et al., 1987), although later studies have contradicted this claiming that it also feed on zooplankton (Gibbons et al., 2002).

Figure 2. A pelagic goby caught in the spring of 2008 off the coast of Namibia.

During the larval stage and as early juveniles they are widely distributed in the upper 50m layer of the ocean (O'Toole, 1978). As older juveniles they are found in all epipelagic depths and as they mature into adults they migrate into deeper waters and can be found in

the demersal zone (close to the bottom) (Bianchi et al., 1993). Studies of the ocean floor off the coast of Namibia have shown that the highest density of the pelagic goby can be found between Walvis Bay and Ludertiz (Hewitson and Cruickshank, 1993). *S. bibarbatus* is also known as the pelagic goby because it spends long periods in the pelagic zone of the water column at night.

1.3 Anoxia and H_2S in the Ocean

As mentioned, it is not uncommon to find areas with hypoxic, or even anoxic ,pockets in the water of the Benguela current, posing a challenge to life in these areas. This is not the only place where conditions like this is found, and one well studied area comparable to this is the oceanic area off the coast of Chile. There are several similarities between the upwelling system in Benguela and the coast of Chile. In both areas there is a strong upwelling current with high plankton activity. The bottom water in these areas is often depleted of oxygen because of intense heterotrophic respiration. Additionally, both areas have sediment-water interface zones with high levels of H_2S (Ferdelman et al., 1999; Fossing, 1990). When measuring H_2S production off the coast of Namibia, Ferdelman found values near zero at the sediment surface, and up to 29 nmol $cm^{-3}d^{-1}$ 2 - 5 cm into the sediment, decreasing at further depths. These values are higher than most teleost fish tested can tolerate (Bagarinao, 1992).

H_2S is a toxic gas produced by bacteria in absence of oxygen and is naturally occurring in both the environment and the gut. Many bacteria produce H_2S by breakdown of dead organic material, like in the muddy bottom of the Benguela. As this is very similar to conditions off the coast of Chile it was expected that it would be caused by the same bacteria (*Thioploca* and *Beggiatoa*), but this was not investigated until 1999 when Schulz et al. (1999) reported the finding of a new bacteria. Their examinations of the sediments off the coast of Namibia show that *Thioploca* and *Beggiatoa* are present in the sediment, but in far lower numbers than expected.

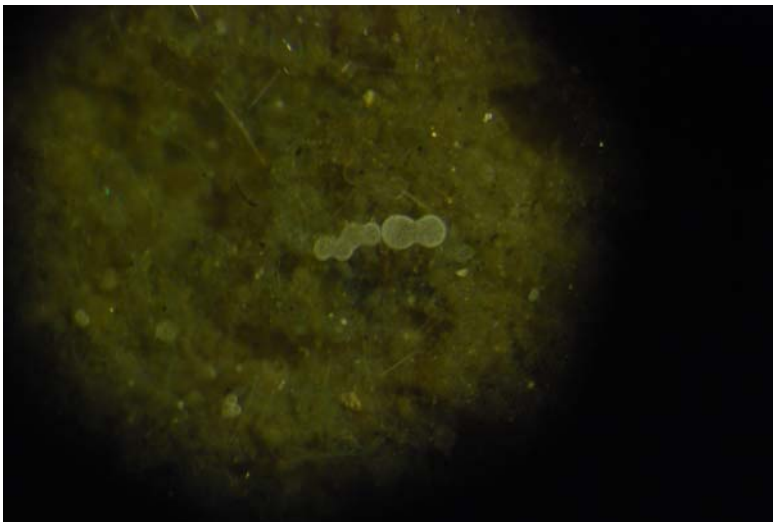

Figure 3. A short chain of *Thiomargarita namibiensis* observed during our cruise in Namibia.

Instead, they found high quantities of *Thiomargarita namibiensis* (”Sulphur Pearl of Namibia”), a sulphur-oxidising bacterium living in low-oxygen water found in the top 3 cm of the sediments. This is the largest known bacterium, large enough to be seen with the naked eye (< 0.75mm) (figure 3).

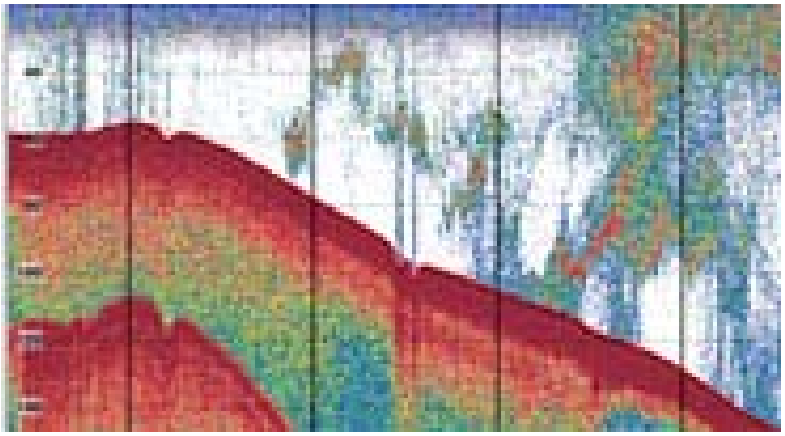

Figure 4. Echogram showing the ocean floor. This echogram has been recorded while the boat was moving into gradually deeper waters, seen in the figure by the gradual lowering of the big red line (ocean floor). Individual fish can be observed as a coloured dot in the picture, and the more fish in one area the more colouring. Some pockets with no fish can be seen, indicating a hypoxic H_2S-area. Most gobies were found in the hypoxic pockets, but cannot be seen on echogram due to hiding in the mud. From Utne-Palm et al. (in review).

Observations of the ocean floor during our research cruise off Namibia showed that there are areas at the bottom where no fish can be detected with sonar (figure 4). These

correspond to pockets of hypoxic water (~10% of air saturation) with high concentration of H_2S, in which most fish cannot survive for more than a few minutes. Surprisingly these are areas where gobies can be caught. Studies done *in vivo* on the research cruise indicated that the goby not only spend long periods of time in these anoxic pockets, but also burrow itself in the mud with only the head visible (Utne-Palm et al, in review). This is a very strong indication of hypoxia tolerance and possibly also a special tolerance to H_2S.

1.4 Maintaining Energy without Oxygen

Energy is needed for all life, and most energy consuming processes in cells are driven by adenosine triphosphate (ATP). Most of the ATP is under normal circumstances produced by oxidative phosphorylation in the mitochondria in all cells, and this process uses oxygen as terminal electron acceptor. As ATP cannot be stored it needs to be continuously synthesised. Oxidative phosphorylation theoretically produces 36 mole of ATP per mole of glucose broken down. When there is no oxygen present ATP is produced through anaerobic glycolysis, yielding only 2 moles of ATP pr mole of glucose. In addition to not producing enough energy to maintain normal function this also leads to a build-up of lactate, which is produced as the end product. Build-up of lactate will lead to acidosis which in turn may cause death by slowing of heart rate, change in ion-concentrations, and depression of glycolysis (for a review, see Nahas, 1970).

Failure to maintain ATP levels will lead to organs failing, which will first involve failure of the heart and brain, and then death. The two options available for keeping ATP supply up during anoxia is to either increase ATP-production through glycolysis, or to reduce ATP consumption by suppressing activity in the various tissues (for a review, see Nilsson and Lutz, 2004). Different animals uses different strategies. Some freshwater turtles (*Trachemys* and *Chrysemys*) that can survive for months in anoxia by suppressing brain activity to a comatose-like state, thus lowering ATP consumption (Fernandes et al., 1997; Hicks and Farrell, 2000). They also buffer the lactate produced by anaerobic glycolysis with calcium carbonate in the shell. Other species (crucian carp and goldfish) survive long periods of anoxia by fermenting lactate to ethanol that leaves the fish through the gills (Shoubridge and Hochachka, 1980).

Species	**Habitat**	**PO_2crit** (mmHg)	**$[O_2]_{crit}$** (mg l-1)	**T** (°C)
Toadfish (*Opsanus tau*)	Atlantic coast of North America	29	1.4	22
Common carp (*Cyprinus carpio*)	European freshwater	30	2.2	10
Crucian carp (*Carassius carassius*)	European freshwater	12 (6)	1.0 (0.5)	8
Goldfish (*Carassius auratus*)	Domesticated (orig. Asian freshwater)	25 40	1.8 2.3	10 20
European eel (*Anguilla anguilla*)	European freshwater	25	1.4	25
Humbug damselfish (*Dascyllus aruanus*)	Great Barrier Reef	29	1.2	30
Coral goby (*Gobiodon ceramensis)*	Great Barrier Reef	22	0.9	30

Table 1. $[O_2]_{crit}$ for 7 hypoxia-tolerant teleosts. Values in parenthesis refer to hypoxia acclimatised individuals. Adapted from Nilsson and Randall (2010 (in press)).

Build-up of lactate during *short* exposures to hypoxia is usually not a problem. When once again able to take up oxygen, lactate can be converted back into pyruvate and be aerobically broken down to ATP. After an anoxic episode, lactate is oxidized, which causes oxygen consumption to rise. The increase of oxygen consumption is a hallmark of an oxygen debt, and will subside once the debt is paid off, meaning when there is no more lactate to break down.

Fish are confronted with lack of oxygen more often than air-breathing animals, due to the much lower solubility and diffusion rate of oxygen in water, lack of photosynthesis at night and in depths with no sunlight, and low mixing of the top layer of water with the lower layers. Furthermore, water fully saturated with oxygen will hold only about 1/30 of the amount found in air. Consequently, numerous fish species show hypoxia tolerance (Nikinmaa and Rees, 2005).

When examining the metabolic rate of fish, it is convenient to do so using respirometry. In this thesis closed respirometry was used, meaning that the gobies were placed in a sealed chamber and the fall in the water oxygen level was recorded. The point where the organism no longer are able extract enough oxygen to maintain their resting oxygen consumption rate is called the critical oxygen tension, PO_{2cri} or critical oxygen concentration $[O_2]_{crit}$ (table 1).

1.5.1 Cytochrome *c* Oxidase

Oxygen is needed as an electron acceptor in the terminal (fourth) complex in the electron transport chain. H_2S mimics the effect of anoxia by blocking complex IV, which is made up of cytochrome *c* oxidase (COX), a transmembrane protein found in the inner membrane of the mitochondria. During oxidative phosphorylation, COX transfers electrons from reduced cytochrome *c* to oxygen, thus creating an electrochemical gradient. It is responsible for almost 90% of oxygen consumption in mammals (Babcock and Wikström, 1992). COX consists of several subunits, and the exact number varies between prokaryots and eukaryots, with an increasing higher degree of complexity between the subunits (Richter and Ludwig, 2003). Schägger (2001) argues strongly for the formation of supercomplexes with Complex I, II and III, further increasing the complexity of COX function.

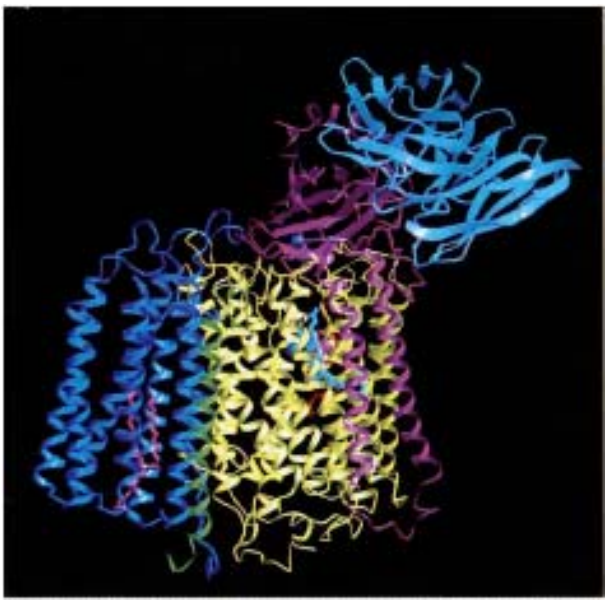

Figure 5. Image of COX. Subunit I (yellow), II (purple) and III (blue) make up the catalytic core. From Itawa et al (1995).

The eukaryotic COX consists of several subunits; 3 are found in the mitochondrial genome (mtDNA) and the last 4-10 subunits are encoded in the nuclear genome, while yeast COX consists of fewer nuclear encoded subunits (Capaldi, 1990; Richter and Ludwig, 2003). The three largest subunits (I, II and III according to the nomenclature introduced by Kadenbach, 1981) are found in mtDNA. According to Barrientos (2002) subunits I, II and III are the subunits that make up the catalytic core reacting to oxygen (figure 5). The rest of the subunits play a role in regulation and formation of the enzyme

(for a review, see Richter and Ludwig, 2003). In eukaryotes subunits I-III are synthesised in the mitochondria and then inserted into the mitochondrial membrane (Ludwig, 1987). Translation, processing and assembly of COX in eukaryotes is a multi-step process, aided by several other gene products (for a detailed review, see Barrientos, 2002). While most organisms have some form of the three main subunits, some species have been found to lack the genes for subunits II and III, such as the green algae Chlamydomonas (Attardi and Schatz, 1988). In spite of this, a striking similarity has been revealed between bacterial and eukaryotic COX, a sure indication of the conservation of this gene during evolution (Moody, 1996; Tsukihara et al., 1995; Tsukihara et al., 1996).

1.5.2 COX Regulation, Anoxia and H_2S.

Regulation of both expression and activity of COX has been intensively studied, but still much remains to be fully explained. It is complicated by the fact that COX is in part expressed from two independently regulated genomes – the nuclear and the mitochondrial genomes, the latter occurring in several copies in each organelle. Not only do each mitochondria contain several copies of its genome, but each cell also contains numerous mitochondria, resulting in 10^3-10^5 copies of mtDNA in a cell (Gross et al., 1969). The level of mutated mitochondrial genomes needed for discovering a change is hard to determine, and a protective threshold is given by the heterogenic nature of mitochondria in a cell. In spite of this it has been shown that mutations in genes for COX subunits show a reduction in enzyme function at very low levels of mutation (D'Aurelio et al., 2001). Trifunovic (2004) also found mitochondrial mutations to be connected to symptoms of old age, and mutated COX seems to be connected to failing cardiomyocytes.

Mitochondria lack the proof-reading systems seen in nuclear transcription and translation, leading to a higher rate of mutation. This is seen in the fact that there are many mitochondria related diseases linked to either mitochondria encoded genes or accessory proteins, for example Parkinson's disease, Leigh's syndrome and Alzheimer's disease to mention a few (Wallace, 1992). In their review Richter and Ludwig (2003) also pointed out that most mitochondrial mutations are either a point mutation or a nonsense (terminal) mutation. The mutations caused by pretermination of translation would lead to a loss of redox-center and subsequent loss of activity for COX. There are also several known cases

of point-mutation leading to reduction of enzyme activity and a lowered maximal respiration rate (Richter and Ludwig, 2003).

Yeast	I	II	III	IV	Va/Vb	VI	VII	VIIa	VIII	VIa	VIb
Bovine	I	II	III	Vb	IV	Va	VIIa	VIc	VIIc	VIa	VIb

The nomenclature of Poyton and McEwen (1996) is used for yeast and that of Kadenbach *et al.* (1983) is used for bovine cytochrome *c* oxidase.

Table 2. Correspondence between yeast and mammalian (bovine) COX nomenclature. From Burke and Poyton (1998)

There are different forms (paralogs) of the subunits encoded in the nucleus in several species of mammals and yeast, including oxygen-dependent/independent paralogs, tissue-specific paralogs and developmental paralogs (for a review, see Burke (1998)). Kwast (1998) discussed the finding of two paralogs of subunit V in yeast (*Saccharomyces cerevisiae*) (a homolog to COX IV in mammals, table 2), where one is expressed in aerobic conditions (Va) and the other one is expressed at hypoxic conditions (Vb). In humans, it is interesting to note that several subunits are expressed at different forms during fetal and adult life (subunits VIa, VIIa and IV) (Bonne et al., 1993). With this in mind it would be interesting to investigate if there are such paralogs that allow oxidative phosphorylation to continue in presence of inhibitory chemicals such as H_2S.

Animal	H_2S concentrations (μM)	
	inhibitory	stimulatory
Acanthamoeba castellanii (soil amoeba)	25–38	15
Tubifex sp. (freshwater annelid)	2	
Solemya reidi (marine clam), gill	14	6
Fundulus parvipinnis (California killifish), liver	14	6
Citharichthys stigmaeus (speckled sanddab), liver	11	6
Rat, liver	19	5–10
Rat, kidney	1–3	

Table 3. Sulphide concentrations that are inhibitory and stimulatory to COX in various species. The low H_2S concentrations stimulate COX activity as it oxidises H_2S. At higher concentration H_2S becomes inhibitory as the enzyme becomes blocked. From Bagarinao (1992)

In spite of eukaryotic COX being expressed from two different genomes, there seems to be no shared regulation of expression between the two genomes. The expression of the nuclear-encoded genes are regulated by transcription factors, and mitochondrial expression seems to be regulated by mitochondrial turn-over (D'Aurelio et al., 2001).

