Molecular and biochemical responses to sand-dwelling in the three-spot wrasse (*Halichoeres trimaculatus*)

By

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I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.I understand that my thesis may be made electronically available to the public.

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Abstract

The three-spot wrasse (*Halichoeres trimaculatus*) is distributed in and around the coral reefs and shallow rocky areas in the tropical and subtropical Indo-Pacific regions. This species displays a distinct diurnal behavior, burrowing under the sand at dusk and emerging out of the sand at dawn, which appears to be synchronized to the photoperiod. In this thesis, the hypothesis tested was that this unique life-style subjected the animal to daily hypoxia exposure while under the sand at night. The measurements of oxygen concentration in the sand around the fish at night confirmed a complete lack of oxygen.

The study had three specific objectives: i) obtain a tissue-specific temporal profile of the hypoxia-related molecular and biochemical responses in wrasse over a 24 h diurnal cycle, ii) determine the responses that were unique to sand dwelling and iii) determine if the responses seen at night in the sand are similar to an anoxic response in this species. Wrasse were maintained in a flow-through seawater aquaria (29 ±1 °C), with sand at the bottom for the fish to hide, and kept under natural photoperiod. The fish were sampled at 10:00, 14:00, 18:00, 21:00, 24:00, 3:00, and 6:00 clock time and plasma and tissue (brain, liver, gill, heart and muscle) were collected to determine the molecular and biochemical responses over a 24 h period. Fish were also sampled from aquaria without sand at night to determine the responses that were specific to hiding in the sand, while fish exposed to nitrogen gas bubbling for 6 and 12 h served as the anoxic group.

A partial cDNA sequence of the hypoxia-inducible factor (HIF)-1α and neuroglobin (two genes that are hypoxia-responsive) were cloned and sequenced from the

liver and brain, respectively, and their expression was determined using real-time quantitative PCR. HIF-1α mRNA abundance was higher in the brain compared to the liver and the gills, while a clear pattern of diurnal change in tissue HIF-1α and brain neuroglobin gene expressions was not observed at night relative to the fish during the day. However, wrasse brain showed a significant reduction in glycogen content at night under the sand and this corresponded with a higher hexokinase activity and increased glucose level suggesting enhanced glycolytic capacity. The plasma glucose and lactate levels were significantly lower at night, while in sand, relative to the day. The lower plasma glucose at night corresponded with a significant drop in liver gluconeogenic capacity (reduction in phosphoenolpyruvate carboxykinase, a key gluconeogenic enzyme, activity), while the lower lactate levels support a lack of activity along with the absence of glycogen breakdown in the muscle. Overall, there was a reduction in the metabolic capacity in the gills, heart, liver and muscle, but not the brain, supporting a tissue-specific metabolic reorganization as an adaptive strategy to cope with sand-dwelling in the wrasse.

The molecular and biochemical responses seen in the wrasse at night in the sand was dissimilar to that seen in fish exposed to anoxia, leading to the conclusion that this species is not experiencing a complete lack of oxygen while under the sand. Also, the lack of muscle movement associated with sand dwelling at night limits anaerobic glycolysis for energy production, thereby eliminating lactate accumulation that was evident in fish exposed to anoxia. Taken together, wrasse showed a tissue-specific difference in metabolic capacity at night while hiding under the sand. While the mechanism involved in this tissue-specific energy repartitioning at night is unclear, one hypothesis involves selective increase in blood flow to the brain, while limiting peripheral circulation, as a means to maintain

oxygen and glucose delivery to this critical tissue while the fish is hiding under the sand. The higher metabolic capacity of the brain, but not other tissues, at night under the sand suggests that maintaining the brain function is essential for the diurnal life-style in this animal.

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List of Acronyms

ANOVA	Analysis of variance
ARNT	Aryl hydrocarbon receptor nuclear translocator
BBB	Blood-brain-barrier
CS	Citrate Synthase
C_T	Threshold cycle
EPO	Erythropoietin
G6PDH	Glucose-6-phosphate dehydrogenase
GK	Glucokinase
HIF	Hypoxia inducible factor
HK	Hexokinase
ICDH	Isocitrate dehydrogenase
LDH	Lactate dehydrogenase
LSD	Least significant differences
MDH	Malate dehydrogenase
ME	Malic enzyme
Ngb	Neuroglobin
NO	Nitric oxide
ODD	Oxygen dependent domain
PEPCK	Phosphoenolpyruvate carboxykinase
PFK	Phosphofructo-1-kinase
PK	Pyruvate kinase
ROS	Reactive oxygen species
S	With sand
TCA cycle	Tricarboxylic acid cycle
VEGF	Vascular endothelial growth factor
WS	Without sand

1 Introduction

1.1 Variations of oxygen availability

Oxygen is essential for most life forms. This is because most animals acquire their energy through respiration, a process of oxygen uptake and release of carbon dioxide. In dry atmospheric air, oxygen composes approximately 21 % of total gas composition. Other gasses include ~78 % of nitrogen, ~0.9 % of argon and less than 0.1 % of carbon dioxide (Schmidt-Nielson, 1997). Despite the current oxygen content in the atmosphere, the first organisms on earth were colonized by prokaryotic anaerobes (Hochachka and Somero, 2002). The dependence on oxygen began with the gradual accumulation of oxygen as a by-product of photosynthesis. The introduction of oxygen modified many redox sensitive elements, including iron, copper and manganese, leading to partitioning of living systems utilizing the aerobic and anaerobic environments (Hochachka and Somero, 2002).

The oxygen level in water is only 1/30 of oxygen in air and the oxygen diffusion is also 10000 times slower than in air (Schmidt-Nielson, 1997). This slower diffusion of oxygen in water decreases the oxygen availability at a fast rate even from modest biological consumption (Nikinmaa and Rees, 2005). For example, there is a large decrease in oxygen availability due to respiration of organisms and breakdown of organic material, especially during the night when photosynthesis does not occur. Other organisms, including green plants, and organic materials use up the oxygen in the eutrophic waters, further hindering the diffusion of oxygen to deeper regions. The slow diffusion rate combined with decreased oxygen tension from other biological consumptions cause the deeper regions of water to be depleted of oxygen due to the lack mixing from the surface. Also, covering of water surface by ice and snow during the wintering season in the temperate, sub-arctic and arctic areas further hinder oxygen diffusion into the water (Nikinmaa, 2002). In the aquatic environment, the term hypoxia refers to oxygen content usually below 4 mg/l,

while normoxia refers to oxygen content around 6-12 mg/l of water, and anoxia refers to complete lack of oxygen (Pihl *et al*, 1991, 1992; Howell and Simpson 1994; Secor and Gunderson 1998; Eby and Crowder 2002).

The level of available oxygen in water can also decrease with an increase in temperature, since temperature affects the solubility of oxygen in water (Schmidt-Nilsson, 1997). However, the temperature changes in aquatic environments are less pronounced, partly owing to the large heat capacity of water (Nikinmaa, 2002). Despite this, the annual water temperature fluctuations range from 0 ^{o}C to 25 ^{o}C in temperate fresh waters, and even larger daily changes between 5 and 35 ^{o}C in temperate tidewater pools (Bridges, 1988). These fluctuations in water temperatures affect cellular processes, leading to higher metabolic rate with elevated temperatures. Generally, oxygen demand for fish doubles with every 10 ^{o}C increase in temperature (Brett and Glass, 1973). Along with increased oxygen consumption, breathing rate in fish is influenced by increases in temperature, such that the ventilation frequency and amplitude as well as the heart rate increase (Barron *et al.,* 1987; Graham and Farrell, 1989; Forstner and Wieser, 1990).

The lack of oxygen leads to death within a few minutes in animals (Smith *et al*, 1996). Aquatic animals including fish are more vulnerable to fluctuations in oxygen availability than air-breathing animals. However, lower vertebrates such as fish have shown more tolerance to variable oxygen availability compared to birds and mammals, which are relatively sensitive to even slight changes in available oxygen (Bickler and Buck, 2007). Some fish species have evolved mechanisms to tolerate variable oxygen exposure, including metabolic depression, enhancing glycogen storage in critical tissues and expression of oxygen-dependent genes.

1.2 Molecular mechanisms involved in hypoxia tolerance

An important adaptation shown by hypoxia tolerant species is transcriptional regulation of genes involved in hypoxia tolerance. In particular, hypoxia inducible factor-1α (HIF-1α) has been

shown to play an important role in the regulation of hypoxia responsive genes, including those that encode for erythropoietin, vascular endothelial growth factor (VEGF), and a number of glycolytic enzymes (Lutz *et al*., 2003).

HIF was discovered during studies on the regulation of erythropoietin (EPO) in mammalian Hep3B cell lines (Semenza and Wang, 1992) and has been studied extensively with regard to gene expression during hypoxia. HIF is a heterodimeric transcription factor, belonging to the PAS-domain family of transcription factors (named for the first members of the family, Per, ARNT, Sim) and consists of the HIF-α and HIF-β subunits. There are three α-subunits, HIF-1α, HIF-2α, and HIF-3α (Wiener *et al*, 1996; Gu *et al*, 1998). The β-subunit is the same as aryl hydrocarbon receptor nuclear translocator (ARNT), which plays a role in hypoxic signaling and gene regulation. ARNT also plays a key role in dioxin-mediated gene transcription by dimerizing with the aryl hydrocarbon receptor and binding to dioxin response elements on the promoter regions of target genes (Hankinson, 1995; Hahn, 2002). HIF is involved in the hypoxic regulation of variety of genes and is also expressed in a variety of cell types (Wenger and Gassmann, 1997; Semenza, 2000; Bracken *et al*, 2003). As HIF is widely expressed and its targets have diverse roles in animal physiology, this transcription factor is considered a "master switch" that regulates molecular responses critical for coping with low oxygen levels in mammals (Semenza, 2002).

The oxygen sensitivity of HIF-mediated gene expressions is, in part, related to oxygen dependence of HIF-1α protein levels. This protein is constitutively expressed during normoxia as well as induced in response to hypoxia. During normoxia, HIF-1α is rapidly degraded, a reaction mediated by the oxygen-dependent degradation (ODD) domain (Nikinmaa and Rees, 2005) in which the conserved proline residues are covalently modified by prolyl hydroxylase enzymes, via the proteasomal pathway. During oxygen limitation, prolyl hydroxylase is inactive allowing HIF-1α to become stable and accumulate in cells. HIF-1α forms a heterodimer with ARNT and

translocates to the nucleus and binds to hypoxia response elements (HREs) in the promoter or enhancer region of hypoxia-inducible genes (Nikinmaa and Rees, 2005).

Fish possess homologs of HIF-α and HIF-β that plays a similar role in hypoxic gene expression as those in mammals. The first fish HIF-α sequence was reported from rainbow trout (*Oncorhynchus mykiss*) (Soitamo *et al., 2001*). The cDNA encoded a protein of 766 amino acids containing regions recognizable as basic-helix-loop-helix, PAS and ODD domains of α-subunits of HIF. The proline and asparagine residues, which are the target hydroxylation sites in mammals, are also conserved in the rainbow trout protein. This protein was found to be similar to HIF-1α from other vertebrates. The second HIF-α was cloned and sequenced from the killifish (*Fundulus heteroclitus)* (Powell and Hahn, 2002) and encoded a protein that is 873 amino acids long. This protein was similar to HIF-2α from other vertebrates. In addition to the deduced HIF protein from rainbow trout, putative HIF-1α has been sequenced from other teleosts, including grass carp (*Ctenopharyngodon idella*) and zebrafish (*Danio rerio*). There is limited data available to conclude whether the HIF-α subunits in fish are orthologs of mammalian HIF-3α or if they represent a distinct gene which is specific to teleosts (Nikinmaa and Rees, 2005).

1.2.1 HIF stability, function and expression in fish

Oxygen dependence of fish HIF stability and function has been studied in salmonids (Soitamo *et al*, 2001; Nikinmaa *et al*, 2004). These studies show that HIF-1α and its DNA binding increases during hypoxic exposure, even though this gene is expressed under normoxic conditions (Soitamo *et al* 2001). In fish cell systems, including rainbow trout hepatocytes, cell lines derived from rainbow trout (RTG-2) or chinook salmon (*Oncorhynchus tshawytscha;* CHSE-214), HIF-1α expression was maximal at 5% oxygen, a level observed in the venous blood of salmonid even under normoxic conditions (Soivio *et al*, 1981; Soitamo *et al*., 2001). As the oxygen tension in

tissues is similar to or lower than the venous oxygen tension, it has been proposed that significant accumulation of HIF-1α protein occurs even in normoxic conditions (Nikinmaa and Rees, 2005).