The activity of COX is regulated by a variety of mechanisms. One main element in inhibiting COX activity is a high intra-mitochondrial ATP/ADP ratio (Napiwotzki and Kadenbach, 1998). Several hormones affect expression of mtDNA, either inhibitory (estrogen) or stimulatory (thyroid hormones, gastrin) (Bettini and Maggi, 1992; Wiesner et al., 1992). In addition several other molecules have been shown to affect the activity of COX. Thus, NO, CO and H_2S are known to inhibit COX activity (Alonso et al., 2003; Winzler, 1943). As reviewed by Bagarinao (1992) H_2S will bind to the catalytic core creating an enzyme-sulphide complex that is non-responsive towards oxygen. However, at low concentrations H_2S actually exerts an *stimulatory* effect on COX, as H_2S is being oxidatively detoxified to thiosulfate (table 3) (Bartholomew et al., 1980; Baxter et al., 1958).

1.6 Aim of Thesis

This thesis forms part of a larger effort aimed at understanding the biology, and thereby the success, of Sufflogobius bibarbatus off the Namibian coast. This effort was based on an 11 day research cruise on RV "G. O. Sars" in April 2008, involving 25 researchers and students, and including sonar surveys of fish movements, trawling at different depths, behavioural studies, sediment studies, and finally physiological measurements. In particular my thesis aims to

1. Investigate the hypoxia tolerance, and the respiratory effects of H_2S, on *Sufflogobius bibarbatus*.
2. Look for any changes in expression of COX when exposed to anoxia and/or H_2S.

2. Methods and Materials:

2.1 Experimental Animals

Fish of the species *S. bibarbatus* were caught by the research vessel "G.O. Sars" off the coast of Namibia in the Benguela region using a bottom trawl and a pelagic trawl at a depth of < 120m. All the respirometry experiments on the pelagic goby were done onboard the ship, while the molecular studies were done on tissues brought back to the University of Oslo.

After bringing the fish on board, the fish were carefully removed from the trawl by hand and kept in a holding tank (1000 L) with seawater at surface water temperature (13ºC ± 1ºC). The fish were kept on deck in a natural day-night cycle, and were not fed after trawling. The fish used for respirometry experiments were kept overnight in a separate tank to reduce stress.

2.2 Respirometry

The respirometry experiments were performed to examine the capacity for oxygen uptake in hypoxic water (as determined by $[O_2]$crit) and to see how H_2S affect the oxygen consumption of the pelagic goby. When doing closed respirometry the fish is placed in a sealed container and the falling level of O_2 is recorded continuously (figure 6).

Figure 6. Goby in a closed respirometer. The oxygen electrode can be seen at the top of the chamber with a magnetic stirrer attached to it to ensure even distribution of oxygen in the chamber.

The respirometer was custom-made from a Perspex cylinder with an inner diameter of 80 mm and an adjustable plunger to allow regulation of volume. This allowed for regulation of volume according to size of the fish. During the experiments the oxygen level in the respirometer was monitored by using a galvanometric oxygen electrode (WTW OXI 340i) placed in the chamber, with a magnetic propeller attached to the tip of the electrode to ensure thorough circulation of water in the chamber and over the electrode. The propeller was driven by a magnetic stirrer placed outside of the chamber. The whole chamber was kept submerged in an aquarium where the temperature was 12°C ± 1°C. The data was recorded with a Powerlab 4/20 and the program Chart 5.0 (both from AD instruments), and the O_2-level was measured as % of air saturation.

The fish were kept in the respirometer for 1-2 hours prior to each experiment to acclimatise them to the chamber. During this period water was continuously fed into the chamber.

2.2.1 Determination of Critical Oxygen Tension and of Metabolic Rate

Three types of experiments were done using closed respirometry. First resting oxygen consumption and critical oxygen tension ($[O_2]_{crit}$) was measured. Oxygen consumption is found by calculating how much O_2 the fish consumes per hour pr kg fish. $[O_2]_{crit}$ is defined as the lowest level where the fish is able to maintain its resting O_2 consumption, and can be found by allowing the fish to consume all the oxygen in the chamber. This initially gives a steady rate of consumption until the O_2 concentration is too low for the fish to efficiently consume O_2 anymore, resulting in a declining rate of consumption. From the graph this can easily be determined by locating the point where the consumption-rate falls off (exemplified in figure 7).

Figure 7. Figure 7. Example of recording of falling oxygen-levels in water during closed respirometry. When oxygen concentration decreases below the critical oxygen tension ($[O_2]_{crit,}$ arrow), the fish will no longer take up oxygen at a constant rate.

2.2.2. Effect of H_2S on Oxygen Consumption

Next the effect of H_2S on the oxygen consumption was measured using the same set-up. Two chambers were run in parallel with one fish in each. The fish were allowed to consume oxygen until the oxygen level reached about 50% of air saturation, whereupon the fish was removed from one of the chambers and both chambers were injected with a H_2S solution prepared from sodium sulphite (NaS) crystals dissolved in deoxygenated seawater. Concentration of total sulphide was measured at the end of the experiment. The empty chamber was run as a blank to record the rate by which H_2S reacts with O_2 dissolved in water. This inorganic disappearance of oxygen was subtracted from the rate of oxygen consumption seen in the chamber with fish. Several experiments were carried out with varying concentrations of total sulphide (100-1200 µM) corresponding to H_2S, concentrations of 3.6 – 43.2 µM.

2.2.3 Build up of Oxygen Debt

Finally the effect of anoxia (O_2 < 0.5%) was measured. This was done by allowing the fish to consume all the oxygen available in the chamber. After approximately 2 hours of subsequent anoxia exposure, the water was replaced with fully aerated water, and the fish

was again left to consume the oxygen. Comparison of the O_2-consumption rate before and after the anoxia exposure revealed if the fish had built up an oxygen-debt.

2.3 Molecular Experiments

The purpose of the molecular experiments was to examine changes in the expression of mRNA for subunits of COX when the fish were exposed to anoxia and/or H_2S.

2.3.1 Cloning and Sequencing of Genes of Interest

The genome of the pelagic goby is not sequenced, so in order to obtain gene specific primers for real-time RT-PCR, the genes of interest need to be sequenced. Because COX I-III make up the catalytic core of COX it was decided to clone and sequence the genes for subunits I-III (COX I-III), while Beta-actin and GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) would be used as reference genes in the real-time RT-PCR assay. Primers used in cloning of all genes were designed based on sequences obtained from other fish species, all found on the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/). Primers were chosen with emphasis on sequences from other gobies, because a close relationship between the species generally means similarity in gene sequence. Due to some variations in the sequences between the species degenerated primers were used, meaning that where the sequences varied a mixture of the bases were used (table 4, for a overview of IUPAC one-letter base abbreviations see appendix C). The primers were designed using the Primer3 tool (http://frodo.wi.mit.edu/primer3/input.htm) (Rozen and Skaletsky, 2000), and purchased from Invitrogen.

2.3.1.1 Cloning Primers:

A mixture of RNA isolated from heart, liver, brain and muscle was extracted with TRIzol (Invitrogen) and then cDNA was synthesized using Superscript III (Invitrogen), all according to manufacturer's instructions. This cDNA was then used as a template for a

polymerase chain reaction (PCR). The target region was then amplified using a Platinum *Taq* DNA polymerase (Invitrogen), and due to the degeneration of the primers, 10 μM of each primer were used. Degenerated primers consist of a mix of different bases and in order to get a high concentration of each of the different bases in the mix, more primer is needed..

Gene	Forward primers	Reverse primers
COXI	CGGIATRRTIGGIACIGVIY	GGGAGCCGAIDGADGAVAYD
	GAGGVTTYGGIAAYTGRYTV	GCAAAGACDGCYCCYATIGA
	TTAGCHTCYTCHGGIGTDGA	GGAGGARTTDGMBARIACRA
	TGTATACCCBCCHYTIKCVG	ACCCTCCRTGIAGRGTRGCI
	CACAYGCIGGDGCHTCYGTH	ATAAAGCCYARIARICCRAT
COXII	CCACCCCTCICAACTAGGWT	GAIRYIGAYCACCGAATGGT
	CATTTYCAYGAYCAYRCHYT	CWGTICCAGGACGCCTAAAC
	ATTATTGTGIYIDTRGTVWC	RTGYTCDGAAATYTGTGGGG
	TCCC CTCTCTACGCATCTTG	GAADBMMTICCITTAAAATACTTC
COXIII	ACCAAGCACAYSCMTAYCAY	CAAAGCCAAARTGRTGBTYD
	TAGTAGAMCCMAGCCCHTGA	AAGTCCATGAAABCCKGTDG
	GGCCATCAYACVCCVCCYGT	TGTGAAGGGGGCYTCRWART
	TTCTGAGCHTTYTWYCAYK	ATGCTRTGRTGDGCYCADGT
Beta-actin	GTTGACAAYGGMTCYGGYA	GCTGGAAGRTRGASAGVGAR
	ATGGGCCAGAARGAYWSCT	AGCACAGTGGTGGCRTAMAG
	GGTGATGARGCHAARAGCAA	TGCTGTTGTAKGTRGTYTC
	CAGGGAGAARATGACMCAGA	CGGGCAACTCRTAGCTCTTC
	GCCCATCTAYGARGGBTAY	CTTGATGTCACGBACRATTT
GAPDH	TGACCCATTCATYGACCTKG	AACCTGGTCCTCHCTGTATC
	CGGTCAAGCHATCACHGTBT	GCTGGCAGGYTTCTCMAGRC
	TCACATTAAGGGYGGTGCHA	ACGGAAGGCCATRCCDGTMA
	GGTGGTGCHAARAGRGTSAT	TCAACGGTCTTCTGDGTDGC
	TGGGTGTCAACCAYSAGAAR	AGGAGGCATTGCTKACMACT

Table 4. Overview of primers used for cloning and sequencing. Due to variations between sequences in other species, degenerated primers were used. (IUPAC one-letter abbreviations can be seen in appendix C).

The PCR was done on a Mastercycler gradient thermal cycler (Eppendorf), starting with a 10 min incubation step on 94˚C before repeating the following steps 35 times: 30sec at

94˚C to denature the cDNA and primers, followed by 1 min at 55˚C for proper annealing of primers to the cDNA and then 1 min/1kb at 72˚C for elongation of the product. In the end there is a final step of 10 min at 72 ˚C to allow all the product to be replicated all the way to the end.

After the PCR the product was visualized by running the samples on a 1% agarose gel and checking if the product had the desired size. PCR-products with the right size were ligated into p-GEM®-T-Easy Vector (Promega) following instructions from manufacturer. Bacterial transformation was used to insert the vector containing the PCR product into calcium competent *E. coli* cells. The cells was plated on LB plates containing ampicilline and IPTG/X-GAL and incubated overnight at 37˚C. After the overnight growth the plates had both white and blue colonies. Each colony consists of identical copies of the plasmid, all identical to the cell starting the colony. Since the plates were covered in ampicilline every cell that survives on the ampicilline-plate will contain a plasmid with ampicilline resistance. The product of breakdown of X-GAL is blue, and blue colonies indicate that the cell has taken up a plasmid without an insert in the region coding for breakdown of the X-GAL. White colonies will also have taken up plasmids, but these plasmids will have an insert in the region coding for breakdown of X-GAL, thus not producing the blue end-product. This is an effective way of screening out the unwanted colonies. Finally some white colonies were chosen and the target region from the plasmids was amplified with vector specific primers in an PCR. The product size was confirmed on an agarose gel, and finally the product was treated with exozap and sent for sequencing at the ABI-lab (http://www.bio.uio.no/ABI-lab/) at University of Oslo. The results were compared to the same genes in other species.

2.3.1.2 Gradient PCR

When confirming the PCR products on an agarose gel it appeared that COXI had not been successfully amplified. In order to test if the melting temperature had been sub-optimal for the primers, a gradient PCR was done. This means running a PCR with triplets of each reaction and using different annealing temperatures for each set of samples, thus testing for different annealing temperatures. The gradient PCR temperature-range was 55˚C ± 2˚C. To further improve results from the gradient-PCR a nested PCR was run. In a nested PCR the products from a previous PCR is used, and primers added to the reaction binds

within the target sequence of the previous PCR. This leads to an amplification of an already amplified region.

2.3.2 Rapid Amplification of cDNA Ends (RACE) to Obtain Full Gene Sequence

RACE was performed to amplify 3' and 5' ends of the previously amplified and sequenced region of all genes, thus obtaining the full cDNA sequence for an unknown mRNA. Although slightly different techniques are used for 5' and 3' RACE, both require that there is a known central sequence of the transcript.

The cDNA was synthesised from RNA purified from total RNA using Dynabeads mRNA Direct Kit (Invitrogen). This kit uses magnetic beads with oligo(dT)-sequences bound to it, which will hybridize to the polyA tail of mRNA, allowing separation of mRNA from other ribonucleotides using a magnet.

Gene	5'RACE	3'RACE primer
COXI	CCCCGGCTAGGTGGAGGGAAA CACCTCCAGCGGGGTCGAAG ACACTTCTGGGTGGCCGAAGAA	CTTCGACCCCGCTGGAGGTG TCTGATTCTTCGGCCACCCAGA TCACGGTAGGGGGCCTAACAGG
COXII	TCAAGATGCAGCCTCCCCCG TGCGAAGGGATGGGAGAGCA CCTTCGATTCTTATATGGTCCCCACCC	GAAGCCCCCGTCCGAGTCCT CCTGGCACCCGGACAATTTCG TGCCTCTCGCCCAGGAGTTTTC
COXIII	CAGATGGCAAGGCCGGAGGT GGTGCCAAGGACAATGAGGAC GA CCTTCTCGGACAATATCCCGTCATCA	TGGAGCCTACTTCACATTCCTGCAA GCAACCGGCTTTCACGGCCTA TGGCACCACCTTCCTGGCTGT

Table 5. Overview of primers used for RACE.

In order to ensure gene-specific amplification of the desired target, gene-specific primers were designed from the sequence obtained in the previous cloning and sequencing experiments (table 5). Once again the Primer3 web resource was used in designing the primers, and the primers were synthesised by Invitrogen.

A SMART RACE cDNA library were created using Superscript RT II (Invitrogen) and SMART RACE cDNA Amplification kit (Clontech) following instructions from the manufacturer.

After amplification by SMART RACE the product size was confirmed by running an agarose gel, and then the product was ligated into the pGEM-T Easy vector (Promega) and transformed into *E.coli* cells in order to amplify the product before sequencing as previously described.

2.3.3 Real-time RT-PCR

2.3.3.1 Exposure

There were four treatment groups; normoxia exposure, anoxia (O_2 < 0.5% of air saturation) exposure, H_2S exposure (~1.0mM of total-sulphide) and a final group of both H_2S and anoxia exposure. Anoxia was achieved by bubbling the closed fish tank with N_2, while the normoxic group was bubbled with air. Each group consisted of 10 fish. After 2 hours of exposure the fish were killed by cutting the spinal cord. From each fish the heart, brain and liver were dissected out and quickly frozen at -80˚C within a few minutes.

2.3.3.2 Isolation of Total RNA Using TRIzol Reagent.

While still frozen the tissues were weighed and transferred to tubes containing TRIzol (Invitrogen) and Teen D lysing Matrix beads (MP Biomedicals). The volume of TRIzol was adjusted according to the weight of the tissue. Then the tissue was homogenized using a homogenizer (Ultra-Turrax T 8, IKA). A 50 pg external standard per mg of tissue was added to the samples that were going to be used for mRNA quantification (see section 2.3.3.4). The rest of the RNA isolation was carried out according to the TRIzol protocol. RNA was stored in -80˚C. After isolation the concentration of RNA in each sample was determined by measuring light absorbance in 1:5 dilutions at 260 nm with a spectrophotometer (Nanodrop ND-1000).