The oxygen effect on HIF-1α stability and activity is associated with proline and asparagine residues and proteins that interact with them. In fish, there is some evidence for the existence of proteins with similar functionality to those seen in mammalian models. For instance, the accumulation of HIF-1α in cells in the presence of proteasome inhibitors implies that the degradation pathway for HIF-α in fish is similar to that of mammals (Soitamo *et al*, 2001). Also, multiple open reading frames similar to that of human prolyl hydroxylase are present in the genome of zebrafish, although more characterization of prolyl hydroxylase needs to be carried out in fish. In addition, an asparaginyl hydroxylase (FIH-1) homolog has been sequenced from zebrafish (Swiss-Prot P59723). These observations suggest that fish have all the components required for reversible stabilization of HIF-1α and the activation of gene expression under hypoxia (Nikinmaa and Rees, 2005).

1.2.1.1 HIF-1α target genes

HIF target genes in mammals have been characterized, including genes involved in red blood cell production, vascularization, apoptosis, and carbohydrate metabolism (Wenger and Gassmann, 1997; Semenza 2000; Wenger, 2000; Pugh *et al*, 2002; Bracken *et al*, 2003). Studies have suggested that similar oxygen-dependent gene expression is also seen in fish.

1.2.1.2 Red blood cell production and oxygen transportation

One of the characteristic responses to low oxygen level is increased hematocrit values in fish. This may be due to swelling of erythrocytes, release of red blood cells, change in plasma volume, and/or new red blood cell formation. In mammals, the increased hematocrit value upon hypoxia involves production of new red blood cells. This production of red blood cells is in response to elevated erythropoietin (EPO) levels, which is regulated by HIF. In fish, human EPO

treatment stimulated the production of red blood cells suggesting a mechanism similar to that in mammals (Taglialatela and Della Carte, 1997). Therefore, it is evident that fish appear to possess homologs of EPO, which can influence the production of red blood cells. However, more studies are required to determine whether HIF plays a role in the expression and function of EPO during hypoxic exposure in fish.

Hemoglobin is an important oxygen-transport protein that is present in fishes as in other vertebrates. Due to selection pressures, including low oxygen levels, teleost fish show a great diversity in hemoglobin components within the erythrocyte of one individual compared to other vertebrate groups. Although studies have suggested that globin chain expression of erythrocytic hemoglobin may be influenced by hypoxic exposure in fish (Marinsky *et al*, 1990), this view was not supported by other studies (Jensen and Weber, 1982). Indeed, the effects of acute or chronic exposure to low-oxygen concentration on globin gene expression is less clear in fish and it remains to be determined if the molecular response is species-specific.

1.2.1.3 Formation of circulatory and respiratory structures

HIF increases vascular endothelial growth factor (VEGF), resulting in the growth of blood vessels in mammals. VEGF has also been sequenced and characterized from zebrafish (Gong *et al*, 2004). Along with VEGF receptors, they play an important role in the vascular development in zebrafish (Lee *et al*, 2002; Ober *et al*, 2004). Also, HIF was shown to play a critical role in developmental defects in Baltic salmon (*Salmo salar*), suggesting that there is correlation between vascular development, VEGF expression and HIF function (Vuori *et al*, 2004). HIF has been shown to regulate vascular programming during normal development by adjusting the level of VEGF. For instance, fetuses lacking HIF-1α result in abnormalities, including disturbances in vasculature, heart and neural tube development, leading to death at mid gestation (Ryan *et al*, 1998; Kotch *et al*, 1999; Compenolle *et al*, 2003).

An important adjustment to oxygen limitation is an increase in respiratory surface area and effective circulation. Studies have shown that African cichlid (*Pseudocrenilabrus multicolor victoriae*) and African cyprinid (*Barbus neumayeri*) exposed to hypoxia show increased fish gill filament length (TGFL), especially in populations that are repeatedly exposed to hypoxic environment (Chapman *et al*, 2000; Schaack and Chapman, 2003). In the case of crucian carp (*Carassius carassius*), a dramatic increase in gill surface area was observed after one week of hypoxic exposure. This is mainly due to secondary lamellae development which is associated with decreased number of cells between lamellae, most likely as a result of apoptosis of interlamellar cells (Solid *et al*, 2003). HIF activation of apoptotic pathways in fish gills could potentially be a molecular link between hypoxia and gill development, but remains to be tested.

1.2.2 Neuroglobin

Neurons are considered one of the most oxygen sensitive types of cells. However, recent studies have shown hypoxia tolerant neurons, with the most tolerant being able to withstand periods of anoxia with a full recovery after return to normoxia (Milton *et al*, 2006). It is well known that not all vertebrate brains are equally sensitive to hypoxia. For example, freshwater turtle (*Trachemys scripta*) survives severe hypoxia and even anoxia by undergoing metabolic depression.

Globins, which are the small proteins that bind oxygen by means of a porphyrin-coordinated Fe^{2+} ion, mediate transportation and storage of oxygen in vertebrate animals (Fuchs *et al*, 2004). Nearly all vertebrates contain the heterotetrameric hemoglobin in red blood cells that transport oxygen in the circulatory system from the respiratory surfaces to the inner organs. The monomeric myoglobin is typically present in the myocytes of cardiac and striated muscles, helping oxygen diffusion to the mitochondria (Flogel *et al*, 2001).

A recently discovered vertebrate globin, the neuroglobin, is thought to be involved in neuronal oxygen homeostasis (Sun *et al*, 2001; Pesce *et al*, 2002; Schmidt *et al*, 2003). Although

neuroglobin was discovered in the neurons of the central nervous system (Fuchs*et al*, 2004), recent studies showed that non-neuronal endocrine tissues as well as peripheral nervous systems also contain neuroglobin (Reuss *et al*, 2002). This suggests that there is a widespread tissue-specific neuroglobin expression in vertebrates, but the functional significance is unknown. The presence of neuroglobin promotes the survival of cultured neuronal cells at low oxygen levels and, therefore, has been hypothesized to protect neurons from hypoxic injury (Sun *et al*, 2001). Investigation of neuroglobin function in non-mammalian species, especially hypoxia and anoxia tolerant animals, will provide a deeper understanding of its role in vertebrate oxygen metabolism (Fuchs, 2004). However, few studies have examined neuroglobin regulation in fish.

It is still unclear if regulation of neuroglobin is affected by varying oxygen levels. Some studies have reported that there is an increased expression of neuroglobin gene and protein in a primary neuronal cell culture from mouse 24 h after anoxia-reperfusion as well as under severe hypoxia (Nicholas, 1997; Sun *et al*., 2001). However, other studies failed to observe a similar response in hypoxic mice *in vivo* (Quandt *et al*, 1995). These conflicting observations suggest that the expression of neuroglobin is involved only during severe hypoxia or anoxia. A study conducted by Roesner *et al* (2006) demonstrated that neuroglobin is differentially regulated in the zebrafish brain and eye. The brain neuroglobin mRNA levels showed a transient upregulation after 24 h anoxia, whereas no effect was observed in the whole eye. A similar response in protein levels was seen in the brain, but not in the eye 48 h after severe hypoxia (Roesner *et al*., 2006).

Previous studies indicate that neuroglobin expressions in mammals are closely linked to the metabolic activity and oxygen consumption (Schmidt *et al*, 2003; Bentmann *et al*, 2005). Therefore, maintenance of oxidative metabolism in the brain of fish will also require high levels of neuroglobin. Studies suggest that neuroglobin function in the brain is similar to that of myoglobin in the heart, which acts as an oxygen supply protein. Also, with several studies suggesting increased level of ROS and NO (nitric oxide) under low oxygen conditions (hypoxia or ischemia),

another potential function of neuroglobin may be an involvement in reactive oxygen species (ROS) metabolism during oxygen deprivation and reperfusion (Herold *et al*., 2004; Brunori *et al*., 2005; Jezek and Hlavata, 2005; Wenger, 2006).

1.3 Biochemical adaptation to hypoxia

While increased oxygen storage and delivery is important during times of hypoxia, a key adaptation to long-term oxygen limitation involves a reduction or near-suspension of many bioenergetic processes (Bickler and Buck, 2006). There are two major mechanisms shown by hypoxia tolerant animals to satisfy both supply and demand of ATP (Bickler and Buck, 2006). One mechanism involves up-regulation of ATP-producing pathways that are energetically efficient to increase supplies, including glycolysis, while the other mechanism involves reduction in ATP turnover (metabolic suppression; Hochachka, 1986; Hochachka and Lutz, 2001). In general, hypoxia adaptation favors pathways that maximize the yield of ATP per mol of O_2, where as anoxia adaptation favors pathways that maximize the yield of ATP per mol of H^+ formed by fermentation (Hochachka, 1994). Hypoxia adaptation involves an increase in the ratio of anaerobic/aerobic metabolic potentials (Hochachka *et al*. 1996). The key element to providing the greatest protection against hypoxia is metabolic depression, including reduction of energy demanding pathways (Storey and Storey, 1990; Hochachka *et al*., 1996).

1.3.1 Metabolic suppression

The mechanisms of metabolic rate reduction by hypoxia-tolerant animals are not well understood. For instance, it is uncertain whether the regulation of pathways of ATP supply (glycolysis, oxidative phosphorylation), ATP demand (protein synthesis, ion pumping) or a combination of the two is involved in the metabolic suppression (Bickler and Buck, 2006). In normoxia, the largest energy sinks (20-30 % of the total ATP-coupled oxygen consumption) are related to ion pumping by the Na^+/K^+ ATPase as well as protein synthesis, while the remaining

oxygen utilization is to drive other energy demanding pathways, including gluconeogensis, Ca^{2+} ATPase, and ureagenesis. During hypoxia and anoxia, the activity of these ATP-demanding pathways significantly decrease, with protein turnover dropping to less than 10% of normoxic rates as shown in turtle liver cells (Hochachka *et al*., 1996). Enzymes involved in the TCA (Tricarboxylic acid) cycle and protein synthesis are suppressed during hypoxia in order to decrease the ATP turnover rates in mammalian muscle cells and goby fish (*Gillichtys mirabilis*) (Murphy *et al*., 1984; Gracey *et al*., 2001). Hypoxia and anoxia tolerant animals, including freshwater turtles (*Trachemys scripta*) and goldfish (*Carassius auratus*), showed a rapid decrease in protein synthesis, which is not seen in hypoxia-intolerant species (Hochachka *et al*, 1996).

In vertebrates, glycogen storage has an important role in tolerating low oxygen level. Compared to those of other vertebrates, anoxia tolerant vertebrates such as crucian carp (*Carassius carassius*), goldfish, and freshwater turtle have large glycogen stores. For example, the crucian carp has the largest glycogen stores of any vertebrate (Lutz and Nilsson, 1997). Similarly, high glycogen concentrations are seen in the brain as well, ranging between 12.8 and 19.5 μmol hexose g^{-1} wet weight of fish in anoxia tolerant vertebrates, whereas anoxia-sensitive species such as rat, mouse and rainbow trout have only 2.2-3.7 μmol g^{-1} wet weight. During anoxia, brain glycogen level falls by 75 and 95 % in the first 2 and 7 h, respectively (Lutz and Nilsson, 1997), suggesting that brain glycolysis provides glucose during the initial phase of anoxia.

1.3.2 Glucose as fuel for brain

Brain represents 0.1 – 1 % of the body weight of vertebrates, but it is responsible for 1.5-8.5 % and 2.7-3.4 % of the total energy consumption in endothermic and ectothermic vertebrates, respectively (Van Ginneken *et al*., 1996). Most of the brain energy consumption is used to maintain ionic gradients across plasma membranes, accounting for approximately 50 – 60 % of the ATP consumption (Hylland *et al*., 1997; Purdon and Rapoport, 1998). Brain energy demands are met

largely via oxidation of glucose, which is an obligatory substrate of brain metabolism of chordates (Kauppinen *et al*., 1989; Hertz and Peng, 1992). Continuous glucose supply from circulation is important for normal functioning of vertebrate brains, since endogenous glucose and glycogen levels are low in most vertebrate brains.

The brain is isolated from the systemic circulation by the blood-brain-barrier (BBB), which limits penetration of substances from blood into the CNS. Therefore, the limiting factor in the cerebral utilization of glucose may be the rate of glucose transport across the BBB or phosphorylation by hexokinase (HK) (Lundquist, 1942; Bernstein and Streicher, 1965; Cserr *et al*., 1978; Cserr and Bimdgaard, 1984; Pardridge, 1991). Under normal conditions, glucose utilization in the brain of mammals is regulated by HK activity more than glucose transporters and this is also the case in rainbow trout brain (Washburn *et al*., 1992; Blasco *et al*., 1996; Aldegunde *et al*., 2000). However, high HK activity in rainbow trout brain suggest that glucose utilization may be limited by phosphorylation of glucose by hexokinase even more in teleost brain compared to mammals (Soengas *et al*., 1998). Teleost brain, therefore, relies heavily on exogenous glucose to fuel energy demanding processes (Sugden and Newsholme, 1973; Knox *et al*., 1980; Lushchak *et al*., 1998).

Teleosts brain show relatively high 6-phosphofructo 1-kinase (PFK) and pyruvate kinase (PK) activities compared to other tissues, suggesting enhanced glycolytic potential (Lushchak *et al*. 1998). Also, the high glycolytic potential is usually associated with an increased flux through the TCA cycle supporting aerobic metabolism of the brain tissue (Yang and Somero, 1993; Lushchak *et al*., 1998; Leary *et al*., 1998).

1.3.3 Glycolysis during oxygen limitation

An important strategy to survive oxygen limitation is increasing the delivery rate of glucose to the brain as well as increasing the tissue glycolytic capacity (Soengas and Aldegunde, 2002). Hypoxia and anoxia-tolerant vertebrates do so by increasing blood glucose concentration

and/or the rate of blood supply to the brain (Lutz and Nilsson, 1997). For example, in goldfish the blood glucose level increases fivefold during anoxia, whereas in freshwater turtles, at room temperature, plasma glucose concentration rises almost eightfold (Lutz and Nilsson, 1997). In crucian carp, the rate of blood flow to the brain doubles during anoxia and remains elevated for the entire anoxic period (Nilsson *et al*. 1994). A similar response is seen in freshwater turtle, although the increase is only temporary, suggesting that it is an emergency response to anoxia in the case of turtle brain (Hylland *et al*. 1994). Therefore, glycogen mobilization under oxygen limiting conditions in fish brain fuels the glycolytic pathway (DiAngelo and Heath, 1987; Lutz and Nilsson, 1997). This increased anaerobic potential is supported by an increase in rates of glycolysis, a large Pasteur effect, increased activities of PFK and PK, and elevated levels of brain fructose 2,6P (Storey, 1987; Heath, 1988; Johansson *et al*., 1995; Lushchak *et al*., 1997). It is thought that the higher potential for anaerobic metabolism in hypoxia-tolerant cells/tissues is not only to produce ATP, but instead is also used to maintain a reduced energy turnover (Karumschnabel *et al*., 1994; Karumschnabel *et al*., 1996).

The increased level of glycolysis causes lactate accumulation, which can lead to self-intoxication and acidosis (Lutz and Nilsson, 1997). Anoxia tolerant species belonging to the *Carassius* genus produce ethanol as the major glycolytic end-product and release it through the gills. This allows them to maintain a high glycolytic rate for longer periods of time without suffering from lactate build up. This is one adaptation seen in some species of anoxia-tolerant fish, including goldfish, but absent in hypoxia-intolerant species (Shoubridge and Hochachka, 1983).

1.4 Summary: Surviving variable oxygen limitation

There are several important metabolic changes which hypoxia tolerating animals must accomplish. First, there must be a rapid switch in metabolism from aerobic to an anaerobic metabolism. This response requires cellular oxygen sensors such as HIF-1α and potentially

neuroglobin. Long-term survival depends on the presence of large glycogen stores and energy conservation via decrease in energy demanding tasks, including ion pumping and protein synthesis. Also, accumulation of metabolic end products, including lactate and ethanol due to anaerobic metabolism associated with prolonged anoxia must be tolerated. In addition, a defense mechanism to fight against free radicals formed especially during reoxygenation must be present.

1.5 Coral reefs and hypoxia: sand-dwelling life-style of the three-spot wrasse (*Halichoeres trimaculatus*)

Tropical coral reefs provide complex environments with a diverse range of habitats for fish (Nilsson *et al*., 2003). The water temperature typically ranges from 25-30 ^{o}C, with hypoxia greatly influencing the biodiversity of the ecosystem. Among the diverse fish groups inhabiting the coral reefs is the three-spot wrasse. This species is distributed in the tropical and subtropical Indo-Pacific regions and can be found in or around the coral reefs or in shallow rocky areas (Kim *et al*., 2002). The three-spot wrasse burrows into the sand at dusk and emerges out of the sand at dawn, a behaviour synchronized by the photoperiod. Measurement of oxygen concentration in the sand (Fiber Optic Oxygen Sensor; Ocean Optics Inc. Dunedin, FL) around the fish at night indicated that the environment was anoxic.

Coral reefs were not generally thought to be hypoxic habitats and coral reef fishes were not known for their hypoxia tolerance. However, recent studies showed that fish in coral reefs do experience hypoxia, including nocturnal hypoxia experienced by animals due to cessation of photosynthesis, hiding from predators at night by moving in to coral colonies, and fish trapped in tidal pool formed during low tides (Munday *et al*., 1997, 2001; Nilsson, 2001; Wise *et al*., 1998; Renshaw *et al*., 2002; Nilsson *et al*., 2003; Nilsson and Renshaw, 2004, Nilsson et al., 2007). There are several other species of fish that are hypoxia tolerant, but most of them inhabit temperate waters where it is less challenging, due to lower water temperatures, to survive hypoxia compared to the tropics because of elevated higher water temperatures (Nilsson *et al*., 2003).

1.6 Experimental rationale and hypothesis

Coral reef fishes show remarkable ability to tolerate hypoxia at water temperature as high as 29 ^{o}C (Nilsson and Nilsson, 2004). The three-spot wrasse has a very interesting life-style in that the fish burrows into the sand at night and remains hidden during the night. Also, it was determined that the sand, where the fish are hiding at night, was anoxic. This led to the hypothesis that the diurnal life-style of sand-dwelling at night subjects the three-spot wrasse to hypoxia daily. To test this hypothesis, this study investigated the diurnal rhythms in some molecular and biochemical end-points that are oxygen-dependent. Specifically, hypoxia inducible factor (HIF)-1α and neuroglobin gene expressions were studied to understand the molecular responses, while the activities of enzymes involved in intermediary metabolism, including glycolytic enzymes and TCA cycle enzymes, were determined to examine the tissue aerobic and anaerobic capacity over a 24 h diurnal cycle. The diurnal molecular and biochemical responses were also compared with fish that had no access to sand at night, and also in fish exposed to anoxia. The rationale for these studies were to address two main questions i) Is the sand-dwelling three-spot wrasse exposed to oxygen limitation?, and ii) Are there any molecular and biochemical strategies that allow this animal to survive this unique life-style?

2 Materials and Methods

2.1 Experimental animals and husbandry

The three-spot wrasse, with body masses ranging from ~ 8 to 55 g were collected by hook and line fishing from the waters surrounding the Sesoko station, Tropical Biosphere Research Center, University of Ryukyus, Okinawa, Japan. Okinawa Prefecture is an island located southwest of mainland Japan (at 24 to 27 degrees north latitude and 122 to 128 degrees east longitude) in the South China Sea. It has a subtropical climate and the average annual temperature is around 22^{o}C, while the winter and summer average temperatures are around 16^{o}C and 28 ^{o}C, respectively. This study was carried out in the summer and fish were maintained in a 200 liter tank with continuous aeration before the start of the experiment.

2.2 Experimental design

2.2.1 Experiment I

Groups of 8 fish were transferred into each of the 11 tanks (30-litre) with constant flow-through seawater at 29 ±1^{o}C. A sand box (7 cm depth) was placed in each of the 11 tanks to mimic their natural habitat. The fish were acclimated for at least one week prior to the commencement of the study. At the start of the experiment, sand boxes were removed from 4 tanks and those were the no sand experimental group sampled at night along with the sand groups. All tanks were maintained under natural photoperiod and the fish were fed daily (Marubeni Nisshin Feed Co., Tokyo) and the feeding was stopped 24 h prior to the start of the experiment. The dissolved oxygen content was measured using a Fiber Optic Oxygen sensor probe (Foxy AL 300; Ocean Optics Inc. Dunedin, FL) with a spectrofluorometer (USB4000-FL preconfigured for fluorescence with USB-LS-450; Ocean Optics Inc. Dunedin, FL). There was 100 % O_2 saturation in the sea water during the day and at night, whereas there was 0% O_2 saturation in the sand.

2.2.2 Experiment II

Two tanks each containing groups of 8 fish were maintained exactly as above. The anoxia experiment was carried out in the morning and consisted of tanks with no sand and bubbled with a gentle stream of nitrogen gas. The dissolved oxygen level reached 0 % O_2 saturation in the tank within 60 min. Fish were sampled at 6 and 12 hr of nitrogen bubbling.

2.2.3 Sampling

Fish were sampled at different time points [3:00, 6:00, 10:00, 14:00, 18:00, 21:00, 24:00 clock time] during the day and at night over a 24 h diurnal cycle (see Fig. 1). The tanks with no sand were sampled only at night time (18:00, 21:00, 24:00, 3:00). Sampling consisted of netting all 8 fish from each tank and anaesthetizing them with an overdose of 2-phenoxyethanol (1:1000) (Kanto Pure chemicals, Tokyo, Japan). Fish were bled by caudal puncture into heparinized capillary tubes and plasma was collected after centrifugation (10,000 rpm; 5 min). Fish were quickly dissected and various tissues including brain, liver, gills, muscle, and heart were collected and immediately frozen in liquid nitrogen. Plasma and tissue samples were stored at -70 ^{o}C for various biochemical and molecular analyses later.

Figure 1. The three-spot wrasse sampling time points

Schematic representation of the diurnal cycle of the three-spot wrasse and the sampling time points (clock time). Wrasse burrows into the sand at around 18:00 and emerges out of the sand at around 6:00 clock time. The black and white bars represent the night and day time, respectively.

2.3 Biochemical analyses

2.3.1 Plasma metabolites

Plasma glucose (modified Trinder method; Raichem, San Diego, CA) and lactate (Trinity Biotech, St. Louis, MO) levels were measured using commercially available colorimetric kits. The absorbance was measured using a microplate spectrophotometer (VersaMax; Molecular Devices Corp., Pao Alto, CA).

2.3.2 Tissue homogenization

Brain, liver, gill and muscle tissues were sonicated (Microson, Farmingdale, NY) in 150 µl of Tris-Hcl buffer (50 mM; pH 7.5) containing protease inhibitor cocktail (Sigma-Aldrich, Oakville, ON). Muscle tissues were pulverized using a mortar and pestle on dry ice prior to sonication. For enzyme determinations, homogenized samples were stored in glycerol buffer (50%

glycerol, 21 mM Na_2HPO_4, 0.5 mM EDTA-Na_2, 0.2% BSA, 5 mM ß-mercaptoethanol and protease inhibitors, pH adjusted to 7.5).

Heart tissues were prepared in Dr. Les Buck's laboratory at the University of Toronto (Department of cells and systems biology). The tissues were homogenized in homogenization buffer (50 mM tris base, 1 mM EDTA, 1 mM dithiothreotol, 0.5 % tween-20, pH adjusted to 7.5) and diluted 10x using glycerol buffer after protein and glycogen contents were measured.

2.3.3 Determination of protein content

Protein concentrations in the homogenates were determined using the bicinchoninic acid (BCA) method (Sigma-Aldrich, Oakville, ON) with bovine serum albumin (BSA) as the standard (Smith *et al*., 1985).

2.3.4 Determination of glycogen content

Tissue glycogen content was analyzed by glucose measurements before and after 2 h of amyloglucosidase hydrolysis at 37 ^{o}C using a commercial colorimetric kit (modified Trinder method; Raichem, Sand Diego, CA) according to established protocol (Vijayan *et al*., 2006). The glycogen content is shown as micromoles glucosyl units per gram protein in the homogenate.