2.3.3.3 cDNA Synthesis

Before cDNA synthesis all RNA samples were treated with TURBO DNase (Ambion) to remove any traces of genomic DNA. This was done according to the manufacturer's instruction with one exception; due to low RNA content in the samples 0.1 µl of DNase Inactivation Reagent were added for each µl of total volume. The cDNA synthesis to be used for real-time quantification was performed using random primers (Invitrogen) and SuperscriptIII (Invitrogen). A 14 µl mix was made containing 1µg totRNA (for liver), 0.5 µg totRNA (for brain) and 0.2 µg totRNA (for heart), 30 ng random primer and 10 mM dNTP (Invitrogen). This mixture was incubated for 5 min at 65˚C and then cooled on ice for 1 min. At this point the volume of the mix was increased to 20 µl by adding 5x Superscript First strand buffer, 200 U of Superscript RT III and DTT (all from Invitrogen). After a 5 min incubation at 25˚C the mixture was incubated for 60 min at 50˚C, followed by 15 min incubation at 70˚C. In the end the cDNA samples were diluted 10 x by adding 180 µl of MQ-water.

2.3.3.4 Real Time RT-PCR

The real-time RT-PCR assay was carried out on a LightCycler 480, using LightCycler 480 SYBR Green 1 Master Kit (Roche). SYBR Green is a fluorescent dye that binds to dsDNA minor groove, emitting fluorescence when bound (Morrison et al., 1998). This allows monitoring of the levels of amplified product when SYBR Green binds to dsDNA. It is during the exponential rise of the PCR product that quantification takes place, as this is the only phase the reaction is exponential. The LightCycler software uses the second derivative method to calculate the point where the increase in fluorescence is highest, called the Cp-value. The more cDNA there is when starting the process the faster this point will be reached, resulting in a lower Cp-value.

The relative expression of a target gene is calculated in comparison with a reference gene, using the primer efficiency (*E*) and the Cp-value as seen in formula 1.

$$relative\ expression = \frac{(E^{CP})_{ref}}{(E^{CP})_{target}} \quad (1)$$

(Pfaffl, 2001)

The primers for real-time RT-PCR were designed based on the gene sequence obtained from cloning and SMART RACE cDNA amplification, and using Primer3. For each gene three sets of gene specific primers were designed, each with a melting-point of ~60˚C, and the efficiency of each primer pair was found by making a dilution curve from the cDNA to be used later in experiments. The dilutions were 1/10, 1/20, 1/40, 1/80 and 1/160, and these dilutions were used in a real-time PCR with the different primers. Based on the Cp-values from each dilution the Light Cycler software calculates the efficiency for each of the primer pairs.

The primer efficiency *E* is calculated by the slope of the dilution curve according to formula 2 given by Rasmussen (2001):

$$E = 10^{(-1/slope)} \quad (2)$$

The efficiency is a measure of how much PCR-product is amplified per cycle. *E* is given as a number between 1 and 2, 1 indicating no PCR product per cycle and 2 indicating that every PCR-product is amplified. The primer pairs with the best results for efficiency and yielding satisfactory Cp-values were selected and are shown in table 6.

The real-time RT-PCR was run using the following program; starting with an incubation period of 10 min at 95 ˚C, then 42 repeats of 10 sec at 95˚C, 10 sec at 60˚C and 10 sec at 72 ˚C. The following settings were used for the melting curve assay: 5 sec at 95 ˚C, followed by 10 sec at 65˚C and a continuous heating to 97˚C.

In this thesis the data have been normalised against an internal control. The advantage of using such a control is that it compensates for differences that would arise from the early steps of treatment, such as variation in starting material, quality of RNA, variations in the efficiency of the RT-reactions, and differences in mRNA isolation. This is because the control would be exposed to the same treatment as the gene (Radonic et al., 2004). None of the traditionally used housekeeping genes are always suitable to use, as

they are expressed at different levels in different experimental exposures (Ellefsen et al., 2008; Tichopad et al., 2004).

Beta actin and GAPDH was chosen as candidate house keeping genes. To test if these house keeping genes were stable in our experiments, we used an external standard when doing real-time RT-PCR. This was added to our tissues in the RNA extraction and was subsequently used to control the expression level of the housekeeping genes (Ellefsen et al., 2008). By combining the use of an external standard and an internal control, the insecurities of unstable house keeping genes are removed. Beta-actin was found to be the best house keeping gene to use, and was subsequently used to normalize the real-time RT-PCR data for the COX genes.

Gene	**Forward primer (5'→ 3')**	**Reverse primer (5'→ 3')**
COXI	GACACCCGAGCCTACTTTACA	GCGGGGGATCATTTGATATT
	CGCAATTCCTACAGGCATA	ATTATACCGCCCCCTAGTA
	CTGCCAGTATTAGCAGCAGGT	GCGGGGTCGAAGAAAGTAGT
	AGCCGGGGTGTCTTCTATCT	TGGGTTATAGCAGGGGGTTT
	ACCCCCTGCTATAACCCAAT	GCTGCTAATACTGGCAGTGAGA
	CCGTACTAATTACAGCCGTCCT	GCGGGGTCGAAGAAAGTAGT
COXII	AATGGACGCAGTACCTGGAC	TCTCGCCCAGGAGTTTTCTA
	GGAGTCAAAATGGACGCAGT	**CCCCACAAATTTCTGAGCAT**
	GACCACCGAATGATTGTTCC	ACTGCGTCCATTTTGACTCC
COXIII	TAACCTGAGCACACCACAGC	TTGCTTGCAGGAATGTGAAG
	ATCGCTGACGGAGTTTATGG	**GAGACAGACAGCCAGGAAGG**
	CCATTCAATCCCTTGCTCTC	GAGCCATAAACTCCGTCAGC
Beta-actin	CAGGCTGTGCTGTCCTTGTA	CATAGATGGGCACTGTGTGG
	AGCCAACAGGGAGAAGATG	GGTGGTACGACCAGAAGCAT
	GAGCACCCTGTCCTGCTTAC	**GGGGTGTTGAAGGTCTCAAA**
GAPDH	AAAGTCATTCCCGAGCTCAA	TCATACTTGGCGGGTTTCTC
	GAGAAACCCGCCAAGTATGA	AAGTCCGTTGAGACGACCTG
	AGCTCAACGGAAAACTGACC	**TAGCCCAGAATTCCCTTCAG**

Table 6. Primers used for real-time RT-PCR amplification. Primer pairs in **bold** were selected as the most optimal based on satisfactory efficiency and Cp-values.

The significance of any changes was tested using Kruskal-Wallis test, as the tested groups often showed differences in their variances. Kruskal-Wallis is an analogue to the one-way analysis-of-variance (ANOVA) for non-normalised populations, and it can be used to test if several independent samples of observations stem from the same original distribution. The null hypothesis stating that the populations are equal was rejected at *P*-values ≤ 0.05.

3. Results

3.1 Respirometry

The aims were to examine how well adapted the fish is to hypoxia and H_2S by measuring changes in oxygen consumption.

3.1.1 Determination of $[O_2]_{crit}$ and Resting Metabolic Rate

The metabolic rate (measured as oxygen consumption, VO_2) in resting gobies was found to be 72.05 ± 2.65 mg O_2 h^{-1} kg^{-1} (mean ± SD) (*N*=11). Further the critical oxygen concentration ($[O_2]_{crit}$), the lowest $[O_2]$ where the fish is able to maintain its resting O_2-consumption, was found to be 5.3 ± 0.88% (mean ± SD) of air saturation (*N*=7), as shown in figure 8.

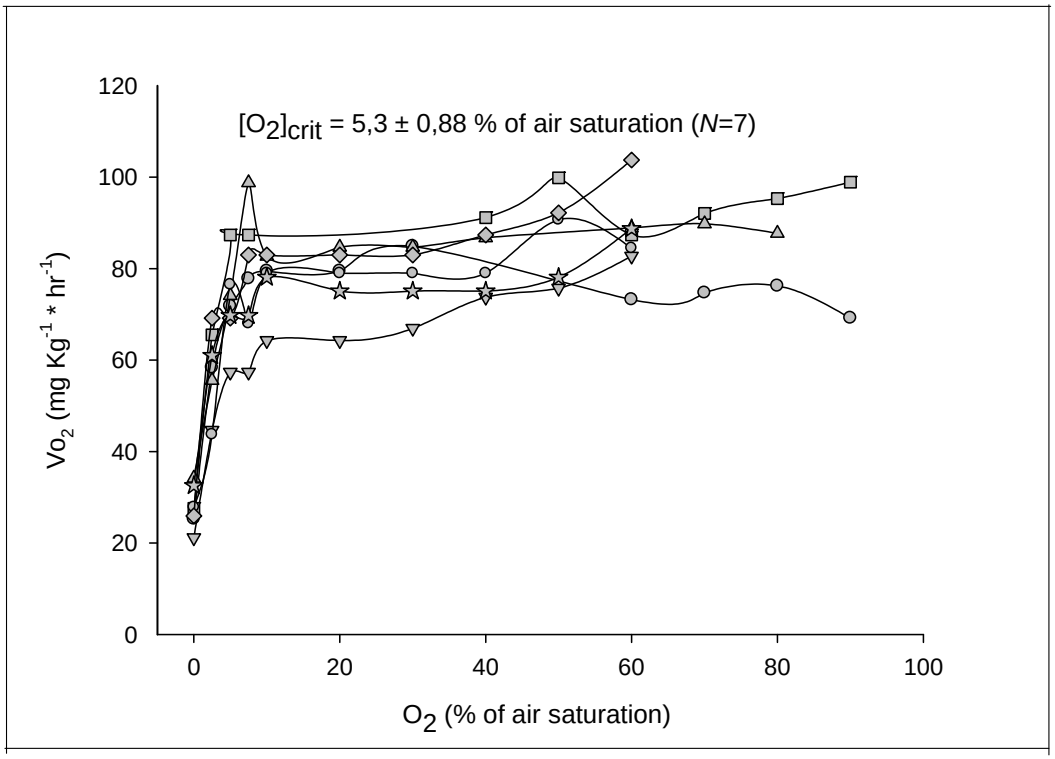

Figure 8. Graph showing a more or less stable VO_2 in 7 fish faced with a decreasing level of oxygen, until $[O_2]_{crit}$ is reached , whereupon a fast drop in VO_2 is seen.

3.1.2 Effect of H_2S on oxygen consumption

Experiments were conducted by running two chambers, one with a fish and one without a fish, as described in the method and materials section. A typical result is illustrated in figure 9.

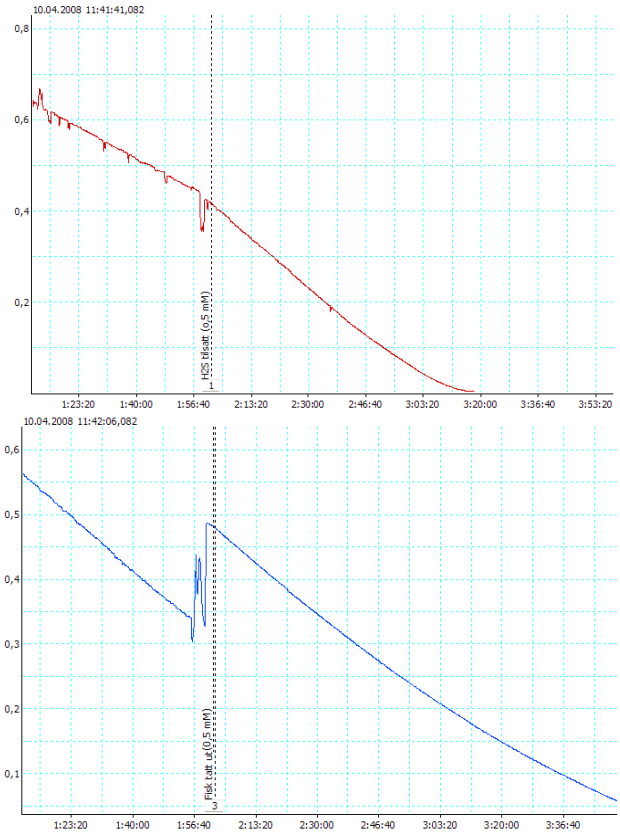

Figure 9. Example of respirometry with addition of H_2S: The graph on the left shows the oxygen tension in the chamber where the fish was kept. The graph on the right shows the chamber where the fish is removed, and gives the rate of the reaction between H_2S and O_2, to be subtracted from the rate of consumption after addition of H_2S in the left graph. In both chambers 0.5mM H_2S was added after ~2 hours. In this example consumption was partly inhibited.

Five different concentrations of H_2S were added to different fish to find the concentration where H_2S blocked respiration. Oxygen consumption was not inhibited by the lower levels of H_2S added (3.6µM and 7.2 µM). At higher concentrations of H_2S, consumption is nearly (18 µM H_2S) and completely (36 µM and 43.2 µM H_2S) inhibited (figure 10). Values for H_2S were calculated to be 3.6% of total sulphur added (at ~15˚C and 3.5% salinity) according to (Millero et al., 1988). All fish survived the experiments.

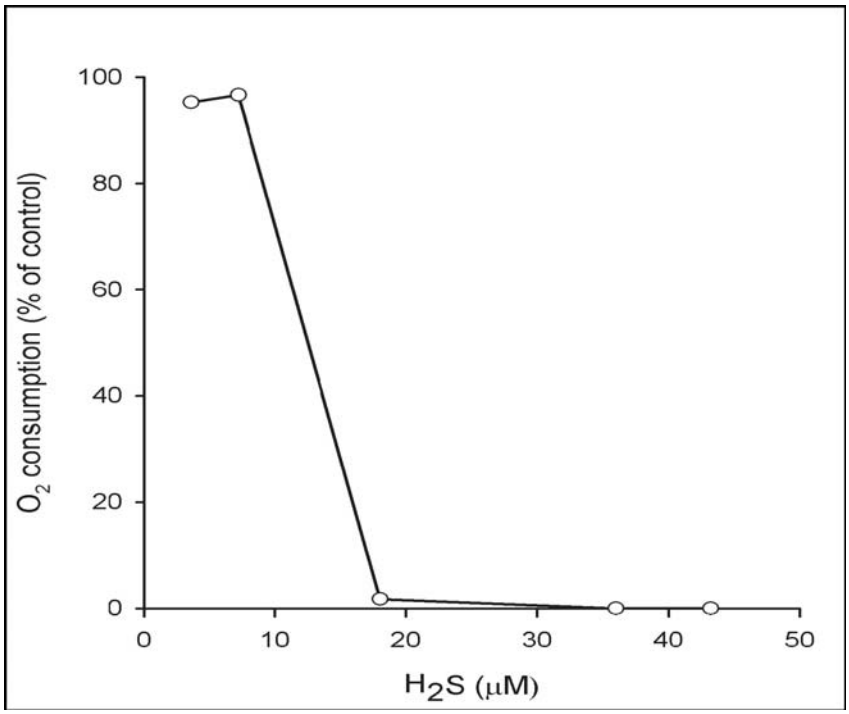

Figure 10. Oxygen consumption when the fish is exposed to varying concentrations of H_2S in % of control. Each point is the result of one fish. Oxygen consumption is not inhibited by low levels of H_2S. At 18μM consumption is almost totally inhibited, and O_2 consumption stops completely at higher values.

3.1.3 Build up of Oxygen Debt

To find out if the gobies built up an oxygen-debt in anoxia we did anoxia exposure experiments, and compared oxygen consumption before and after the exposure. Fish that can produce ethanol has no oxygen debt when reoxygenated and this will indicate whether or not the pelagic goby can produce ethanol or another alternative metabolic end product than lactate. The results showed a significant increase in oxygen consumption after anoxia. Average oxygen consumption before exposure to anoxia was at 58.5 ± 9.9 mg kg^{-1} h^{-1} (mean ± SD) , and after an average of 2 hours in anoxia the O_2 consumption average rose to 89.9 ± 16.4 mg kg^{-1} h^{-1}(mean ± SD, n = 7) (exemplified for one fish in figure 11).

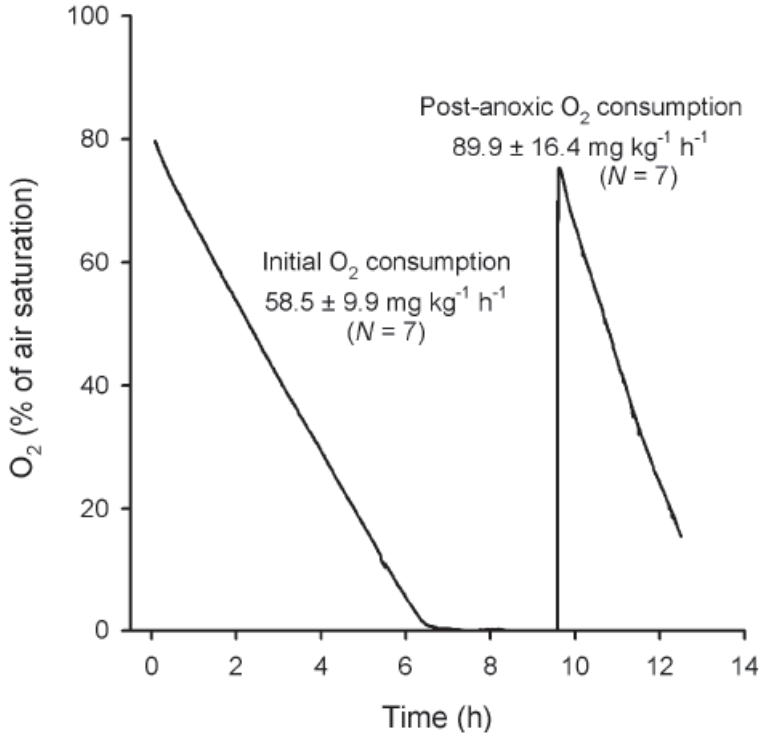

Figure 11. Oxygen consumption before and after exposure to anoxia. The trace is from one fish while the values given are from 6 fish. The more steep line to the right in the graph reveals an increase in O_2-consumption after exposure to anoxia.