2.3.5 Enzyme Activities

Enzyme activities were measured in 50 mM imidazole buffered (pH 7.4) assay buffer in a final volume of 250 µl at room temperature by continuous spectrophotometry at 340 nm (unless indicated) on a microplate reader (VersaMax; Molecular Devices Corp., Palo Alto, CA) according to established protocols (Vijayan *et al*., 2006). The sample volume (ranging from 1-20 µl in a total of 250 µl reaction mixture) for each enzyme activity measurement was determined after preliminary testing. The following assay conditions were used for optimal activities:

- Citrate synthase (EC 4.1.3.7): 0.5 mM oxaloacetate, 0.1 mM dithionitrobezoic acid, 0.3 mM acetylcoenzyme A in 50 mM Tris-HCl buffer, pH 8.0, measured at 410 nm

- Glucose-6-phosphate dehydrogenase (EC 1.1.1.49): 1 mM Glucose-6-phosphate, 4 mM $MgCl_2$, 0.4 mM NADP

- Glucokinase (EC 2.7.1.2): 1 mM ATP, 20 mM Glucose, 5 mM $MgCl_2$, 0.25 mM NADH, 10 mM KCl, 2.5 mM phosphoenol pyruvate, 1 unit/reaction lactate dehydrogenase, 1 unit/reaction pyruvate kinase

- Hexokinase (EC 2.7.1.1): 1 mM ATP, 1 mM Glucose, 5 mM $MgCl_2$, 0.25 mM NADH, 10 mM KCl, 2.5 mM phosphoenol pyruvate, 1 unit/ reaction lactate dehydrogenase, 1 units/reaction pyruvate kinase

- Isocitrate dehydrogenase (EC 1.1.1.42): 0.6 mM Isocitrate (threo-D(+)-iso), 4 mM $MgCl_2$, 0.4 mM NADP

- Lactate dehydrogenase (LDH: EC 1.1.1.27): 1 mM pyruvate and 0.12 mM NADH

- Malic enzyme (malate dehydrogeanse-decarboxylating) (EC 1.1.1.40): 1 mM malate, 4 $MgCl_2$, 0.4 mM NADP

- Malate dehydrogenase (EC 1.1.1.37): 0.5 mM oxaloacetate, 0.12 mM NADH

- Na^+/K^+ ATPase (EC 3.6.3.9): With and without 0.5 mM ouabain, 45 mM NaCl, 10 mM KCl, 2.5 mM $MgCl_2$, 2 mM PEP, 0,5 mM ATP, 0.16 mM NADH, 1 mM NaN_3, 1 mM EDTA, 5 unit/ reaction Pyruvate kinase, 5 unit/reaction lactate dehydrogenase

- Phosphoenol pyruvate carboxykinase (EC 4.1.1.32): 0.2 mM deoxyGuanosine diphosphate, 20 mM sodium-bicarbonate, 0.5 mM PEP, 1 mM $MnCl_2$, 0.12 mM NADH

- Pyruvate kinase (PK: EC 2.7.1.11): 2.5 mM phosphoenol pyruvate, 30 mM KCl, 10 mM $MgCl_2$, 0.12 mM NADH, 2.5 mM ADP, 1 unit/ reaction lactate dehydrogenase

The enzyme activity is represented as micromoles of substrate consumed or product liberated per minute (U) per gram protein in the homogenate.

2.4 Cloning and sequencing of partial neuroglobin and hypoxia inducible factor (HIF)-1α cDNA

2.4.1 RNA isolation and first strand cDNA synthesis

Total RNA (DNase treated) was isolated from brain, gills and liver tissues using RNeasy Mini Kit (Qiagen, Missisauga, ON) using the manufacturer's protocol and quantified by spectrophotometer reading at 260 nm (Varian, CARY 50 Bio UV-visible Spectrometer). The RNA

integrity was determined by performing 1 % agarose gel electrophoresis containing ethidium bromide. First Strand cDNA Synthesis kit (MBI Fermentas, Burlington, ON) was used for synthesis of first strand cDNA from 1 µg total RNA. Briefly, total RNA was denatured for 5 min at 70 ^{o}C, then cooled on ice for 10 min. The sample was used in 20 µl reverse transcriptase reaction using 0.5 µg oligo d(T) primers, 1 mM each dNTP, 20 U ribonuclease inhibitors, 40 U of Moloney murine leukemia virus reverse transcriptase (M-MuLV). The reaction was incubated for 1 hr at 37 ^{o}C and stopped by heating for 10 min at 70 ^{o}C.

2.4.2 Cloning and sequencing partial neuroglobin

2.4.2.1 Neuroglobin primer design

Neuroglobin primers were designed using the conserved regions of neuroglobin sequences from zebrafish (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss*), and pufferfish (*Tetraodon nigroviridis*).

2.4.2.2 PCR and cloning of neuroglobin

The PCR reaction was performed with 1µl of brain cDNA as a template, 15 pmol each of neuroglobin forward and reverse primer (Table 1), 10 mM dNTPs, 1.5 mM $MgCl_2$ and 1U Taq polymerase in a 25 µl total volume. The PCR cycles and conditions include: Initial denaturation at 94 ^{o}C for 1 min; 40 cycles of 94 ^{o}C for 30 s, 61.5 ^{o}C for 30 s, 72 ^{o}C at 30 s; final elongation at 72 ^{o}C for 10 min then cooled at 4 ^{o}C. The resulting single PCR product of 160 bp was detected by running a 1.5 % agarose gel electrophoresis containing ethidium bromide. After the gel extraction (QIAquick Gel Extraction Kit; Qiagen, Missisauga, ON) of the PCR product, it was ligated and cloned using pGEM-T easy vector systems (Promega, Madison, WI) and introduced into DH5α *Escherichia coli* cells. After LB-ampicillin selection, transformed cells were cultured and plasmids were isolated using GenElute^{TM} Plasmid Miniprep kit (Sigma-Aldrich, Oakville, ON). The isolated plasmid was sequenced at York University Core Molecular biology and DNA Sequencing Facility

(Toronto, ON, Canada) using an Applied Biosystems 337 DNA Sequencer (Perkin Elmer, Foster City, CA). The partial neuroglobin sequence obtained was subjected to Basic Local Alignment Search Tool (BLAST) and aligned with the known sequence of other species (*Danio rerio:* NM_131853*, Oncorhynchus mykiss*: NM_001124389, NM_001124388*, Tetraodon nigroviridis:* AJ315608) using multiple sequence alignment tool, ClustalW.

2.4.2.3 Quantitative real-time PCR (qPCR) of neuroglobin and HIF-1α

Tissue-specific mRNA abundance of neuroglobin in the brain and HIF-1α in brain, liver and gills, were determined by qPCR. Prior to qPCR, PCR was performed to amplify the predicted neuroglobin and HIF-1α products under the following conditions: Initial denaturation at 94 ^{o}C for 1 min; 40 cycles of: 94 ^{o}C for 30 s, annealing for 30 s, 72 ^{o}C at 30 s; final elongation at 72 ^{o}C for 10 min then cooled at 4 ^{o}C. Primers used, annealing temperatures, and the product sizes for neuroglobin, HIF-1α (*Halichoeres trimaculatus*: DQ340563) and β-actin (*Onchorhyncus mykiss*: NM_001124235) are shown in table 1.

Table 1. Primers used for neuroglobin and HIF-1α quantitative real-time PCR (qPCR)

Gene	Primers	Annealing Temperature (^{o}C)	Product size (bp)
HIF-1α	Forward: 5'-AAGAGCTGAGGGAGATGCTG Reverse: 5'-ACACTGTGGCTGGGAGTTCT	57.5	575
Neuroglobin	Forward: 5’ CTCCAGCCCAGAGTTCCTC Reverse: 5’ CCTTCCCAGGTTCAACAAGA	61.5	160
β-actin	Forward: 5’ TGTCCCTGTATGCCTCTGGT Reverse: 5’ AAGTCCAGACGGAGGATGG	49	121

2.4.3 Standard Curve

The PCR products were each detected by running a 1.5 % agarose gel electrophoresis containing ethidium bromide. To construct a plasmid stock, the gel extracted PCR product was ligated using pGEM-T easy vector systems (Promega, Madison, WI) and introduced into DH5α *Escherichia coli* cells for cloning. After LB-ampicillin selection, transformed cells were cultured and plasmids with HIF-1α inserts were isolated using GenElute^{TM} Plasmid Miniprep kit (Sigma-Aldrich, Oakville, ON).

The plasmid stock was serially diluted 10 X using nuclease free water (Qiagen, Missisauga, ON). Each standard reaction mix contained 1 µl of standard, 4 pmol of each primer and 2X concentrated iQ^{TM} SYBR Green Supermix (Bio-Rad, Hercules, CA). Each of 25 µl reactions had 100 mM KC, 20 mM Tris-HCl, pH 8.4, 0.4 mM each dNTP (dATP, dCTP, dGTP,

dTTP), 50 U/ml iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR Green I, 20 nM fluorescein, and stabilizers. To prevent pipetting errors, master mixes of triplicate reactions (3 x 25 µl) were prepared. The background subtracted threshold cycles (C_T) for the standards were determined using the iCycler IQ™ real time detection software (Bio-Rad, Hercules, CA) with PCR conditions including: initial denaturation at 94 °C for 3 min; 40 cycles of: 94 °C for 30 s, annealing for 30 s, 72 °C at 30 s; final elongation at 72 °C for 8 min then cool at 4 °C. PCR products were subjected to melt curve analysis to confirm the presence of a single amplicon. Neuroglobin threshold cycle (C_T) values were plotted against log of standard copy numbers to obtain standard curves. The PCR efficiency (E) ranged from 97-100%, determined using the equation, $E = [10^{-(1/slope)} * 100]$, automatically calculated in the iCycler IQ™ real time detection software (Bio-Rad). The HIF-1α threshold cycles (C_T) values were plotted against log of standard plasmid concentrations to obtain a standard curve.

2.4.4 Quantification of samples

1 µl cDNA was used in a reaction of 25 µl. Both gene of interest and β-actin were measured for each sample. The reaction components, PCR conditions and melt curve analysis for all the samples were kept constant as the standards. The gene of interest, and β-actin mRNA abundances were determined using the background subtracted threshold cycle (C_T) values and their respective standard curves. Both neuroglobin and HIF-1α mRNA abundance were normalized and expressed as a ratio of gene of interest to β-actin. β-actin was used as the house-keeping gene (internal control) for quantification since the C_T values were similar for all samples in the study.

2.5 Statistical Analysis

All statistical analysis were performed with SPSS version 15.01 (SPSS Inc., Chicago, IL) and data are shown as mean ± standard error of mean (S.E.M). The data were transformed (logarithmic), wherever necessary, for homogeneity of variance, but non-transformed values are

shown in the figures and tables. One way Analysis of variance (ANOVA) followed by Least Significant Difference (LSD) post hoc test was used to determine temporal changes in HIF-1α and neuroglobin mRNA abundances, metabolite levels, and enzyme activities over the 24 h diurnal cycle, as well as anoxia exposure. Two-way ANOVA followed by LSD post hoc test was used to determine the effects of sand-dwelling on neuroglobin and HIF-1α mRNA abundances at night. A probability level of $P \leq 0.05$ was considered significant.

3 Results

3.1 Plasma:

Plasma metabolites were measured during the 24 h diurnal cycle. The highest glucose level was seen at 14:00 compared to all other time points over a 24 h diurnal cycle ($P \leq 0.05$; One-way ANOVA). The glucose level dropped significantly at 18:00 attaining the lowest level at 21:00, and the low levels were maintained until 3:00, after which it increased to a similar level seen at day time points (Fig. 2A). There were no significant changes in plasma lactate levels between 10:00 and 14:00, after which the levels dropped and were significantly lower at 18:00 and decreased even further at 21:00 and 24:00. At 6:00, the level increased significantly compared to the night time values, attaining levels similar to that at 10:00 and 14:00 (Fig 2B). To determine the effects of sand at night, the groups without sand were compared to groups with sand. Statistical comparison between groups with sand and no sand at night times (21:00, 24:00, 3:00, 6:00) revealed significant differences in both glucose and lactate levels; both levels were significantly higher in the group with sand compared to without sand (Table 2; $P \leq 0.05$; Two-way ANOVA). Anoxia response was determined in the wrasse by bubbling nitrogen gas; the plasma lactate level increased significantly after 6 h of anoxia, while there was no change in glucose level without anoxia (Table 3; $P \leq 0.05$; One-way ANOVA).