3.2 Molecular Experiments

Here, the aim was to use real-time RT-PCR to test for anoxia and H_2S induced changes in gene expression in *S. bibarbatus.*

3.2.1 Cloning and Sequencing

The sequences of COX I-III in the pelagic goby were obtained by PCR, RACE-PCR and subsequent sequencing as described in the Materials and Methods section.

3.2.2 COXI

Sequencing of the cloned COXI gene is compared to others species in figure 12. Hs is *Homo sapiens*, while the other three are fish species (*Oryzias latipes, Gillichthys mirabilis and Gobiodon histrio*). Comparison of nucleotide sequence gave a 79.6 % similarity between the COXI subunit of *S. bibarbatus* and *Oryzias latipes*, while comparison to another goby (*Gobiodon histrio)* gave a 78.5% similarity.

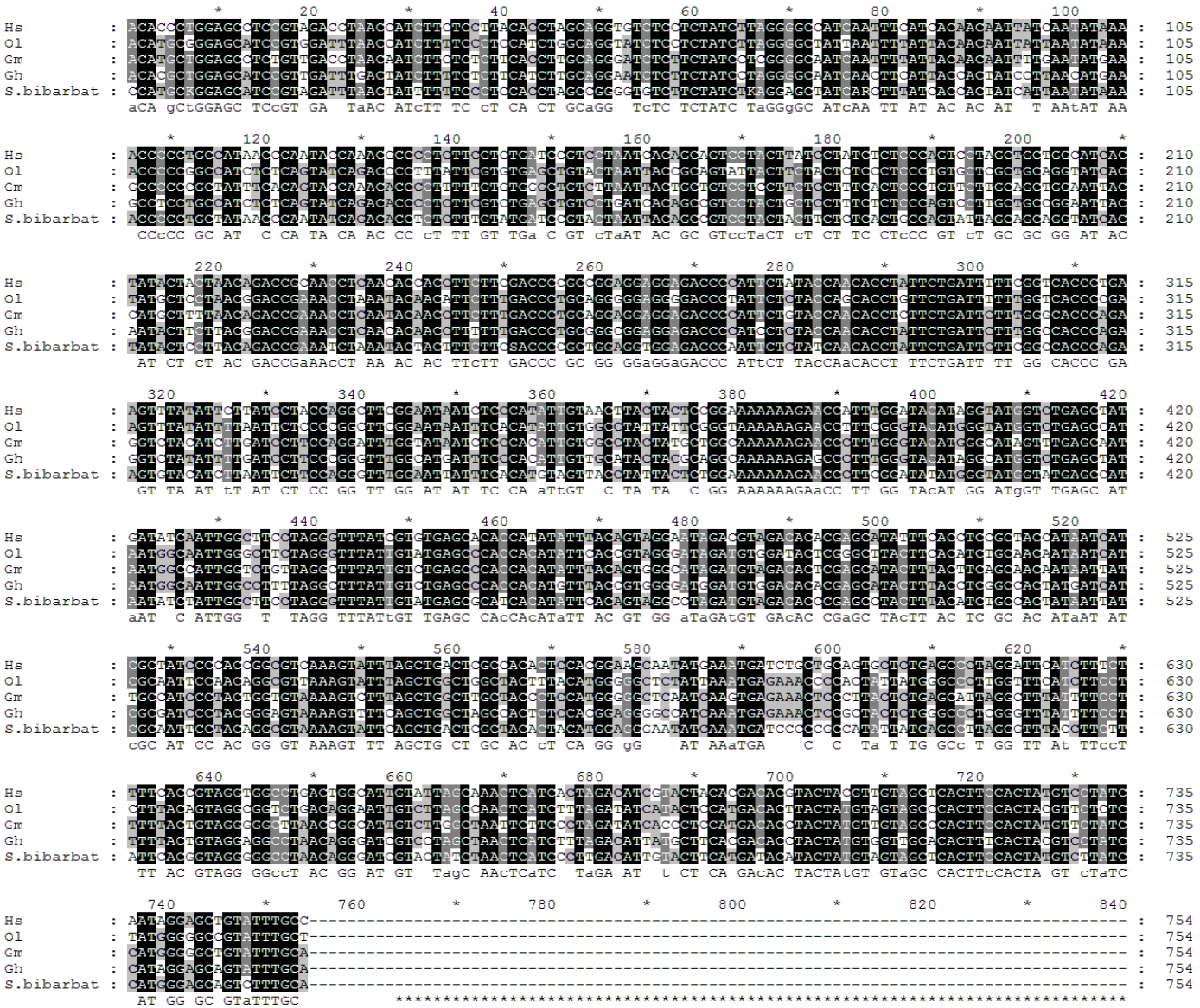

Figure 12. Comparison of nucleotide sequence of COXI in five species. From this we can see there is a 78.5% similarity between *S. bibarbatus* COXI and the same gene in *Gobiodon histrio* (Gh), another goby.

3.2.3 COXII

Sequencing of the gene for COXII in *S. bibarbatus* gave a 79.5% similarity to the same gene in *Oryzias latipes* (figure 13). Other species in comparison are *Carassius carassius*, *Homo sapiens* and *Gadus morhua.*

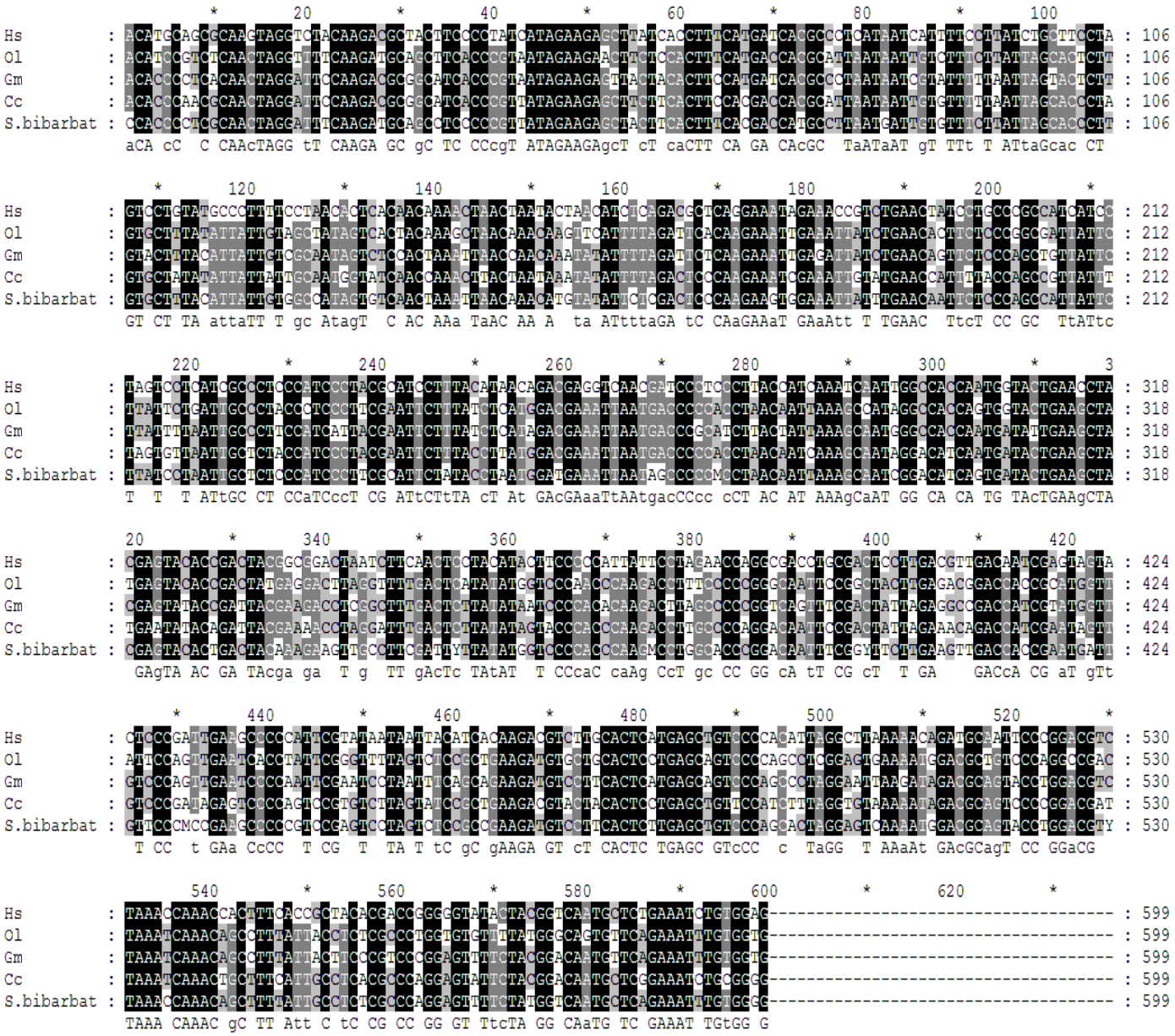

Figure 13. Comparison of nucleotide sequence of COXII in five species. From this we can see there is a 79.5.6% similarity between the *S. bibarbatus* COXII and the same gene in *Oryzias latipes*.

3.2.4 COXIII

The nucleotide sequence of COXIII in *S. bibarbatus* compared to the same sequence in three other species can be seen in figure 14. When comparing the nucleotide sequence to *Oryzias latipes* a 80.2% similarity was found, while comparison to another goby, *Gymnogobius pteschiliensis*, gave a 78.2% similarity between them. Other species are *Homo sapiens* and *Oplegnathus fasciatus*.

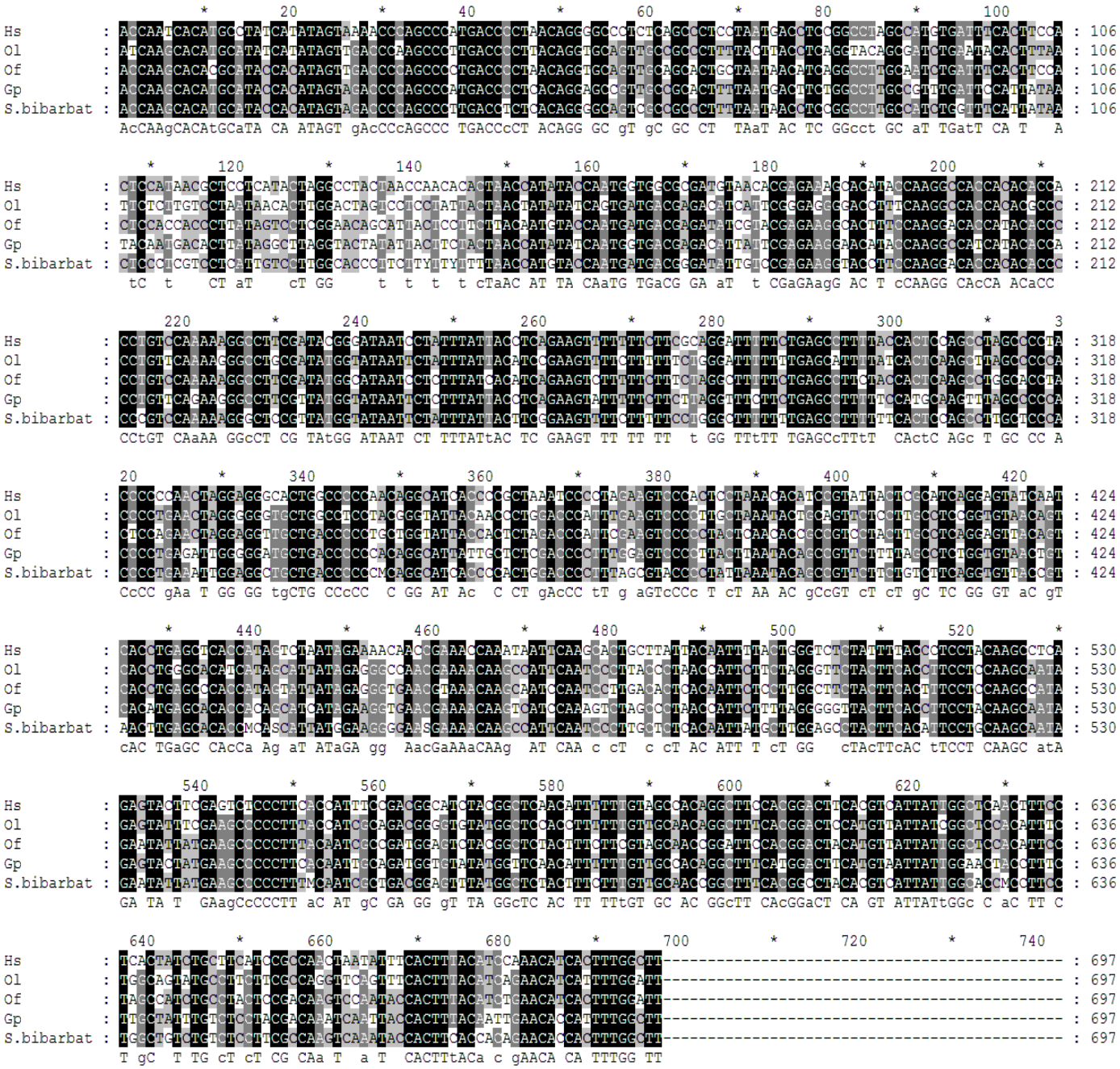

Figure 14. Comparison of nucleotide sequence of COXIII in five species. From this it can be seen that *S. bibarbatus* COXIII is 78.2% similar to the same gene in another goby (*G. pteschiliensi. Gp))*

3.3 Real-time RT-PCR

Studies of the expression of COX genes in heart, brain and liver of the pelagic goby were done using real-time RT-PCR. The gene expression was measured in four groups; normoxia (N), anoxia (A), H_2S (H) and anoxia combined with H_2S (HA).

3.3.1 Reference Genes

In order to confirm that our reference genes were expressed at a stable level when treated with anoxia and/or H_2S, we normalised expression of beta-actin to the levels of known amounts of an added external standard 2A4 (figure 15).

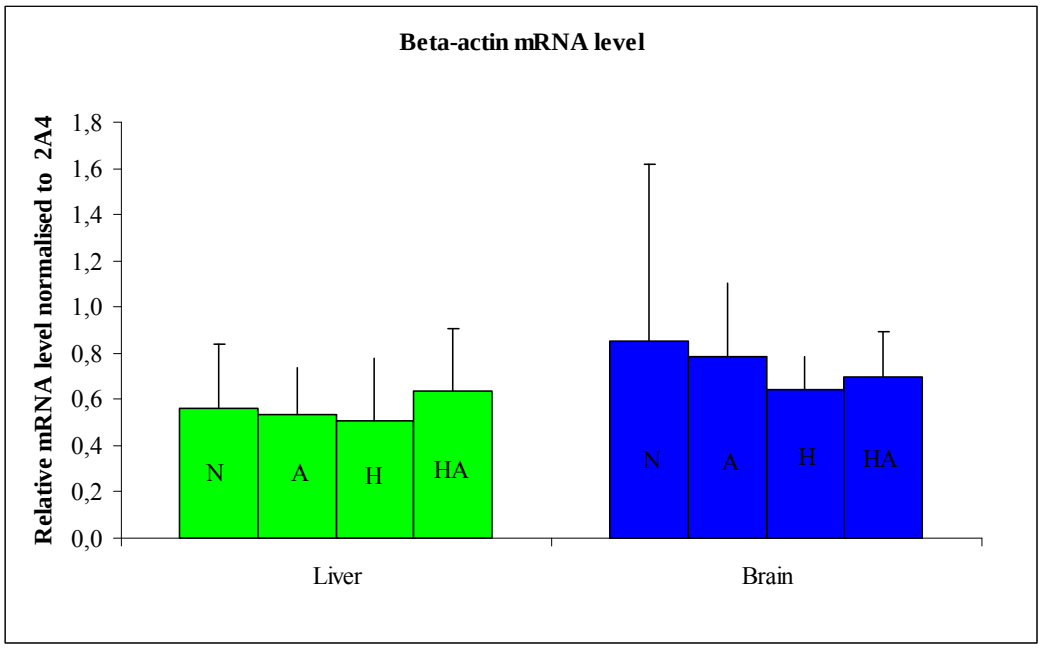

Figure 15. Beta-actin mRNA levels in liver and brain of *S. bibarbatus* normalised to an external standard. No statistically significant difference in mRNA levels was found (p=0.69 for both groups calculated separately), so beta-actin was used as a reference gene.

In addition statistical calculations were done comparing expression levels of beta-actin (without any normalization) in all three tissues and all exposure-groups, and this also gave no statistically significant difference in expression levels ($p = 0.22$ for heart, $p = 0.75$ for liver and $p = 0.31$ for brain) (figure 16). Because of the stable expression of beta-actin, we

decided to use beta-actin as reference gene for the COX gene expression, as this gave a smaller sample-to-sample variation than using the externally added control gene as reference.

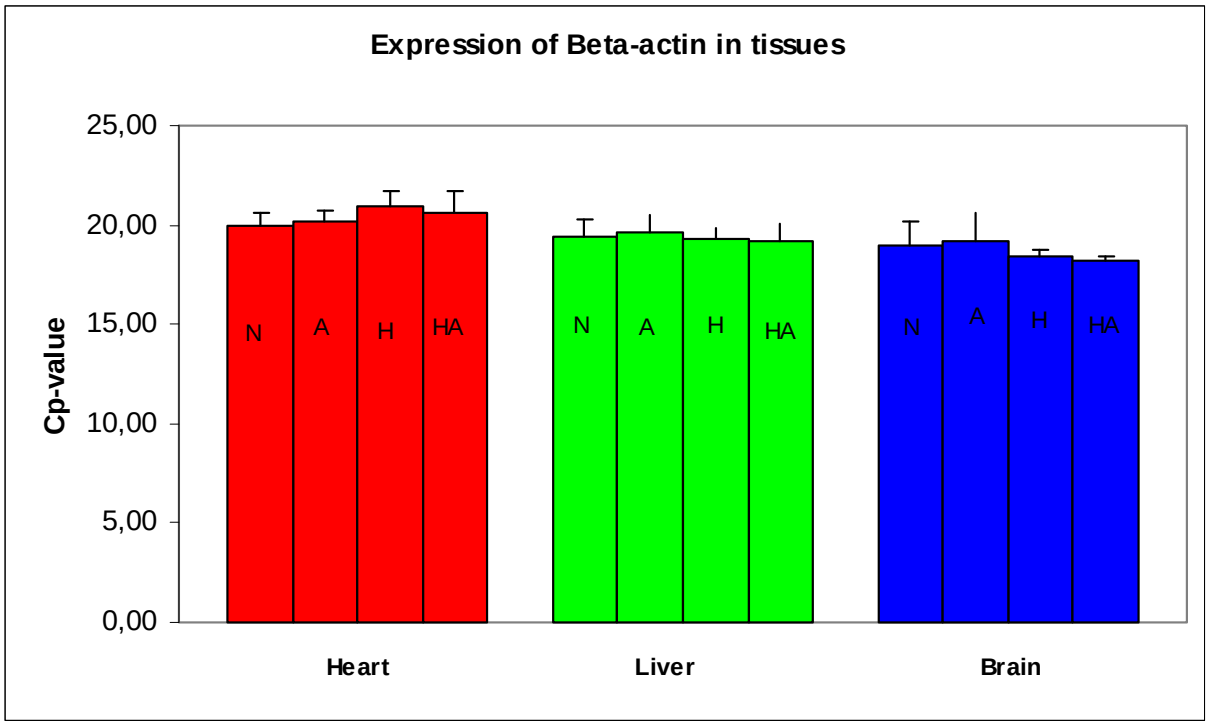

Figure 16. . Beta-actin mRNA levels in heart, liver and brain. All three groups showed similar levels, Cp = 19.48 ± 0.81 (mean ± SD), and no statistical significant differences in between groups was found.

3.3.2 COXI

When running real-time RT-PCR on COXI there was no detectable expression. Six different primer pairs were tested and neither gave a positive result. We concluded that the expression of this gene was too low to give an accurate result in this assay and it was thus omitted from subsequent experiments.

3.3.3 COXII

There was no statistically significant differences between either of the groups in heart ($p = 0.35$), liver ($p = 0.40$) or brain ($p = 0.70$) when comparing the treatments to the normoxic control (figure 17). This suggests that expression of COXII in these tissues was not effected by anoxia or H_2S.

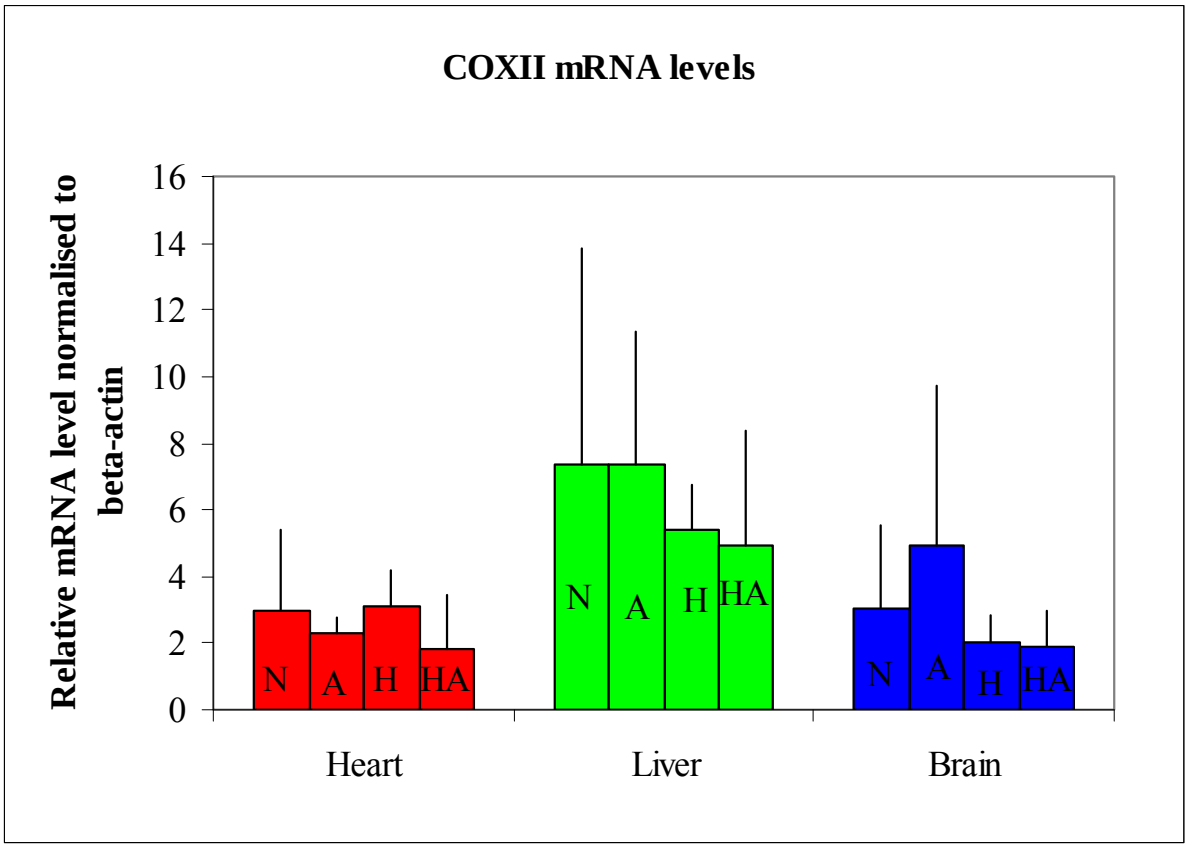

Figure 17. COXII mRNA levels in the pelagic goby, normalized to Beta-actin. Neither heart, liver or brain showed any statistically significant change in expression of COXII in response to the treatments.

3.3.4 COXIII

The results of comparing the expression of COXIII in the tissues when exposed to the various treatments. There was no statistically significant change in expression of COXIII. (p= 0.96 for heart; p = 0.85 for liver; p = 0.48 for brain, figure 18).

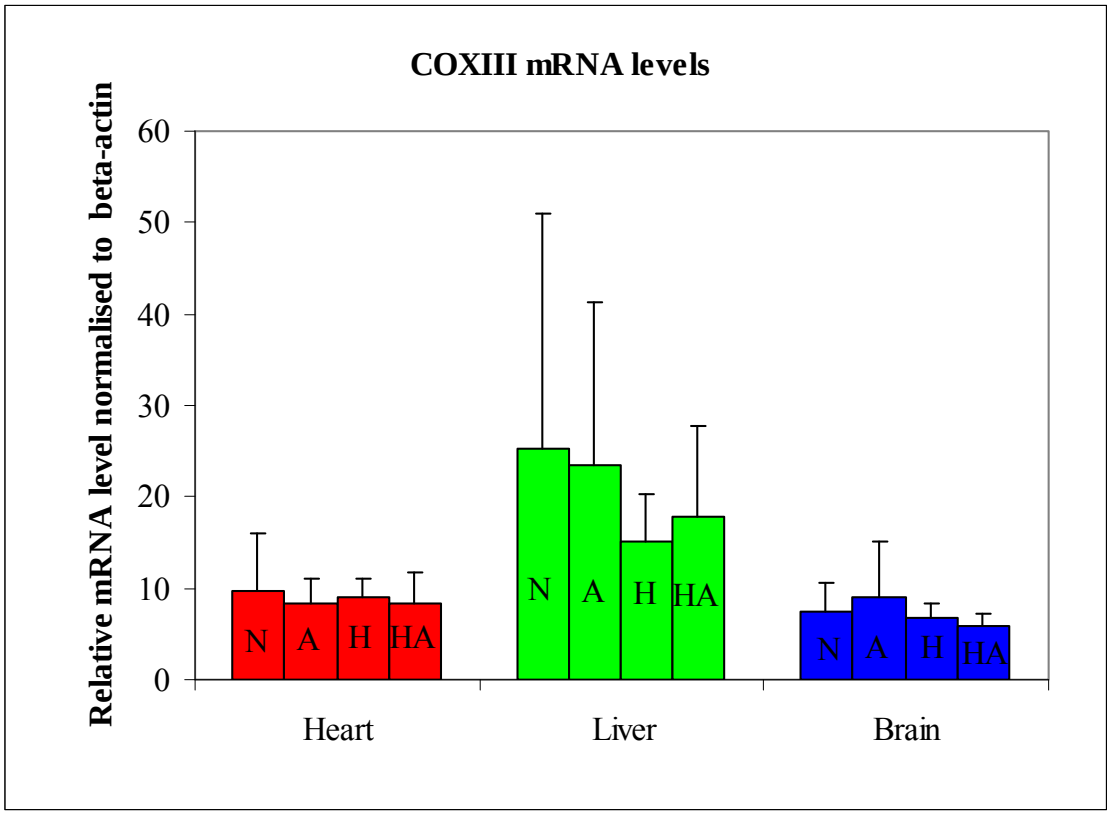

Figure 18. COXIII mRNA levels normalized to Beta-actin. Each of the four exposure groups tested is shown here, grouped by tissue. There was no statistically significant change in expression of either group in either tissue.

Thus the treatments did not appear to affect the expression of COXIII in the tissues examined.

4. Discussion

4.1 Respirometry

Metabolic rate, and therefore oxygen consumption, is dependent on activity level, size and temperature. Thus, small highly active fish in tropical waters have the highest rates, as exemplified by the record high rate of oxygen consumption of swimming coral reef larvae, which consume up to 6000 mg O_2 kg^{-1} h^{-1} (Nilsson et al., 2007). A high metabolic rate will also lead to a high $[O_2]_{crit}$ due to higher demands for oxygen uptake. Because of this it is very important to compare experiments done at similar temperatures.

A resting metabolism of 72.05 ± 2.65 mg O_2 kg^{-1} h^{-1} (mean ± SD) which was found in the pelagic goby corresponds well with other similar measurements, for example the resting metabolism found in another goby *Gobiusculus flavescens* of 88 mg O_2 Kg^{-1} h^{-1} (similar in size, and temperature of 15 ˚C) (Thetmeyer, 1997).

The pelagic goby was found to have an $[O_2]_{crit}$ = 5.3 ± 0.88 % of air saturation (mean ± SD, figure 8). When comparing to other hypoxia-tolerant species this is an extremely low $[O_2]_{crit}$. Generally, species that are tolerant to hypoxia have a lower critical oxygen tension than species that are not hypoxia tolerant (Nilsson and Randall, 2010 (in press)); and when compared to the overview presented by Nilsson and Randall (2010 (in press)), the goby has the lowest $[O_2]_{crit}$ found (5.3% of air saturation = 0.44 mg O_2 l^{-1} compared to 1.0 mg O_2 l^{-1} for crucian carp at similar temperature, see table 1, section 1.4.). That the *Sufflogobius bibarbatus* appears to display the lowest $[O_2]_{crit}$ ever measured in a fish suggest it is very well adapted to survive the low oxygen levels often found on the ocean floor off the Namibian coast. This is thus highly likely to be one of the prerequisites for its success in this habitat.

4.1.1 Effect of H_2S on Oxygen Consumption

The effect of H_2S on respiration was measured using varying concentrations, from 3.6μM to 43.6μM. The two lowest concentrations do not inhibit oxygen consumption (figure 10), but as concentrations increased above 18 μM, respiration was completely inhibited.

However, the fish still survived at least 2 hours at these high H_2S concentrations. Comparing this to table 2 we see that these concentrations are similar to the inhibitory limit for isolated mitochondria studied in other vertebrates (including mammals). Thus, *S. bibarbatus* does not have a COX that is insensitive to H_2S, and therefore its ability to survive sulphide exposure must relate to its ability to survive anoxia, because having an inhibited COX will be functionally identical to anoxia. Indeed, anoxia tolerance has been suggested to be the key adaptation to survive high H_2S concentrations (Bagarinao and Vetter, 1992), and it could be mentioned that the H_2S tolerance of *S. bibarbatus* is similar to that of other highly sulphide tolerant species, such as *Fundulus parvipinnis* (Bagarinao and Vetter, 1992).

4.1.2 Build up of Oxygen Debt

The pelagic goby practices DVM (diel vertical migration) spending the day-time in the benthic zone on the bottom, and migrating into the pelagic zone at nightfall, remaining there till dawn when it returns to the bottom (shown in figure 19). One task for the Namibia expedition was to find out if the pelagic goby resolves the problem of lactate build-up during hypoxia at the bottom by producing ethanol, like the crucian carp does.

Crucian carp does not experience an increase in oxygen consumption after an exposure to anoxia, because it expels its anaerobic end-product (ethanol) from the organism. Our measurements comparing oxygen consumption of the pelagic goby before and after a lengthy exposure to anoxia gave a clear increase in consumption, as seen in figure 11 (see Results, 3.1.3.). This indicates that the goby accumulates an oxygen debt and does not produce a significant amount of ethanol during anoxia. Indeed, measurements made by others involved in the project showed that no ethanol could be detected in the blood of anoxic gobies, and that there was a significant rise in its blood lactate levels during anoxia exposure (see Appendix A) Thus, after anoxia and H_2S exposure, the goby will need to pay off an oxygen debt by consuming more oxygen than the basal resting metabolism would indicate. This ties in well with its diurnal migration pattern, indicating that one reason for its ascent from the bottom at night is to pay off an oxygen debt acquired at the bottom.