3.2 Brain:

HIF-1α mRNA abundance was used as an indicator of molecular response to oxygen limitation. The HIF-1α transcript level was significantly lower at 18:00 compared to all other time points (Fig 3; $P \leq 0.05$; One-way ANOVA). The mRNA abundance of neuroglobin in the brain was also measured as an indicator of oxygen limitation. However, neuroglobin gene expression was not significantly different between any time points over the 24 h cycle (Table 4).

Metabolites (glucose and glycogen), as well as glycolytic enzyme (hexokinase, HK; pyruvate kinase, PK; lactate dehydrogenase, LDH) activities in the brain were measured as indicators of glycolytic capacity of the tissue. The glucose levels in the brain increased significantly at 12:00 compared to 10:00, 14:00 and 18:00, maintaining the level until 6:00 and dropping significantly at 10:00 (Fig 4A). This corresponded with an increase in HK activity at 21:00 compared to the day time points (10:00 and 14:00 and 18:00). The maximal HK activity was attained at 24:00 after which the activity decreased to day time values (Fig 4B). Brain glycogen levels were significantly lower at night compared to day samples. Specifically, the levels were significantly lower at 24:00 compared to all other time points. The low levels were maintained until 6:00, after which the levels increased significantly at 10:00 and 14:00 (Fig 4C).

The activities of PK, a glycolytic enzyme, and citrate synthase (CS) and isocitrate dehydrogenase (ICDH), two enzymes of the TCA cycle, showed no significant changes over the 24h diurnal cycle (Table 4). Similarly, the activities of malic enzyme (ME), glucose-6-phosphate dehydrogenase (G6PDH), and malate dehydrogenase (MDH) measured as indicators of oxidative pathways including fatty acid synthesis and reactive oxygen species (ROS) scavenging, did not show significant difference over a 24 h diurnal cycle (Table 4).

Statistical comparison between the sand and no sand groups at night revealed significant differences in glucose and glycogen levels, as well as enzyme activities, except for ME (Table 5; $P \leq 0.05$; Two-way ANOVA). Overall, glucose and glycogen levels and all the enzyme activities were significantly higher in the group with sand compared to no sand. G6PDH and MDH activities in the group with sand showed significantly higher activities compared to group without sand at 3:00 and 6:00, whereas HK activity in the sand group was significantly higher compared to no sand group at 3:00 (Table 5).

With anoxia, the HIF-1α mRNA abundance increased significantly at 6 and 12 h and neuroglobin mRNA abundance increased significantly at 12 h of exposure compared to 0 h group (Table 6; $P \leq 0.05$; One-way ANOVA). The glucose level increased significantly at 6 h of anoxia compared to 0 h and remained high until 12 h. However, CS activity decreased significantly after 12 h anoxia compared to 0 and 6 h of exposure (Table 6).

3.3 Liver:

HIF-1α mRNA abundance in the liver was highest at 10:00 followed by a significant decrease until 18:00. The level increased at 24:00, but decreased significantly at 6:00 compared to 10:00, 24:00, and 3:00, but not with other time points (Fig 3; $P \leq 0.05$; One-way ANOVA). While metabolites including glycogen and lactate did not show significant changes, the glucose level decreased at 14:00 and 18:00 compared to 10:00, which showed the highest level, and reached the lowest level at 21:00 over the 24 h diurnal cycle (Table 7; $P \leq 0.05$; One-way ANOVA). The level increased significantly at 24:00 attaining level similar to that at 14:00 and 18:00 and this was maintained until 10:00. The HK activity showed an increasing trend during the day and a decreasing trend at night, with a significant drop only at 21:00 compared to all the other time points except at 10:00. The activity of phosphoenolpyruvate carboxykinase (PEPCK), a measure of gluconeogenic capacity, was maintained throughout the day. However, the activity of this enzyme showed a decreasing trend at 21:00 and reached the lowest activity at 3:00, after which the activity increased at 6:00 and was not significantly different from the day time value. MDH was maintained during the day and the activity was significantly higher only at 24:00 compared to all the time points. There were no significant changes in the activities of ME, CS, ICDH, G6PDH, LDH, and GK over a 24 h time period (Table 7).

Statistical comparison of sand and no sand groups at night revealed significant differences in HIF-1α mRNA abundance, glucose and glycogen levels and enzyme activities except

ME, MDH, and GK (Table 8; $P \leq 0.05$; Two-way ANOVA). Overall, all enzyme activities were significantly lower in the groups with sand compared to without sand. However, glucose and glycogen levels were significantly higher in the sand group compared to no sand group. The highest HIF-1α mRNA abundance was seen at 3:00, while 21:00 and 6:00 showed the lowest abundance compared to the other sampling times at night. MDH activity was significantly lower in group with sand compared to group without sand at 21:00. However, the highest MDH activity was at 24:00 compared to the other night time points. GK activity was significantly lower in the group with sand compared to group without sand at 3:00 and 6:00 (Table 8).

In the anoxia study, lactate level increased significantly at 6 h of anoxia exposure and stayed elevated even at 12 h compared to 0 h group (Table 9; $P \leq 0.05$; One-way ANOVA). The activity of G6PDH significantly increased at 12 h compared to 0 h of anoxia. HIF-1α mRNA abundance as well as glucose, glycogen, and enzyme activities showed no significant changes in response to 6 and 12 h of anoxia exposure (Table 9).

3.4 Gills:

HIF-1α mRNA abundance was significantly higher at 14:00, 21:00 and 6:00, compared to 10:00, 18:00, and 24:00. The lowest gene expression of HIF-1α was at 3:00 compared to all other time points (Fig 3; $P \leq 0.05$; One-way ANOVA). The HK activity in the gill was highest at 14:00, which decreased significantly until 6:00, reaching the lowest activity compared to the other time points (Table 10; $P \leq 0.05$; One-way ANOVA). The highest ME activity was seen at 14:00 over the 24 h diurnal cycle; the ME activities at 24:00, 3:00 and 6:00 were significantly lower compared to other time points. An increasing trend was seen in G6PDH activity during the day, with the highest activity was seen at 18:00, after which the activity significantly dropped at 24:00 compared to day time points. The low activity was maintained during the night time points. No significant changes

in the activities of CS ICDH, MDH, LDH, PK and Na^+/K^+-ATPase were observed over a 24 h time period (Table 10).

Statistical comparison between groups with sand and no sand at night revealed significant differences in ME, ICDH, G6PDH, and PK activities (Table 11; $P \leq 0.05$; Two-way ANOVA). Overall, these enzyme activities were significantly lower in groups with sand compared to no sand, except in PK activity, which showed the opposite response. G6PDH and Na^+/K^+-ATPase activities were significantly higher at 21:00 in the group with sand compared to the group without sand, whereas HK activity was significantly higher at 24:00 in the sand group. However, at 6:00 both G6PDH and HK activities were significantly higher in the groups without sand compared with sand. No significant differences were observed in HIF-1α mRNA abundance, as well as CS, LDH and MDH activities between groups with sand and no sand. There was no significant difference in any of the parameters with anoxia exposure (Table 12).

3.5 Heart:

The highest glucose level was seen at 14:00 compared to all time points over a 24 h diurnal cycle. The glucose level decreased significantly at night time until 3:00, compared to 14:00, after which the levels increased during day time (Fig 5A; $P \leq 0.05$; One-way ANOVA). The HK activities at 10:00, 21:00 and 6:00 were significantly higher than other time points, while the lowest activity was seen at 14:00 (Fig 5B). The lactate level at 18:00, 21:00 and 3:00 were significantly lower than other time points, while the highest level was seen at 24:00. Glycogen levels as well as LDH activities did not show significant differences over a 24 h diurnal cycle. ME activity in the heart maintained similar activity in all time points except for a significant drop at 14:00, while the lowest CS activity was seen at 6:00 compared to 10:00 and 18:00, but not with any other time points.

Statistical comparison between night time groups with sand and no sand revealed significant differences in glucose level and CS activity. Glucose level was significantly higher in the group with sand compared to no sand, while CS activity was lower in the group with sand compared to no sand (Table 14; $P \leq 0.05$; Two-way ANOVA). Glucose level at 24:00 was the lowest compared to other night time points, while lactate level showed the highest level at 24:00 compared to other night time points (Table 14). There were no significant differences between night time groups with sand and no sand in LDH activities and glycogen levels.

In the anoxia study, glucose and lactate levels increased significantly at 6 h of anoxia exposure compared to 0 h group, and this level was maintained even at 12 h (Table 15; $P \leq 0.05$; One-way ANOVA). HK activity decreased significantly at 6 h, compared to 0 h group, but the activity increased significantly at 12 h compared to 6 h group. Similarly, both LDH and ME activities decreased significantly at 6 h compared to 0 h anoxia exposure, while CS activity decreased significantly at 12 h of anoxia exposure compared to 0 and 6 h (Table 15).

3.6 Muscle:

Changes in muscle glucose and glycogen levels were not observed during the 24 h diurnal cycle (Fig 6A and B; $P \leq 0.05$; One-way ANOVA). The lactate levels at night time were significantly lower compared to the day time points, with the highest level seen at 6:00 compared to all other time points (Fig 6C). Statistical comparison between groups with sand and no sand at night showed a significant increase in lactate level at 6:00 compared to other night time points, while no significant changes were observed in glucose and glycogen levels (Table 16; $P \leq 0.05$; Two-way ANOVA). In anoxia study, both glucose and lactate level showed significant increase after 12 h anoxia compared to 0 h group (Table 17; $P \leq 0.05$; One-way ANOVA). There were no significant changes in glycogen levels with anoxia exposure.

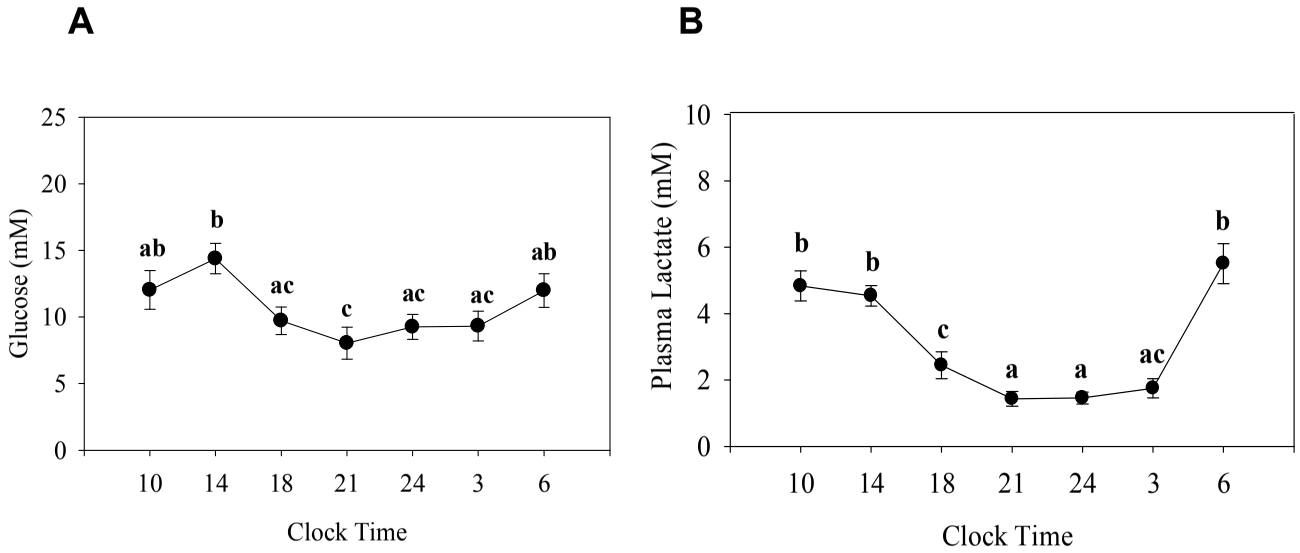
A
B
Glucose (mM)
Plasma Lactate (mM)
Clock Time
Clock Time
ab
b
ac
c
ac
ac
ab
b
b
c
a
a
ac
b
10
14
18
21
24
3
6

Figure 2. Plasma metabolite measurement during 24 h diurnal cycle.