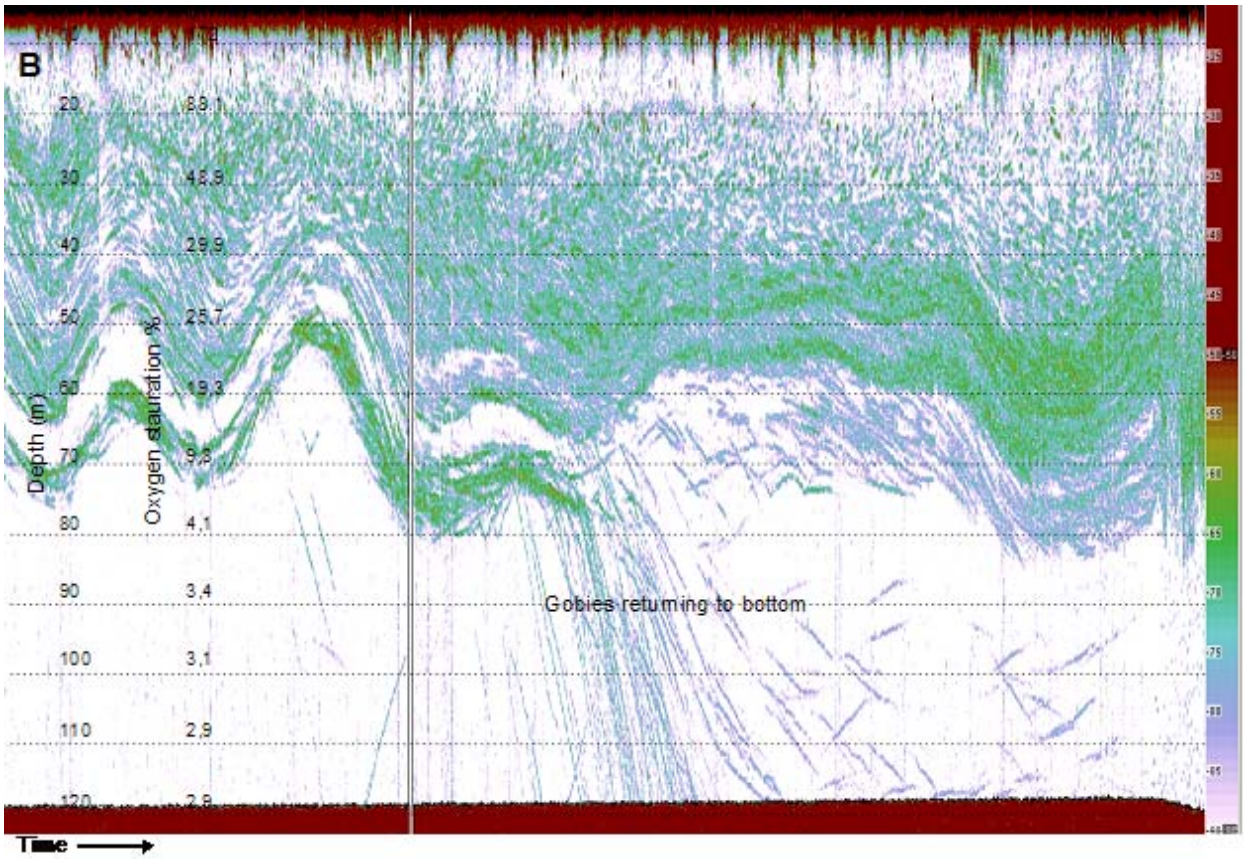

Figure 19. Echogram of the ocean. This figure shows an echogram taken while the boat was in a fixed position, over an extended period of time. We can clearly see that there are several layers of fish moving with the current, and at a given point in time (dawn) suddenly the lower layer of fish dissolve and the fish return to the hypoxic water. From Utne-Palm et al (in review).

Combining trawling and echograms gave the expedition the opportunity to study the feeding habits of the goby, and analysis of stomach content showed that it feeds mainly upon benthic polychaetes and diatoms. This contradicts the previous belief that the goby performs DVM in order to feed at night. Further analysis of the stomach-contents showed that the contents remained largely undigested during the day, and it becomes gradually digested as it migrated into the pelagic. From this we concluded that the *S. bibarbatus* enters the pelagic both to digest the food eaten while it is sheltered in the muddy bottom, and to re-oxygenate the blood and pay off the oxygen debt.

4.2 Molecular Experiments

4.2.1 Cloning and Sequencing

An initial aim for cloning COX was to investigate if it was different from other COX in a way that could help the pelagic goby survive exposure to H_2S. However, as our respirometry results, combined with literature data on mitochondrial H_2S sensitivity in other vertebrates, suggested that the goby's capacity to tolerate H_2S stems from anoxia tolerance rather than having an especially H_2S-tolerant COX, this possibility was rejected. Indeed, the cloning of all three subunits of COX revealed a relatively high degree of similarity to the same genes in other species (79%-80% correlation to *Oryzias latipes*). Thus, COX appears to be a highly conserved gene, found in a genome without an internal correction system, the mitochondrial genome. This indicates that there is a strong evolutionary selection for keeping COX un-mutated.

4.2.2 Real-time RT-PCR

Expression of COXI could not be quantified with real-time RT-PCR. This could indicate one of three things. One reason could be that none of the primers tested were good enough and did not bind to any area of COXI mRNA. However, after having tried six different primers this seems unlikely. More likely, it could be that COXI mRNA is expressed at a very low level, possibly because the protein is very stable and therefore does not need to be synthesised very often. Finally, the mRNA itself could be very unstable and therefore we did not manage to isolate it.

mRNA expression of COX II and III seemed unaffected by anoxia and/or H_2S (figure 17 and 18). This is contradictory to the findings of Poyton and McEven (1996) done at protein level where they found that both COX I and II were regulated by the presence of oxygen. If such a regulation exists in the pelagic goby, we could have neglected to discover it because of post-transcriptional regulation of expression. This would give the same amount of mRNA without it being translated into protein, falsely indicating a maintained expression level (as reviewed by Greenbaum et al, 2003). Experiments have been done where an reduction in mtDNA did not cause a noticeable

reduction in mRNA of COX genes, supporting that regulation occur post-transcriptionally (Trifunovic et al., 2004). However, it does seem wasteful to be producing mRNA that will not be used during an energy crisis such as anoxia. A second way of having higher amounts of COX II and III proteins during anoxia would be if the degradation was reduced, giving each protein a longer lifespan. This would not require any increase in production of mRNA, but would give higher amount of protein found. An investigation into protein-expression of COX in *S. bibarbatus* could possibly yield different results. Another possibility, maybe the most likely one, is that the gobies studied already had their COX genes fully induced, as they were caught in an environment where they would be regularly exposed to hypoxia. Indeed, this could be a factor behind their low $[O_2]_{crit}$ and future experiments could be aimed at comparing *S. bibarbatus* from different habitats, as well as studying COX expression in individuals caught in well oxygenated areas and exposing these to hypoxia, anoxia and H_2S.

It is also possible that changes in COX expression could be occurring in other subunits presently not studied. Kwast and Burke (1998) reviewed the effects of hypoxia in yeast stating that subunit V seems to be regulated by anoxia. The nomenclature surrounding the subunits of COX is a little confusing, but this is a paralog to subunit IV in mammals. Subunit V in the yeast *S. cerevisiae* can be expressed in one of two paralogs, expression of Va at normal oxygen levels and when oxygen concentration drops below 1μmol L^{-1} switches to expression of Vb. Studies of the different subunit V paralogs show that Vb stimulates a higher maximum turnover number (TN_{max}) giving a higher *in vivo* rate of electron transfer (Allen et al., 1995). Allen et al suggest that the Vb paralog increase the catalytic rate of COXI in order to prevent the formation of dangerous partially reduced products like hydrogen peroxide and fully exploit the minute amounts of oxygen present.

Other subunits that are regulated by oxygen have been found in the amoeba *Dictyostelium discoideum*, but this involves subunit VII (homolog of subunit VIc in mammals and VIIa in yeast) (Schiavo and Bisson, 1989). Similarly to regulation of subunit V in yeast one of these paralogs are expressed at normal conditions (VIIe) and switching occurred at hypoxic conditions (to VIIs), although the oxygen threshold for switching is much higher in *D.discoideum* than in yeast.

These findings of several paralogs to several subunits in COX argues for further studies of the *S. bibarbatus* COX, in particular the subunit homologous to yeast subunit V.

4.3. Conclusion

The experiments done in this thesis, combined with other experiments carried out by the expedition, show that the goby has a record low $[O_2]_{crit}$ making it possible for the goby to continue its routine resting metabolism even at very low oxygen concentrations. In addition it can survive several hours of anoxia and H_2S exposure by surviving on anaerobic, lactate-producing metabolism, thereby building up an oxygen debt. This oxygen debt could be an important reason for its nocturnal ascent from the bottom of the ocean to more oxygen rich waters. The ability to survive for an extended time in conditions deadly to most vertebrates gives the goby a great advantage by making it able to seek out food on the anoxic and sulphide rich bottom, while at the same time being out of reach from predators. This may be the key to its success, making it the dominant fish species in this ecosystem. From the molecular studies I can conclude that there is no significant change in the expression of COX subunits II and III, at least not at transcriptional level (COX subunit I could not be quantified), suggesting that a possible induction of these enzymes does not form a part of its H_2S tolerance or hypoxia tolerance.

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Appendix A

Catch me if you can!

A multi niche extremist thriving in the Benguela upwelling system

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Abstract The northern Benguela upwelling system is amongst the most productive in the world[1] . Yet much of the production is not fully utilised and accumulates over the inner shelf as diatomaceous anoxic mud, rich in hydrogen sulphide and methane gas. Kills of fish and invertebrates occur during "sulphur eruptions" when even surface water is affected by hydrogen sulphide (H_2S) from the sediment[2,3]. Few metazoan life forms are capable of surviving the inhospitable seabed environment, which is dominated by various bacteria fuelled by the hydrogen sulphide, both on the mud where white mats of large sulphide-reducing bacteria visibly cover the surface[4] and in the lower water column where blooms of chemolithotrophic bacteria oxidise hydrogen sulphide[5]. Here, however, we report how a remarkable species of fish, the bearded goby *Sufflogobius bibarbatus*, thrives in this inhospitable environment through a combination of unusual physiological and behavioural adaptations: it not only lives within the deadly mud and consumes it as food, but it uses the hypoxic bottom water as a refuge from predators. Nightly vertical migrations allow it to digest the food and pay off the oxygen debt built up at the bottom. Away from the seafloor, it swims with jellyfish, probably to avoid predatory fish. These traits in combination may have allowed *S. bibarbatus* to assume a pivotal role in the Benguela ecosystem following the demise of pelagic fisheries at the end of the 1960s.

Introduction Sulphidic and anoxic marine sediments occur worldwide in areas of natural eutrophication[6]. Intense decay processes in the mud belt off Namibia produce high concentrations of hydrogen sulphide in the surface sediments[7], and evidence of sulphide eruptions date back to the early 1900s[8,9,10,3]. Although chemolithotrophs play a role in detoxifying the overlying sulphidic water[5], we know little of the physiological and behavioural adaptations of higher life forms in this environment. Anticipated climate change effects could lead to more frequent low oxygen events[11] and sulphide eruptions off Namibia[12,13].This scenario poses key survival challenges to organisms and affects, sustainable harvesting of marine resources, which is of major importance in the region[14].

Small gobiid fishes are numerous in nearshore marine environments and they are often the major prey for locally harvested fish[15]. In northern Benguela, however, the bearded goby *S. bibarbatus* plays a far bigger, ecosystem-wide role, where it now occupies

a pivotal position in the shelf's food web[16]. Until the mid 1960s, sardines (*Sardinops sagax*) were the most abundant pelagic fish, but over-exploitation at the end of that decade coupled with environmental changes led to the fishery's collapse[17,14]. Seabirds, penguins, gannets, cormorants, fur seals and piscivorous fish,such as hake (*Merluccius* sp.) and horse mackerel (*Trachurus trachurus*), were forced to find alternative food sources, and gobies have now become their staple diet[16]. Paradoxically, despite the sharp increase in predation, *S. bibarbatus* has increased in abundance[18], even though it is considered a slow-growing, late-maturing and relatively long-lived species with a low fecundity[19]. Here, we present reasons for the success of the bearded goby.

Using the Norwegian research vessel G. O. Sars, we conducted a cross-shelf acoustic survey off Namibia in April 2008 (23º20`S-14º12`E to 23º 40`S-13º15`E), coupled with pelagic and demersal trawling, a full suite of environmental sampling stations, and on-board behavioural and physiological experiments. This allowed us to determine diel patterns of movement and feeding of *S. bibarbatus*, and achieve a comprehensive understanding of the behavioural and physiological strategies underlying the success of this fish in this extreme environment.

Field Observations

S. bibarbatus was most abundant over the inner shelf, down to depths of 200 m, where the seafloor comprised a thick layer of diatomaceous mud characterised by millimolar concentrations of sulphide close to the sediment surface. No other vertebrates were found in the overlying lower water column (see Fig. 1a-b), with oxygen levels below 5 % of air saturation (or 0.3 ml O_2 l^{-1}). Although some *S. bibarbatus* were recorded in the water column during day and night, most of the population was on the seabed during daylight hours, as documented from demersal and pelagic trawls and acoustic records. Video observations have previously shown *S. bibarbatus* to be closely associated with sediments[20], and gobies were caught in multi-core tubes whilst sampling the mud.

Acoustic records coupled with pelagic trawling show gobies ascending from the bottom in the evening to join an existing acoustic scattering layer (SL) in the water column (Fig. 1a), and then returning to the bottom throughout the night, or in the morning (Fig. 1b). These pelagic SLs are dominated by two species of large jellyfish (*Aequorea forskalea* and *Chrysaora hysoscella*), the biomass of which is currently estimated to exceed that of finfish off Namibia[21]. Data from over 11 000 samples of pelagic fish landings at Walvis Bay (1991 – 2006) show that gobies are significantly more likely to be caught with jellyfish than other fish species (Kruskal-Wallis ANOVA by Ranks; H (4, N= 40) =18.18, p=.001), and six times more likely to be found with jellyfish than its predator, the horse mackerel (Table 1). The behavioural experiments were designed to test the hypothesis that *S. bibarbatus* swims with jellyfish to reduce predation risk, as has been shown for juvenile whiting (*Merlangius merlangus*) in other ecosystems[22].

The goby's nocturnal ascent does not appear to be for feeding. Gut fullness was significantly higher, and the state of content's digestion was significantly lower, in ascending than descending fish (Prop. odds logistic regression, $p < 0.001$, see Supplementary Table 1) (Fig. 3a). Thus, gobies appear to feed on the bottom during the day and then digest their food whilst away from the sea-floor at night. This observation is reinforced by the gut contents themselves: benthic polychaetes and diatomaceous mud (Fig. 3b). The branchiospines of the gill are too far apart (0.3 - 1.1 mm) to allow this species to filter diatoms from the water, and they appear to be ingested (directly and/or indirectly) from the benthos, which is rich in giant sulphur bacteria (0.1 to 0.75 mm) (Schulz et al.,

1999), of a size that could be filtered out. Mud surface feeding is known from the pacific goby (*Gobionellus sagittula*), whose gut content consists of diatoms, algal debris and flocculate detritus[23]. Sulphide bacteria are an important prey for tubeworm, clam and snail in seep areas, but not in fish from the same stations[24]. As digestion requires energy, it is suppressed in the hypoxic environment occupied by fish during the day[25], so we propose that *S. bibarbatus* ascends at night in order to digest its food and to repay its oxygen debt. *This goby is the first species ever shown to perform diel vertical migration (DVM) in order to digest food and to re-oxygenate its body.*

On-board experiments

On-board experiments were used to test hypotheses formed during the *in situ* observations, *viz*: (i) that *S. bibarbatus* tolerate anoxia (O_2 < 0.5 % air saturation) and hydrogen sulphide, (ii) has benthic habitat preference, (iii) has a preference for associating with jellyfish to avoid predators. We also tested their predators' ability to cope with hypoxia (using hake), or associate with jellyfish (using horse mackerel).

Physiological studies confirmed our predictions that S. bibarbatus tolerates hypoxia, anoxia and hydrogen sulphide astonishingly well. Thus, it was found to have an extremely low critical oxygen level ($[O_2]_{crit}$ = 5.3 ± 0.3 % of air saturation = the lowest $[O_2]$ at which resting oxygen uptake can be maintained) (Fig. 2a). This should allow it to sustain aerobic metabolism at water depths below the main SL (found at ca 10 % of air saturation; Fig. 1a,b). While *S. bibarbatus* builds up an oxygen debt during anoxia (Fig. 2b), which is probably largely related to their need to oxidize accumulated lactate, the rate of lactate accumulation in the blood declined markedly after 1 h of anoxia (Fig. 2c), suggesting metabolic depression. Metabolic depression is also indicated by their suppressed ventilation rate at $[O_2]$ below $[O_2]_{crit}$ (Fig. 2d). This ability to limit lactate production should increase the time that can be endured on the anoxic mud.

Hypoxia may impair escape responses, and therefore increase the vulnerability of fish when attacked by predators[26]. However, if gobies were touched (using a lever mounted in the lid of the sealed aquaria) all fish reacted with an immediate, fast escape response even after 7-9 h below their $[O_2]_{crit}$ followed by 4 - 5 h in anoxia, (n=7). This shows that anoxic gobies retain neural responsiveness, which is probably needed not only for predator avoidance but also for initiating the ascent at night.