Plasma glucose (A), and lactate (B) levels in the three-spot wrasse. The x-axis represents the clock time, and the y-axis represents the units of measurement (mM). Values represent means ± SEM (*N*=5-9); Different letters indicate significant difference (P≤0.05, One-way ANOVA).

Table 2. Wrasse plasma measurements of metabolites during night time sampling points, with and without sand

Plasma metabolite measurements at night sampling time points (21:00, 24:00, 3:00, 6:00 clock time) with sand (S) and without sand (WS). Values represent mean ± SEM (*N*=5-7). Significant sand effects and the interaction are shown (P≤0.05; Two-way-ANOVA); Metabolite measurements are expressed as μmol/g protein.

Wrasse Plasma Measurements: Night Time									P≤0.05
	21:00		24:00		3:00		6:00		
	With Sand	Without Sand	With Sand	Without Sand	With Sand	Without Sand	With Sand	Without Sand	
Glucose	8.03 ± 1.21	8.48 ± 1.81	9.25 ± 0.94	6.52 ± 0.72	9.31 ± 1.11	5.69 ± 1.01	11.98 ± 1.26	8.86 ± 1.27	S>WS
Lactate	1.43 ± 0.22	0.92 ± 0.45	1.46 ± 0.18	1.67 ± 0.22	1.75 ± 0.29	1.28 ± 0.30	5.51 ± 0.60	3.06 ± 0.19	S> WS 6>24,3>21

Table 3. Wrasse plasma measurements of glucose and lactate during anoxia exposure

Plasma enzyme activities and metabolite measurements at 0, 6, and 12 h of anoxia in three-spot wrasse. Values represent mean ± SEM (*N*=4-9). Significant time effects are shown as superscripts ($P \leq 0.05$, one-way-ANOVA); Values with same superscripts are not significantly different. The metabolites are expressed as mM.

Plasma Anoxia		
	0 h	**6 h**
Glucose	12.03 ± 1.45	20.04 ± 10.95
Lactate	4.83 ± 0.46 [a]	46.10 ± 1.77 [b]

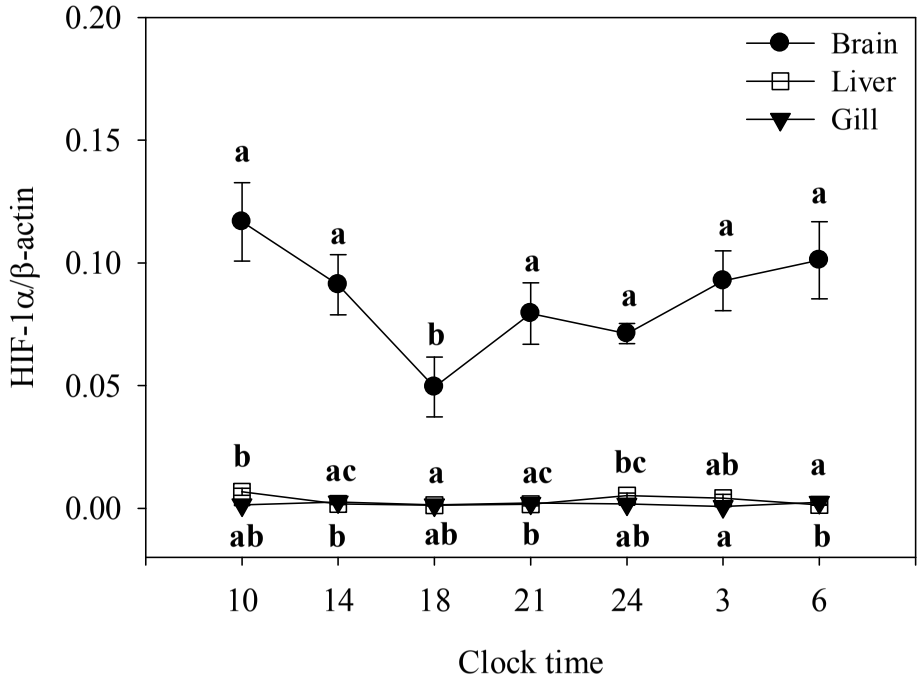
0.20
0.15
0.10
0.05
0.00
HIF-1α/β-actin
Brain
Liver
Gill
a
a
b
a
a
a
a
b
ac
a
ac
bc
ab
a
ab
b
ab
b
ab
a
b
10
14
18
21
24
3
6
Clock time

Figure 3. Hypoxia inducible factor (HIF)-1α mRNA abundance in the brain, liver and gills during the 24 h diurnal cycle.

Hypoxia inducible factor (HIF)-1α mRNA abundance in the brain, liver and gills of three-spot wrasse quantified using real-time PCR. The x-axis represents the clock time and the y-axis represents the normalized ratio of HIF-1α over housekeeping gene, β-actin mRNA abundance. Values represent means ± SEM (*N*=4-8); Different letters indicate significant difference (P≤0.05, One-way ANOVA).

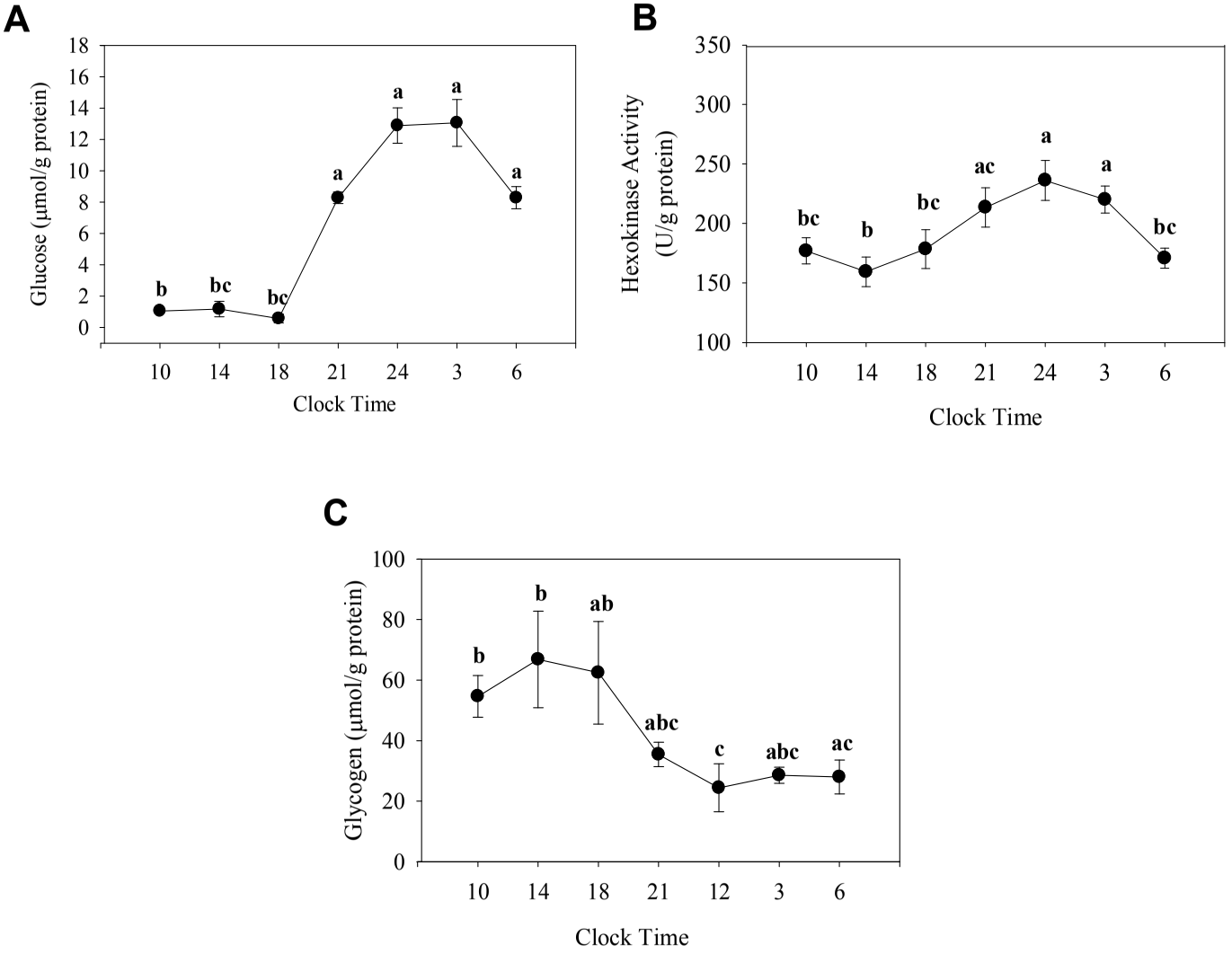
A
Glucose (µmol/g protein)
18
16
14
12
10
8
6
4
2
0
b
bc
bc
a
a
a
10
14
18
21
24
3
6
Clock Time
B
Hexokinase Activity (U/g protein)
350
300
250
200
150
100
bc
b
bc
ac
a
a
bc
10
14
18
21
24
3
6
Clock Time
C
Glycogen (µmol/g protein)
100
80
60
40
20
0
b
b
ab
abc
c
abc
ac
10
14
18
21
12
3
6
Clock Time

Figure 4. Brain glucose, glycogen and hexokinase activity during the 24 h diurnal cycle.

Glucose (A), glycogen (C) level, and hexokinase activity (B) in the brain of the three-spot wrasse. The x-axis represents the clock time, and y-axis represents the unit of measurement (glucose and glycogen in μmol/g protein; hexokinase activity in U/g protein) Values represent means ± SEM (*N*=5-8); Different letters indicate significant difference (P≤0.05, One-way ANOVA).

Table 4. Wrasse brain measurements of neuroglobin mRNA abundance and enzyme activities during diurnal cycle

Brain neuroglobin (Ngb) mRNA abundance, and enzyme activities at different sampling time points (clock time) over a 24 h diurnal cycle. Values represent mean ± SEM (*N*=5-8). The mRNA abundance represents normalized ratio of target gene (Ngb) over house-keeping gene (β-actin); enzyme activities are expressed as U/ g protein. Citrate synthase (CS), malic enzyme (ME), isocitrate dehydrogenase (ICDH), glucose-6-phosphate dehydrogenase (G6PDH), malate dehydrogenase (MDH), lactate dehydrogenase (LDH), pyruvate kinase (PK).

Wrasse Brain Measurements: Diurnal Cycle							
	10:00	**14:00**	**18:00**	**21:00**	**24:00**	**3:00**	**6:00**
Ngb mRNA	0.45 ± 0.01	0.42 ± 0.02	0.42 ± 0.01	0.43 ± 0.02	0.44 ± 0.03	0.44 ± 0.02	0.43 ± 0.02
CS	92.98 ± 2.15	n/a	102.26 ± 2.49	n/a	94.15 ± 2.63	95.83 ± 3.17	100.94 ± 7.56
ME	1.08 ± 0.12	1.09 ± 0.13	1.10 ± 0.21	1.38 ± 0.23	1.53 ± 0.10	1.28 ± 0.17	1.11 ± 0.19
ICDH	23.14 ± 1.50	24.42 ± 1.75	29.01 ± 3.73	26.50 ± 2.06	25.28 ± 0.66	28.75 ± 1.81	28.39 ± 2.44
G6PDH	5.75 ± 0.44	4.84 ± 0.62	6.20 ± 0.81	5.12 ± 0.62	5.43 ± 0.26	6.10 ± 0.41	5.93 ± 0.30
MDH	858.74 ± 41.37	840.01 ± 50.36	952.73 ± 71.73	841.68 ± 61.36	847.61 ± 14.93	921.41 ± 33.27	960.70 ± 48.51
LDH	439.16 ± 19.52	389.05 ± 25.01	441.01 ± 26.58	427.78 ± 22.24	471.37 ± 10.25	426.10 ± 26.69	459.30 ± 26.43
PK	607.88 ± 23.02	632.70 ± 28.81	634.33 ± 48.92	625.38 ± 30.85	664.53 ± 14.88	586.97 ± 45.24	670.32 ± 36.01

Table 5. Wrasse brain measurements of hypoxia inducible factor (HIF)-1α, neuroglobin (Ngb) mRNA abundance, metabolites and enzyme activities during night time sampling points, with and without sand

Brain hypoxia inducible factor (HIF)-1α, neuroglobin (Ngb) mRNA abundance, enzyme activities and metabolite measurements at night time sampling points (21:00, 24:00, 3:00, 6:00 clock time) with sand (S) and without sand (WS). Values represent mean ± SEM (*N*=5-6). Significant sand, time effects and the interaction are shown (P≤0.05, Two-way-ANOVA); * represents significantly higher level between groups with sand and without sand at that time point (P≤0.05, Two-way-ANOVA). The mRNA abundance values represent the normalized ratio of target gene (HIF-1α and Ngb) over housekeeping gene (β-actin) mRNA abundance; enzyme activities are expressed as U/ g protein; metabolites are expressed as μmol/ g wet weight. Citrate synthase (CS), malic enzyme (ME), isocitrate dehydrogenase (ICDH), glucose-6-phosphate dehydrogenase (G6PDH), malate dehydrogenase (MDH), lactate dehydrogenase (LDH), hexokinase (HK), and pyruvate kinase (PK). n/s indicates no significant treatment effect.