We compared anoxia tolerance in hake (*M. capensis*) and *S. bibarabatus* by measuring the performance of their excised hearts. These experiments showed that the pumping capacity (heart rate x contraction force[27]) of both species' hearts was reduced by ~80 % after 20 min of anoxia. For the goby this likely reflected anoxia-induced metabolic depression. However, for the hake, it probably revealed an inability to cope with anoxia; as after 40 min of subsequent re-oxygenation, only the goby heart recovered to pre-anoxic values, suggesting that the hake heart had sustained permanent damage (Fig. 2e). The implication of this is that hake are not able to coexist with the gobies on the anoxic seafloor, thereby providing the gobies with a refuge from these predators.

Hydrogen sulphide is a respiratory poison that blocks mitochondrial respiration by inhibiting cytochrome c [28]. The rate of oxygen consumption by *S. bibarbatus* was virtually unaffected by 100 - 200 µM total sulphide (corresponding to 6 – 12 µM H_2S [29,28]), while it became 98 % suppressed at a total sulphide level of 500 µM (= 30 µM H_2S) (Fig. 2f). These data correspond well with measurements of the effect of H_2S on isolated vertebrate mitochondria (from both relatively H_2S tolerant fish and mammals), showing that H_2S levels below 6 µM stimulates mitochondrial oxygen consumption (probably because mitochondria utilize oxygen to detoxify H_2S to thiosulphate), while H_2S levels above 11-

14 μM inhibits mitochondrial respiration[28]. This suggests that fish surviving high H_2S levels do not rely on H_2S tolerant cytochrome c [28]. Rather, the key to surviving high [H_2S] is anoxia tolerance, which makes the fish temporarily independent of mitochondrial respiration through a sufficiently high capacity for anaerobic (glycolytic) ATP production. The anoxia tolerance of *S. bibarbatus* gives it an ability to survive the extremely high [H_2S] that peak in the mud where these fish feed and hide during daylight hours (Fig. 4). While the bearded goby's tolerance of H_2S matches the world's most tolerant marine fishes (see Table 2 in[30]), which all are marine species inhabiting muddy coastal waters (see Table 2 in[30]), it is the first fish reported to survive such extremes in an open coast habitat.

In individual, onboard habitat choice experiments, *S. bibarbatus* showed a significant preference for sediments of diatomaceous mud (collected on station from benthic cores) rather than aerated sand, (2-sample t-test on normalized arc-sine transformed data: t=3.29; p=0.0017, n=30). We even observed the fish burrowing into the sulphur-rich mud, especially if threatened or disturbed. These findings suggest *S. bibarbatus* deliberately exposes itself to a more extreme environment in order to avoid predation.

Interactions between jellyfish and horse mackerel, and jellyfish and gobies were examined by allowing fish to choose between two chambers during a 300 sec trial. The chambers, one with and one without a jellyfish (*C. hysoscella),* were separated by a wide mesh screen. Horse mackerel strongly avoided jellyfish and either fled the chamber these were in (within 18 sec ± 11 sec, mean ± s.e.), or never moved across the mesh divider to associate with jellyfish. In contrast, gobies took significantly (2-sided t-test: $p<0.001$; df = 25, n=14) longer (210 sec ± 37 sec, mean ± s.e.) to leave the jellyfish chamber, and they frequently moved across the divider. *This confirms our prediction from field observations and fish landings that gobies in the water column choose to associate with jellyfish, but their predators do not.*

Conclusion

The marine ecosystem off Namibia has witnessed a number of profound ecological and environmental changes since the collapse of the commercial pelagic fisheries at the end of the 1960s, including a proliferation of jellyfish[21], a change in the fish community structure[16], a possible increase in hypoxia and toxic gas eruptions[13] and consequently a change in the food web dynamics[17]. These changes are all symptoms of the same malaise – the loss of a supremely successful filter feeder (sardine) has resulted in the liberation of ecological space within the pelagos for opportunistic jellyfish and a less efficient utilisation of primary production, which in turn has resulted in increased sedimentation of phytoplankton. The bearded goby, by virtue of tolerating the low oxygen and high H_2S levels at the bottom, by deriving their nutriment from the benthos, by utilizing DVM to digest food and pay off their oxygen debt, and by using jellyfish as a refuge, have come out amongst the winners.

Climate change will likely increase coastal upwelling[12], which in an area like the Benguela could lead to an increase in the frequency of sulphide eruptions and anoxic water masses[13]. Organisms able to tolerate such extreme conditions are likely to be successful in these future environments. Furthermore, *S. bibarbatus* which feeds low in the food chain (feeding on diatom and bacteria rich mud on the seabed) represents a species that efficiently contributes to trophic energy transfer within these waters. *S. bibarbatus*, with its remarkable suite of adaptations, looks set to play a critical role in the ecosystem off Namibia. Thus caution should be exercised in any plans for its exploitation[19].

Full methods description and any associated references are presented in the supplementary information.

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Author Contribution A.C.U-P., M.J.G., A.G.V.S., B.C. and S.K. designed the research, S.K. and T.A.K performed acoustic interpretations, M.H., A.C.U-P. and M.J.G. performed feeding and gut contents analysis, G.E.N., B.C., J.A.V.S., A.C.U-P., A.G.V.S., I.L., G.K.S, R.A.U.S. performed the physiological studies; A.C.U-P., A.G.V.S. and V.B. performed the behavioural studies, B.C. and K.R.P. contributed with environmental data, M.J.G. and B.F. analysed fisheries and catch data. A.C.U-P., S.K., M.J.G., A.G.V.S., V.B., B.C., G.E.N., J.A.V.S. interpreted the data, A.C.U.P. wrote the manuscript with text inputs from M.J.G., A.G.V.S., V.B., S.K., G.E.N. and K.R.P. All authors discussed the concepts and results, and commented on the manuscript. . ..,.

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Month	N	*Sufflogobius bibarbatus*	*Sardinops sagax*	*Engraulis encrasicolus*	*Trachurus trachurus capensis*	*Etrumeus whitheadi*	
January	1066	18	23	13	22	75	
February	1757	17	26	10	19		151
March	1982	19	28	14	20		508
April	1749	18	18	10	14		130
May	2088	38	27	13	35	80	
June	1113	49	124	48	103		182
July	1038	73	131	95	147		175
August	318	48	40	44	70		233
Average		**35**	**52**	**31**	**54**		**192**

Table 1. Temporal changes in the frequency at which the dominant species of pelagic fish were caught with jellyfish, expressed as: Total number of catches of fish species X with jellyfish, divided by the total number of catches of the same species X without jellyfish: by month. Data collected from randomly selected samples of the landings of pelagic fleet in Walvis Bay, Namibia, for the period 1990-2007. Only months where the total number of samples (N) was greater than 100 are shown. Information for juvenile hake (*Merluccius* sp.) not available as this species is not a routine part of the pelagic fishery.

Figure legend

Figure 1 Acoustic record (38 kHz). a, Showing gobies ascending from the sediment in the afternoon, joining an acoustic scattering layer of jellyfish and **b,** returning to the sediment in the morning. Sequential near-bottom pelagic trawling gave no catches prior to the ascent and a unispecific catch of gobies during the ascent. The vessel was stationary during the descent, therefore many echoes were returned from each fish, then being depicted by "lines". Depth in meters and average oxygen level (% oxygen saturation) is given at ten meters intervals. Temperature decreased gradually from 18 ºC at the surface to 13 ºC above the bottom.

Figure 2 Physiological studies on *Sufflogobius bibarbatus* (a,b,c,d,f) and *Merluccius capensis* (e). a, Gobies maintained a constant oxygen consumption rate until the critical oxygen concentration ($[O_2]_{crit}$) of 5.3 % ± 0.3 % (mean + s.d.) of air saturation (~0.3 ml O_2 l^{-1}) was reached. $[O_2]_{crit}$ is the lowest $[O_2]$ in which the animal is able to maintain its resting rate of O_2 consumption (Prosser & Brown, 1961). Seven replicate fish marked with individual symbols. **b,** Representative trace showing that 3 h exposure to anoxia caused an oxygen debt as post-anoxia oxygen-consumption rate was increased by ~35% (n=6). **c,** Accumulation of blood lactate during 3 h of anoxia (O_2 < 0.5 % air saturation). Note that the rate of increase slowed after 1 h, indicating metabolic depression. Significant differences (P<0.05; one-way analysis of variance; Student-Newman-Keuls post-test) between time points are indicated by dissimilar letters. n=33 fish in total and 3 – 6 at each time point. Values are means ± s.e.m. **d,** Ventilation rate increased in response to falling water $[O_2]$ until $[O_2]_{crit}$, then ceased (regression line obtained by locally weighted scatterplot smoothing (LOESS)) (n=7). **e,** Isolated, spontaneously contracting heart preparations of *S. bibarbatus* (n=6) successfully recovered from 20 minutes of anoxia, whereas hearts of its predator, *M. capensis* (n=6), were irreversibly damaged by anoxia. Asterisks indicate a statistically significant difference (P < 0.05; one-way repeated measures analysis of variance performed on non-normalized data; Student-Newman-Keuls post-tests) from the control normoxic level (i.e., 0%). Values are means ± s.e.m. **f,** Oxygen consumption of gobies exposed to different sulphide concentrations at a normoxic oxygen level (> 50 % of air saturation) (n=5).

Figure 3 Gut analysis. a, Box and whisker plot showing difference in degree of gut fullness and digestion, for fish ascending from bottom to the pelagic at dusk or returning to the bottom at dawn (n=99 gut fullness, and n=75 digestion grade). Gut fullness score is graded 1 to 5, 5 being completely full and 1 being empty, while digestion score is graded 1 to 4 (4 being fully digested and 1 being undigested). Tick line is the median; Box is the interquartile range; Whiskers are maximum and minimum; Dots are outliers. Where no box or whiskers there is little variability in the data. **b,** Diet composition based on the percentage of fish that have consumed a particular prey category (y-axis) related to whether caught in the pelagic (red bars) or on the bottom (blue bars).

Figure 4 Profile of pore water hydrogen sulphide concentration [H_2S] in a sediment core sampled off the Namibian coast (23º23'S - 14º12'E; 120 m water depth).

Figure 1.

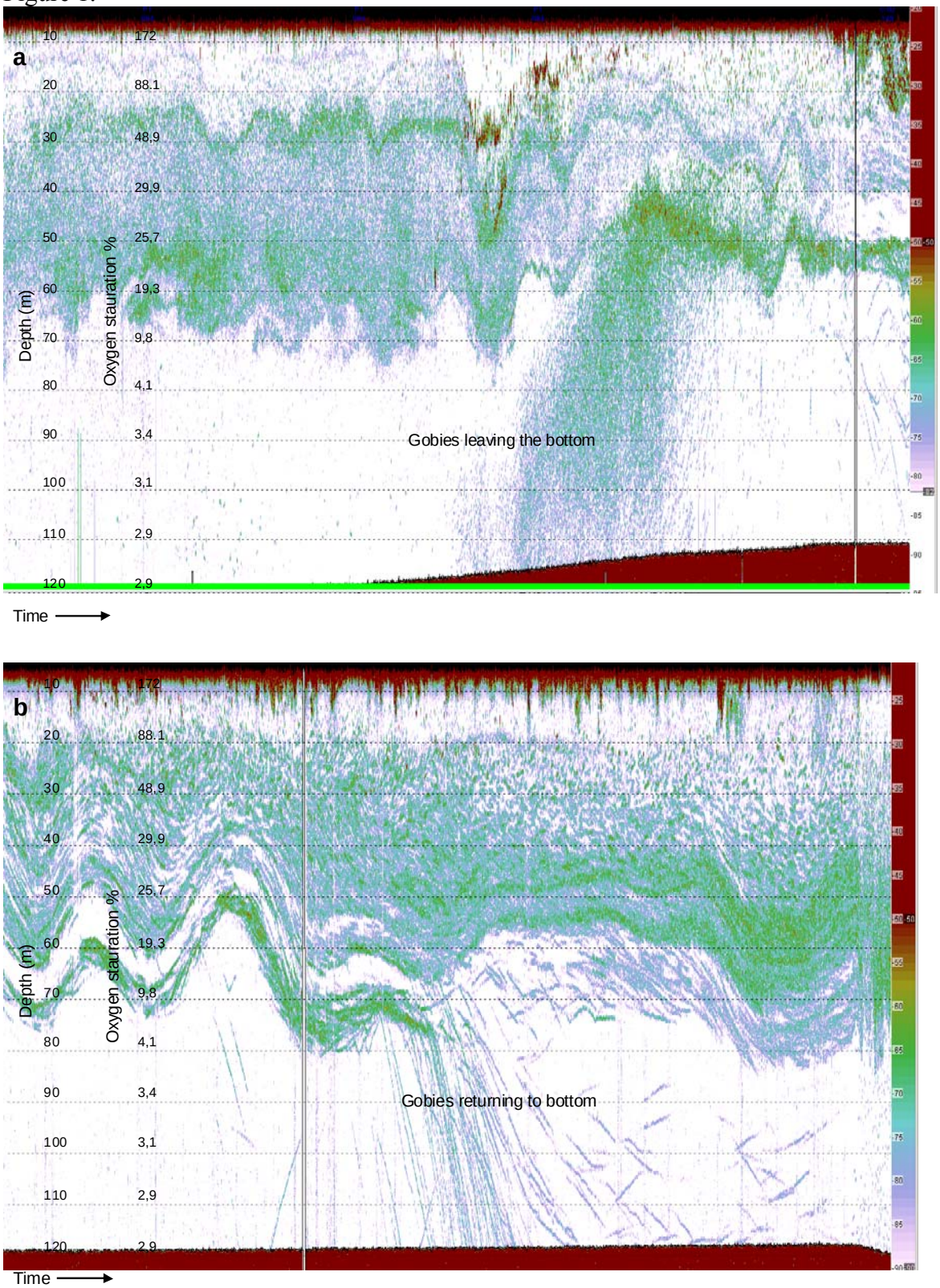
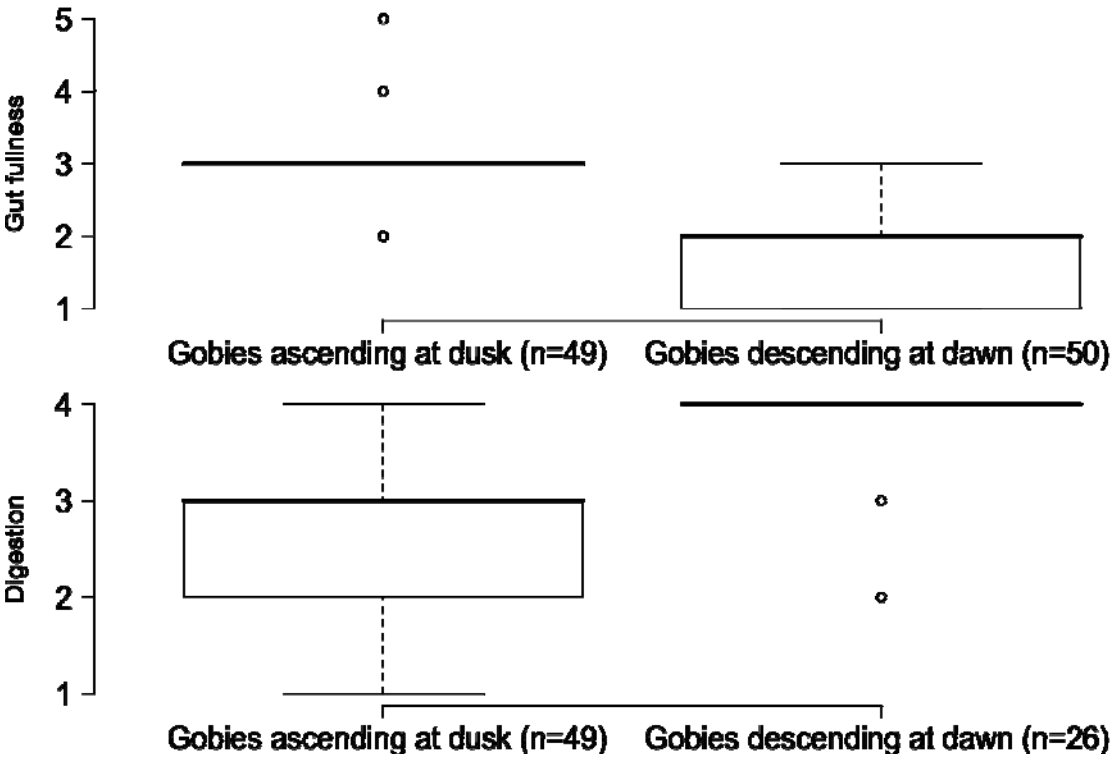
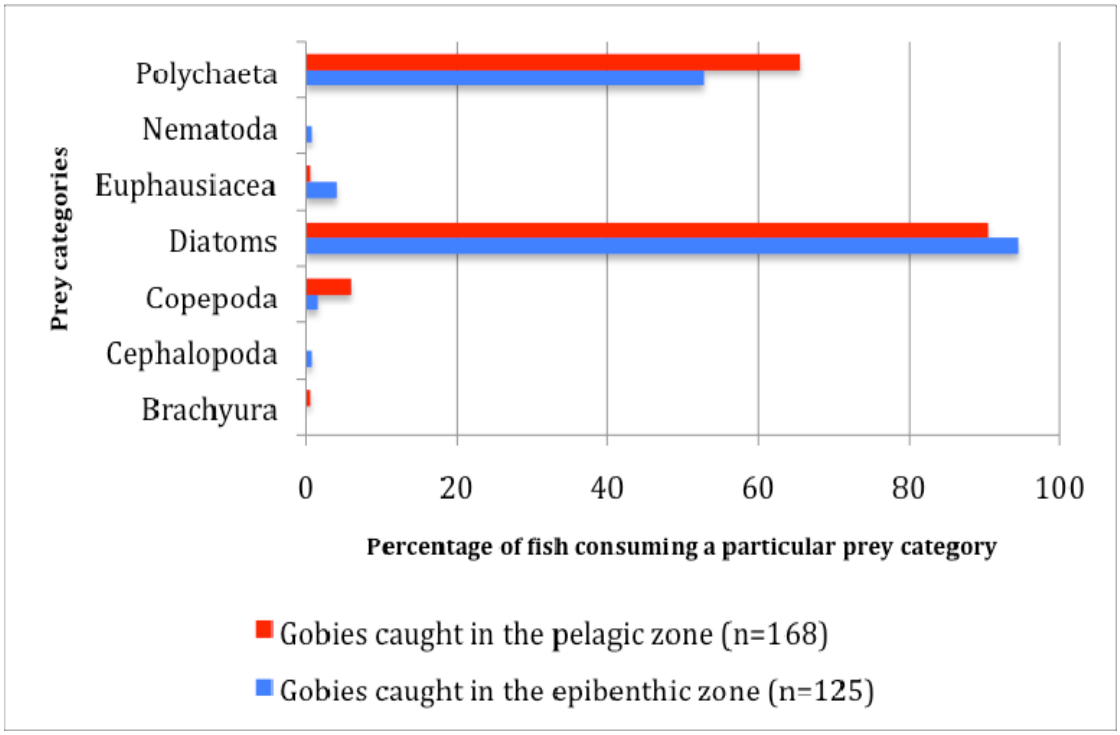

a
Depth (m)
Oxygen staturation %
10
20
30
40
50
60
70
80
90
100
110
120
172
88.1
48,9
29,9
25,7
19,3
9,8
4,1
3,4
3,1
2,9
2,9
Gobies leaving the bottom
Time
b
Depth (m)
Oxygen staturation %
Gobies returning to bottom
Time

Figure 3.

a


Gut fullness
5
4
3
2
1
Gobies ascending at dusk (n=49)
Gobies descending at dawn (n=50)
Digestion
4
3
2
1
Gobies ascending at dusk (n=49)
Gobies descending at dawn (n=26)

b


Prey categories
Polychaeta
Nematoda
Euphausiacea
Diatoms
Copepoda
Cephalopoda
Brachyura
0
20
40
60
80
100
Percentage of fish consuming a particular prey category
Gobies caught in the pelagic zone (n=168)
Gobies caught in the epibenthic zone (n=125)

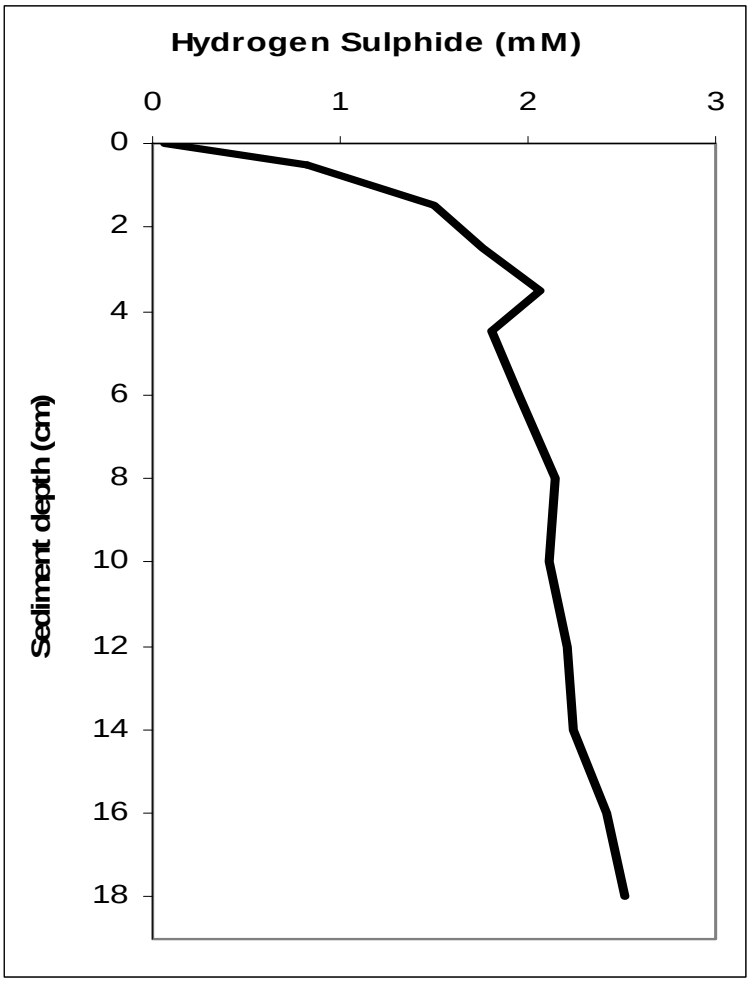

Hydrogen Sulphide (mM)
0
1
2
3
Sediment depth (cm)
0
2
4
6
8
10
12
14
16
18

Figure 4.

Appendix B

Real-time RT-PCR

In the molecular part of this thesis real-time RT-PCR (or reverse transcriptase real-time PCR) is used for quantifying the expression level of genes. Real-time RT-PCR includes two main steps: RT-reaction being reverse transcription to make cDNA from mRNA isolated from the tissues of interest, and real-time PCR is the reaction that allows for quantification. The real-time RT-PCR technique is sensitive enough to be used for quantifying expression in a single cell (Liss, 2002), and specific enough to separate between closely related homologues if the primers are correctly designed (Ginzinger, 2002). It is also the most powerful method for amplifying small amounts of mRNA (Wang et al., 1989).

The synthesis of cDNA is the cause for many problems in regards to RT-PCR, as the efficiency of each synthesis can vary. The gene(s) of interest is amplified in a PCR reaction using gene-specific primers and is measured by using a DNA-binding flourescent dye like SYBR-green or ethidium bromide. The usage of DNA-binding dyes combined with the amplification of the gene of interest in the real-time RT-PCR reaction gives a high degree of specificity. One major drawback to using a non-specific dye is that it will bind to any dsDNA, including primer-dimers, therefore its is very important to verify that it is only the amplicon of interest that get amplified.

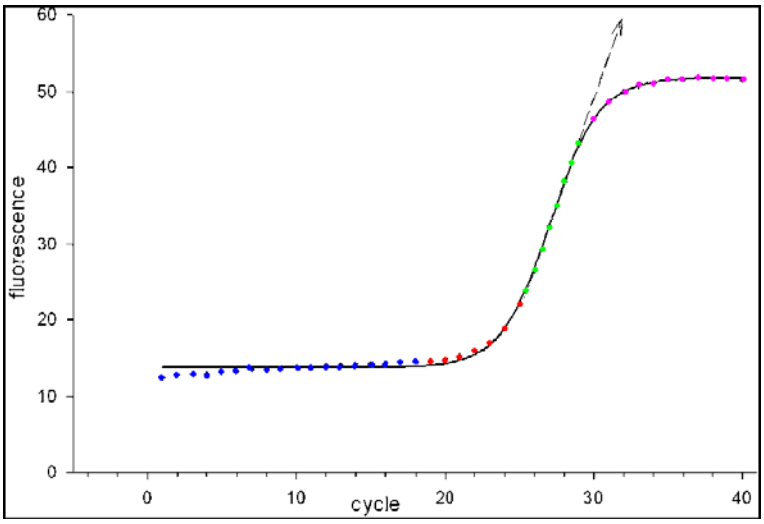


Figure 5: Real-time RT-PCR fluorescence: Figure showing the four phases of real-time RT-PCR amplification, starting with the flat initial phase (blue). In this stage the amplification of the desired region is indistinguisable from the background fluorescence. After that an exponential growth-phase follows(red), quickly turning into a linear growth-phase(green). Towards the end of the PCR a platau-phase is reached(purple), and no further increase can be detected. Figure adapted from (Tichopad et al., 2002).

When running real-time RT-PCR and plotting the product against cycles run, four distinct stages can be observed. In the beginning of amplification it will not be possible to distinguish the fluorescence from the amplicon of interest from the fluorescence from other DNA's in the reaction. This can be seen as a flat, starting region of an sigmoid curve. The second part of the amplification-curve is an exponential stage of amplification, where the desired amplicon is doubled during each cycle of the PCR. In the third stage of amplification the increas has slowed down, and is now showing a linear growth of product. The lower amplification rate is due to a combination of shortage of primers,

dNTP's and product-product annealing. The final stage is where amplicon-growth comes to a halt forming a platau-phase, and no traceable new growth of product occurs (Figure 5) (Kainz, 2000; Tichopad et al., 2002).

For this thesis the LightCycler machine and software was used for measuring fluorescence emitted during the PCR-reaction. This is just one of several thermal flourimeters that can be used for measuring the fluorescence SYBR-green emits during the PCR. The results of the real-time PCR run will be plotted by the LightCycler Data Analysis Software to give a graphic dispay of cycles vs fluorescence. In order to calculate the amount of initial product we use a point known as Cp (crossing point). According to Rasmussen (2001) the method least subject to errors would be the second derivative method, due to the fact that it is the most automated method. By using the second derivative method it is the exponential part of the curve that is used to calculate the amount of initial sample. In this part of the curve the **growth** of fluorescence is at its highest, and the Cp is set to be the point where the growth of fluorescence is at its maximum, i.e. just before the linear part of the curve. This means the lower the Cp-value, the higher amount of starting template that was present.

Quantification can be done in one of two ways. The first method of quantification is absolute quantification, and is based on either an internal or external calibration curve made using known amounts of DNA in a dilution curve (Pfaffl, 2001). This gives an exact number of copies of mRNA in a cell. The second method of quantification is relative quantification, based on the expression of one gene versus another one. When doing relative quantification it is important that the reference gene is expressed at a stable level so that comparison to it will not be disturbed by regulation of the reference gene. In most cases using a housekeeping gene is recommended(Pfaffl, 2001), although some treatments cause regulation of commonly used housekeeping genes such as β-actin and GADPH and may need other additional controls (Ellefsen et al., 2008; Radonic et al., 2004). Relative quantification is the most commonly used quantification for experiments examining physiological changes in gene expression.

In this thesis the data have been normalised against an internal control. The advantage of using such a control is that it compensates for differences that would arise from the early steps of treatment. This is because the control is exposed to the same treatment as the gene (Radonic et al., 2004). Adding an external standard when doing real-time RT-PCR removes any concerns about the constitutively expression of internal control (usually housekeeping genes) when exposed to any treatments (Ellefsen et al., 2008). By combining an external standard and an internal control, the insecurities of differential treatment of the samples is removed, and the expression level of the reference gene is controlled against the external standard.

Extractions of different tissues should still not be compared to each other. This is because there may be variations in efficiancy between extractions and PCR-amplifications from inhibitory factors possibly found in different tissues (Tichopad et al., 2004). My results are comparing one tissue at a time to remove any such insecurities.

Appendix C

Bases	IUPAC Code
Deoxyadenine	A
Deoxycytosine	C
Deoxyguanidine	G
DeoxyThymidine	T
Deoxyuracil	U
Deoxyinosine	I
Phosphorothioate-A	F
Phosphorothioate-C	O
Phosphorothioate-G	E
Phosphorothioate-T	Z
A+C+G	V
A+T+G	D
T+C+G	B
A+T+C	H
A+T	W
C+G	S
T+G	K
A+C	M
C+T	Y
A+G	R
A+G+C+T	N

IUPAC one-letter abbreviations used for degenerated bases in primers. This have been used where the sequence varies between species, and in order to have primers that will base-pair to several options it is made up of a mix of several of the bases.

Appendix D

Abbreviations:

A: Anoxia

ATP: Adenosine triphosphate

Cc: *Carassius carassius*

Cp: Crossing point, the cycle number needed to reach the second derivative maximum (PCR)

COX – Cytochrome *c* oxidase

dNTP: deoxyribonucleotide triphosphate

DTT: Dithiothreitol

DVM: Diel vertical migration

EtBr: Ethidium bromide

E. coli: *Escherichia coli*

F: Forward primer

GAPDH - glyceraldehyde-3-phosphate dehydrogenase

Gh: *Gobiodon histrio*

Gm: *Gillichthys mirabilis*

gm: *Gadus morhua*

Gp: *Gymnogobius pteschiliensis*

GSP: gene specific primer

H: H_2S

HA: H_2S + anoxia

H_2S: Hydrogen sulphide

Hs: *Homo sapiens*

Kb: Kilobase (1000 basepairs)

LB: Lysogeny broth

MtDNA: Mitochondrial DNA

N: Normoxia

NaS: Sodium sulphite

Of: *Oplegnathus fasciatus*

Ol: *Oryzias latipes*

PCR: Polymerase chain reaction

R: Reverse primer

RACE: rapid amplification of cDNA ends

RT: Reverse transcription

RV: Research vessel

Sb: *Sufflogobius bibarbatus*

SD: Standard deviation

TPP: Thiamine Pyrophosphate

X-gal: 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

Appendix E

Reagents (product, catalog number and manufacturer):

Advantage 2 polymerase mix, 639202, Clontech Laboratories Inc
Agarose, SeaKem®, 50004, Cambrex
Ampicillin (D[-]-α-Aminobenzylpenicillin), A-9518, Sigma
Chloroform, C2432, Sigma
DEPC (diethyl pyrocarbonate), SD5758, Sigma
Dithiothreitol, Y00147, Invitrogen
DNAfree Kit, AM1906, Ambion
dNTP-mix, 10297-018, Invitrogen
Dynabeads mRNA Direct Kit, 610.01, Invitrogen
Ethidium Bromide, 443922U, BDH-Electron
IPTG (isopropyl-β-D-thio-galactoside), 21727117, Promega
Nuclease-Free Water, AM9932, Ambion
LightCycler FastStart DNA Master^{PLUS} SYBR Green I, 04707516001, Roche Diagnostics
pGEM®-T Easy Vector systems, A1360, Promega
Platinum Taq Polymerase, 10966, Invitrogen
RNA 6000 Nano Lab Chip ® Kit, 5065-4474, Agilent
SMART RACE cDNA Amplification Kit, 634914, Clontech
SOC medium, 15544-034, Invitrogen
Superscript First strand buffer, Invitrogen
SuperScript ™ III Reverse Transcriptase, 18080-(044), Invitrogen
Teen D lysing Matrix beads, MP Biomedicals
TRIzol ® Reagent, 15596-018, Invitrogen
TURBO DNA-free Kit, Cat #1907, Ambion
X-gal, V3941, Promega

Equipment (product, catalogue number/model and manufacturer):

Eppendorf centrifuge, 5417R, Eppendorf
Finnpipettes, 0.5-10 µL, U23386; 2-20 µL, T27033; 20-200 µL, T27388; 100-1000 µL, T28301,
Galvanometric oxygen electrode OXI 340i, WTW
Homogenizer, Ultra-Turrax T8, IKA
LightCycler 480 Instrument, 03531414201, Roche Diagnostics
Mastercycler gradient, 5331, Eppendorf
NanoDrop ®, Model ND-1000, NanoDrop Technologies
Powerlab 4/20, ADInstruments

Software (freeware is listed with websites)

BioEdit, version 7.0.5.1 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html)
Chart 5.0, ADInstruments
ClustalX, version 2.0 (http://bips.u-strasbg.fr/fr/Documentation/ClustalX/)
EndNote X, Thomson
GeneDoc, version 2.7. (http://psc.edu/biomed/genedoc)
LightCycler® Software, version 4.0, Roche Applied Science
NanoDrop, version 3.0.1, Coleman Technologies Inc.
Netblast, version 2.2.17 (http://www.ncbi.nlm.nih.gov/blast/download.shtml)
Past, version 1.89 (http://folk.uio.no/ohammer/past)
Primer3 primer design program, version 0.4.0 (http://frodo.wi.mit.edu/primer3/input.htm)
SigmaPlot 10, Systate Software Inc.