Wrasse Brain Measurements: Night Time									P≤0.05
	21:00		24:00		3:00		6:00		
	With Sand	Without Sand	With Sand	Without Sand	With Sand	Without Sand	With Sand	Without Sand	
HIF-1α mRNA	7.94E-02 ± 1.25E-02	8.45E-02 ± 5.27E-03	7.12E-02 ± 4.07E-03	9.24E-02 ± 1.97E-02	9.27E-02 ± 1.22E-02	1.28E-01 ± 2.55E-02	1.01E-01 ± 1.57E-02	9.30E-02 ± 3.53E-02	n/s
Ngb mRNA	0.43 ± 0.02	0.44 ± 0.04	0.44 ± 0.03	0.42 ± 0.01	0.44 ± 0.02	0.48 ± 0.05	0.43 ± 0.02	0.45 ± 0.06	n/s
CS	n/a	n/a	94.15 ± 2.63	n/a	95.83 ± 3.17	74.30 ± 2.51	100.94 ± 7.56	79.12 ± 2.85	S>WS
ME	1.38 ± 0.23	1.39 ± 0.11	1.53 ± 0.10	1.35 ± 0.13	1.28 ± 0.17	0.92 ± 0.10	1.11 ± 0.19	1.15 ± 0.16	24≥21≥3,6
ICDH	26.50 ± 2.06	25.54 ± 1.59	25.28 ± 0.66	24.40 ± 1.32	28.75 ± 1.81	22.64 ± 0.66	28.39 ± 2.44	26.03 ± 1.25	S> WS
G6PDH	5.12 ± 0.62	5.64 ± 0.46	5.43 ± 0.26	5.13 ± 0.47	6.10 ± 0.41*	4.42 ± 0.23	5.93 ± 0.30 *	4.07 ± 0.10	S> WS
MDH	841.68 ± 61.36	931.22 ± 41.28	847.61 ± 14.93	881.46 ± 35.89	921.41 ± 33.27 *	730.46 ± 32.76	960.70 ± 48.51 *	765.73 ± 9.47	S> WS
LDH	427.78 ± 22.24	380.86 ± 25.71	471.37 ± 10.25	370.84 ± 18.86	426.10 ± 26.69	361.20 ± 21.79	459.30 ± 26.43	388.25 ± 23.03	S> WS
HK	213.48 ± 16.50	231.53 ± 10.39	236.13 ± 16.83	196.68 ± 15.36	220.02 ± 11.37 *	149.43 ± 11.14	170.87 ± 8.53	163.99 ± 17.71	S> WS 21≥24,3≥6
PK	625.38 ± 30.85	583.15 ± 25.22	664.53 ± 14.88	511.82 ± 35.37	586.97 ± 45.24	513.82 ± 11.83	670.32 ± 36.01	556.45 ± 5.99	S> WS
Glucose	8.27 ± 0.36	7.88 ± 1.04	12.88 ± 1.14	6.60 ± 1.30	13.05 ± 1.50	6.71 ± 2.89	8.28 ± 0.71	8.73 ± 2.71	S> WS
Glycogen	35.43 ± 4.05	14.89 ± 1.62	24.41 ± 7.93	13.88 ± 3.56	28.54 ± 2.63	12.55 ± 7.21	28.00 ± 5.59	20.42 ± 2.96	S> WS

Table 6. Wrasse brain measurements of hypoxia inducible factor (HIF)-1α and neuroglobin mRNA abundance, enzyme activities and metabolites during anoxia exposure

Brain hypoxia inducible factor (HIF)-1α and neuroglobin (Ngb) mRNA abundance, enzyme activities and metabolite measurements at 0, 6, and 12 h of anoxia in three-spot wrasse. Values represent mean ± SEM (*N*=3-8). Significant time effects are shown as superscripts ($P \leq 0.05$, one-way-ANOVA); Values with same superscripts are not significantly different. The mRNA abundance is expressed as normalized ratio of target gene (HIF- 1α, or Ngb) over house-keeping gene (β-actin); metabolites are expressed as μmol/ g protein; enzyme activities are expressed as U/ g protein. Citrate synthase (CS), malic enzyme (ME), isocitrate dehydrogenase (ICDH), glucose-6-phosphate dehydrogenase (G6PDH), malate dehydrogenase (MDH), lactate dehydrogenase (LDH), hexokinase (HK), pyruvate kinase (PK).

Wrasse Brain Anoxia			
	0 h	**6 h**	**12 h**
HIF-1α mRNA	1.17E-01 ± 1.60E-02 [a]	1.92E-01 ± 1.77E-02 [b]	3.21E-01 ± 1.65E-02 [c]
Ngb mRNA	0.45 ± 0.01 [a]	0.46 ± 0.02 [a]	0.55 ± 0.05 [b]
CS	92.98 ± 2.15 [a]	94.34 ± 3.72 [a]	70.58 ± 6.03 [b]
ME	1.08 ± 0.12	1.04 ± 0.11	0.60 ± 0.36
ICDH	23.14 ± 1.50	29.38 ± 1.63	26.69 ± 3.36
G6PDH	5.75 ± 0.44	5.84 ± 0.44	6.83 ± 0.56
MDH	858.74 ± 41.37	860.65 ± 60.85	787.14 ± 62.44
LDH	439.16 ± 19.52	378.36 ± 13.24	396.90 ± 48.78
HK	176.95 ± 11.00	162.72 ± 9.93	176.10 ± 16.88
PK	607.88 ± 23.02	573.19 ± 28.27	576.47 ± 22.23
Glucose	1.06 ± 0.10 [a]	18.03 ± 8.01 [b]	24.43 ± 5.67 [b]
Glycogen	54.62 ± 6.87	65.83 ± 11.87	40.03 ± 11.49

Table 7. Wrasse liver measurements of enzyme activities and metabolites during diurnal cycle

Liver enzyme activities and metabolite measurements at different sampling time points (clock time) over a 24 h diurnal cycle. Values represent mean ± SEM (*N*=2-8). Significant time effects are shown as superscripts (P≤0.05, One-way-ANOVA); Values with same superscripts are not significantly different. Metabolites are expressed as µmol/ g wet weight; enzyme activities are expressed as U/ g protein. Citrate synthase (CS), malic enzyme (ME), isocitrate dehydrogenase (ICDH), glucose-6-phosphate dehydrogenase (G6PDH), malate dehydrogenase (MDH), lactate dehydrogenase (LDH), hexokinase (HK), glucokinase (GK), phosphoenolpyruvate carboxykinase (PEPCK).

Wrasse Liver Measurements: Diurnal Cycle							
	10:00	**14:00**	**18:00**	**21:00**	**24:00**	**3:00**	**6:00**
CS	24.34 ± 2.63	n/a	24.51 ± 3.04	n/a	19.63 ± 1.85	18.43 ± 3.15	19.18 ± 1.10
ME	0.27 ± 0.12	0.27 ± 0.06	0.45 ± 0.14	0.39 ± 0.14	0.29 ± 0.05	0.32 ± 0.07	0.34 ± 0.09
ICDH	3.21 ± 0.72	7.52 ± 2.43	11.28 ± 1.07	7.75 ± 2.34	9.53 ± 0.81	7.72 ± 1.63	8.73 ± 2.25
G6PDH	2.18 ± 0.66	4.03 ± 0.43	4.40 ± 0.93	3.73 ± 1.14	5.07 ± 0.62	2.55 ± 0.53	4.40 ± 0.71
MDH	1159.25 ± 198.98 [a]	992.76 ± 143.29 [a]	1170.86 ± 220.08 [a]	873.27 ± 117.40 [a]	2349.58 ± 330.96 [b]	1606.41 ± 302.04 [ab]	1374.64 ± 196.33 [ab]
LDH	27.89 ± 2.86	22.81 ± 1.57	26.16 ± 2.79	29.05 ± 6.15	26.35 ± 3.92	24.02 ± 2.00	28.11 ± 3.50
HK	15.63 ± 2.88 [b]	20.03 ± 2.03 [abc]	22.92 ± 2.03 [ac]	16.80 ± 3.18 [bc]	25.85 ± 3.02 [a]	24.12 ± 3.03 [ac]	20.39 ± 1.45 [abc]
GK	7.34 ± 1.05	11.46 ± 1.05	10.66 ± 1.17	6.90 ± 1.99	8.90 ± 2.22	9.07 ± 0.99	7.06 ± 0.76
PEPCK	0.88 ± 0.31 [b]	0.79 ± 0.12 [b]	0.81 ± 0.15 [b]	0.54 ± 0.19 [b]	0.47 ± 0.16 [ab]	0.16 ± 0.07 [a]	0.64 ± 0.15 [b]
Glucose	50.01 ± 5.89 [a]	39.75 ± 3.11 [b]	30.14 ± 3.61 [b]	20.54 ± 3.01 [c]	38.12 ± 3.17 [b]	29.86 ± 1.62 [b]	38.72 ± 1.76 [b]
Glycogen	246.68 ± 37.85	318.84 ± 57.34	283.70 ± 78.03	347.83 ± 95.24	398.90 ± 88.69	438.50 ± 97.69	361.26 ± 65.85
Lactate	1.58 ± 0.50	2.16 ± 0.66	2.06 ± 1.61	1.42 ± 0.52	2.29 ± 0.40	3.31 ± 0.54	1.44 ± 0.34

Table 8.Wrasse liver measurements of hypoxia inducible factor (HIF)-1α and neuroglobin (Ngb) mRNA abundance, enzyme activities, and metabolites during night time sampling points, with and without sand

Liver hypoxia inducible factor (HIF)-1α mRNA abundance, enzyme activities and metabolite measurements at night time sampling points (21:00, 24:00, 3:00, 6:00 clock time) with sand (S) and without sand (WS). Values represent mean ± SEM (*N*=2-6). Significant sand and time effects and the interaction are shown (P≤0.05, two-way-ANOVA); * represents significantly higher level between groups with sand and without sand at that time point (P≤0.05, Two-way-ANOVA). The mRNA abundance values represent the normalized ratio of HIF-1α over housekeeping gene (β-actin) mRNA abundance; metabolites are expressed as μmol/ g wet weight; enzyme activities are expressed as U/ g protein. Citrate synthase (CS), malic enzyme (ME), isocitrate dehydrogenase (ICDH), glucose-6-phosphate dehydrogenase (G6PDH), malate dehydrogenase (MDH), lactate dehydrogenase (LDH), hexokinase (HK), glucokinase (GK), phosphoenolpyruvate carboxykinase (PEPCK). n/s indicates no significant treatment effect.

Wrasse Liver Measurements: Night Time									P≤0.05
	21:00		24:00		3:00		6:00		
	With Sand	Without Sand	With Sand	Without Sand	With Sand	Without Sand	With Sand	Without Sand	
HIF-1α mRNA	1.65E-03 ± 6.66E-04	1.92E-03 ± 5.05E-04	5.14E-03 ± 1.05E-03	3.72E-03 ± 8.43E-04	4.12E-03 ± 1.64E-03	7.35E-03 ± 3.97E-03	1.43E-03 ± 1.40E-04	3.42E-03 ± 8.20E-04	3≥24>21,6
CS	n/a	n/a	19.63 ± 1.85	n/a	18.43 ± 3.15	26.66 ± 3.20	19.18 ± 1.10	26.48 ± 3.08	S < WS
ME	0.39 ± 0.14	0.40 ± 0.03	0.29 ± 0.05	0.32 ± 0.06	0.32 ± 0.07	0.45 ± 0.20	0.34 ± 0.09	0.43 ± 0.06	n/s
ICDH	7.75 ± 2.34	9.64 ± 1.38	9.53 ± 0.81	15.23 ± 1.95	7.72 ± 1.63	12.32 ± 3.41	8.73 ± 2.25	12.44 ± 1.23	S < WS
G6PDH	3.73 ± 1.14	6.21 ± 0.76	5.07 ± 0.62	6.29 ± 0.41	2.55 ± 0.53	3.94 ± 0.52	4.40 ± 0.71	5.07 ± 1.18	S < WS
MDH	873.27 ± 117.40	1637.67 ± 233.60	2349.58 ± 330.96	1931.55 ± 222.54	1606.41 ± 302.04	1420.31 ± 226.28	1374.64 ± 196.33	1133.15 ± 207.10	24>21,3,6
LDH	29.05 ± 6.15	26.35 ± 3.62	26.35 ± 3.92	30.60 ± 3.48	24.02 ± 2.00	29.27 ± 0.42	28.11 ± 3.50	30.26 ± 1.71	S < WS
HK	16.80 ± 3.18	28.99 ± 3.12	25.85 ± 3.02	30.90 ± 4.03	24.12 ± 3.03	39.07 ± 9.71	20.39 ± 1.45	26.66 ± 2.69	S < WS
GK	6.90 ± 1.99	9.13 ± 1.40	8.90 ± 2.22	10.68 ± 1.67	9.07 ± 0.99	22.58 ± 5.52 *	7.06 ± 0.76	15.07 ± 1.16 *	3>6>21,24
PEPCK	0.54 ± 0.19	0.37 ± 0.12	0.47 ± 0.16	0.45 ± 0.14	0.16 ± 0.07	0.55 ± 0.17	0.64 ± 0.15	0.72 ± 0.32	S < WS 6 ≥21,24≥3
Glucose	20.54 ± 3.01	19.33 ± 5.30	38.12 ± 3.17	22.52 ± 5.28	29.86 ± 1.62	17.15 ± 6.24	38.72 ± 1.76	29.70 ± 4.73	S > WS
Glycogen	347.83 ± 95.24	176.17 ± 70.89	398.90 ± 88.69	100.85 ± 44.91	438.50 ± 97.69	88.31 ± 56.22	361.26 ± 65.85	64.81 ± 44.90	S > WS
Lactate	1.42 ± 0.52	1.84 ± 0.34	2.29 ± 0.40	2.39 ± 0.37	3.31 ± 0.54	1.48 ± 0.60	1.44 ± 0.34	2.72 ± 0.67	n/s

Table 9. Wrasse liver measurements of hypoxia inducible factor (HIF)-1α and neuroglobin mRNA abundance, enzyme activities and metabolites during anoxia exposure

Liver hypoxia inducible factor (HIF)-1α mRNA abundance, enzyme activities and metabolite measurements at 0, 6, and 12 h of anoxia in three-spot wrasse. Values represent mean ± SEM (*N*=4-8). Significant time effect are shown as superscripts (P≤0.05, one-way-ANOVA); Values with same superscripts are not significantly different. The mRNA abundance is expressed as ratio of target gene (HIF- 1α) over house-keeping gene (β-actin); metabolites are expressed as μmol/ g tissue; enzyme activities are expressed as U/ g protein, Citrate synthase (CS), malic enzyme (ME), isocitrate dehydrogenase (ICDH), glucose-6-phosphate dehydrogenase (G6PDH), malate dehydrogenase (MDH), lactate dehydrogenase (LDH), hexokinase (HK), glucokinase (GK), phosphoenolpyruvate carboxykinase (PEPCK).

Wrasse Liver Anoxia			
	0 h	**6 h**	**12 h**
HIF-1α mRNA	6.75E-03 ± 1.48E-03	1.16E-02 ± 1.90E-03	1.37E-02 ± 2.78E-03
CS	24.34 ± 2.63 [a]	21.71 ± 1.26 [ab]	16.48 ± 0.63 [b]
ME	0.27 ± 0.12	2.05 ± 1.51	1.40 ± 0.44
ICDH	3.21 ± 0.72	3.57 ± 1.42	2.80 ± 2.53
G6PDH	2.20 ± 0.65 [a]	3.44 ± 0.47 [ab]	5.66 ± 1.21 [b]
MDH	1159.25 ± 198.98	787.79 ± 99.39	851.90 ± 210.71
LDH	27.89 ± 2.86	33.30 ± 3.11	38.69 ± 3.40
HK	15.63 ± 2.88	9.58 ± 1.80	12.01 ± 2.18
GK	7.34 ± 1.05	5.38 ± 1.12	7.03 ± 0.91
PEPCK	0.88 ± 0.31	0.75 ± 0.15	0.99 ± 0.01
Glucose	50.01 ± 5.89	47.95 ± 10.72	29.95 ± 11.23
Glycogen	246.68 ± 37.85	391.29 ± 105.16	96.68 ± 57.32
Lactate	1.58 ± 0.50 [a]	13.06 ± 1.64 [b]	19.51 ± 2.05 [b]

Table 10. Wrasse gill measurements of enzyme activities during diurnal cycle

Gill enzyme activities at different sampling time points (clock time) over a 24 h diurnal cycle. Values represent mean ± SEM (*N*=4-8). Significant time effect are shown as superscripts (P≤0.05, One-way-ANOVA); Values with same superscripts are not significantly different. Enzyme activities are expressed as U/ g protein. Citrate synthase (CS), malic enzyme (ME), isocitrate dehydrogenase (ICDH), glucose-6-phosphate dehydrogenase (G6PDH), malate dehydrogenase (MDH), lactate dehydrogenase (LDH), hexokinase (HK), pyruvate kinase (PK), sodium potassium adenotriphosphatase (ATPase).

Wrasse Gill Measurements: Diurnal Cycle							
	10:00	**14:00**	**18:00**	**21:00**	**24:00**	**3:00**	**6:00**
CS	23.74 ± 1.78	n/a	22.01 ± 1.72	n/a	18.41 ± 2.17	19.97 ± 3.82	19.61 ± 3.79
ME	5.34 ± 0.66 [bc]	11.49 ± 2.06 [c]	7.00 ± 0.98 [bc]	6.94 ± 1.77 [bc]	2.95 ± 1.60 [a]	2.57 ± 0.83 [a]	2.72 ± 2.02 [a]
ICDH	15.22 ± 2.96	22.09 ± 6.06	21.73 ± 1.68	22.07 ± 3.32	9.71 ± 2.26	13.07 ± 2.82	12.01 ± 3.15
G6PDH	21.04 ± 3.44 [ab]	24.01 ± 4.96 [a]	34.07 ± 3.16 [b]	27.54 ± 1.21 [b]	14.64 ± 3.09 [a]	14.06 ± 3.53 [a]	12.43 ± 2.48 [a]
MDH	287.09 ± 43.96	416.73 ± 72.90	377.15 ± 33.08	457.60 ± 38.37	459.98 ± 69.40	346.72 ± 58.26	410.78 ± 49.52
LDH	455.67 ± 72.35	613.18 ± 65.20	480.75 ± 36.51	524.50 ± 65.26	475.32 ± 40.58	416.61 ± 21.86	478.61 ± 21.76
HK	67.53 ± 8.04 [ab]	91.38 ± 12.04 [c]	80.14 ± 6.95 [bc]	63.62 ± 4.19 [abc]	77.35 ± 6.30 [bc]	59.58 ± 8.26 [ab]	51.23 ± 4.24 [a]
PK	151.83 ± 9.68	162.22 ± 16.92	162.36 ± 7.55	154.93 ± 11.84	179.37 ± 12.87	182.77 ± 16.47	157.51 ± 6.94
ATPase	19.80 ± 3.02	21.48 ± 4.11	20.15 ± 2.23	28.06 ± 4.41	30.13 ± 3.06	15.50 ± 3.11	17.44 ± 5.43

Table 11. Wrasse gill measurements of hypoxia inducible factor (HIF)-1α mRNA abundance, and enzyme activities during night time sampling points, with and without sand

Gill hypoxia inducible factor (HIF)-1α mRNA abundance and enzyme activities at night time sampling points (21:00, 24:00, 3:00, 6:00 clock time) with sand (S) and without sand (WS). Values represent mean ± SEM (*N*=3-6). Significant sand effects and the interactions are shown (P≤0.05; two-way-ANOVA); * represents significantly higher level between groups with sand and without sand at that time point (P≤0.05, Two-way-ANOVA). The mRNA abundance is expressed as normalized ratio of HIF-1α over house-keeping gene (β-actin); enzyme activities are expressed as U/ g protein. Citrate synthase (CS), malic enzyme (ME), isocitrate dehydrogenase (ICDH), glucose-6-phosphate dehydrogenase (G6PDH), malate dehydrogenase (MDH), lactate dehydrogenase (LDH), hexokinase (HK), pyruvate kinase (PK), Na^+/K^+-ATPase (ATPase). n/s indicates no significant treatment effect.

Wrasse Gill Measurements: Night Time									P≤0.05
	21:00		24:00		3:00		6:00		
	With Sand	Without Sand	With Sand	Without Sand	With Sand	Without Sand	With Sand	Without Sand	
HIF-1α mRNA	2.10E-03 ± 4.30E-04	7.80E-04 ± 8.23E-05	1.71E-03 ± 4.11E-04	9.15E-04 ± 1.42E-04	7.73E-04 ± 7.60E-05	1.35E-03 ± 4.42E-04	2.44E-03 ± 7.55E-04	1.60E-03 ± 3.42E-04	n/s
CS	n/a	n/a	18.41 ± 2.17	n/a	19.97 ± 3.82	16.51 ± 4.25	19.61 ± 3.79	30.09 ± 3.23	n/s
ME	6.94 ± 1.77	7.23 ± 1.72	2.95 ± 1.60	5.53 ± 0.81	2.57 ± 0.83	5.37 ± 1.42	2.72 ± 2.02	5.95 ± 0.84	S < WS
ICDH	22.07 ± 3.32	15.93 ± 2.23	9.71 ± 2.26	18.56 ± 1.13	13.07 ± 2.82	18.45 ± 1.27	12.01 ± 3.15	18.61 ± 1.25	S < WS
G6PDH	27.54 ± 1.21 *	21.21 ± 2.57	14.64 ± 3.09	25.23 ± 3.05	14.06 ± 3.53	23.94 ± 2.97	12.43 ± 2.48	21.46 ± 1.74 *	S < WS
MDH	457.60 ± 38.37	378.73 ± 36.19	459.98 ± 69.40	353.51 ± 21.27	346.72 ± 58.26	369.95 ± 37.35	410.78 ± 49.52	411.21 ± 18.89	n/s
LDH	524.50 ± 65.26	450.20 ± 55.19	475.32 ± 40.58	417.02 ± 31.68	416.61 ± 21.86	434.10 ± 39.51	478.61 ± 21.76	495.06 ± 29.09	n/s
HK	63.62 ± 4.19	62.19 ± 5.67	77.35 ± 6.30 *	57.53 ± 4.78	59.58 ± 8.26	68.04 ± 5.22	51.23 ± 4.24	72.43 ± 1.93 *	n/s
PK	154.93 ± 11.84	151.38 ± 14.79	179.37 ± 12.87	135.03 ± 11.65	182.77 ± 16.47	138.51 ± 9.79	157.51 ± 6.94	155.43 ± 8.26	S > WS
ATPase	28.06 ± 4.41 *	14.85 ± 2.30	30.13 ± 3.06	18.08 ± 4.89	15.50 ± 3.11	26.00 ± 7.54	17.44 ± 5.43	24.97 ± 3.41	n/s

Table 12. Wrasse gill measurements of hypoxia inducible factor (HIF)-1α mRNA abundance, enzyme activities during anoxia exposure

Gill hypoxia inducible factor (HIF)-1α mRNA abundance, enzyme activities and metabolite measurements at 0, 6, and 12 h of anoxia in three-spot wrasse. Values represent mean ± SEM (*N*=3-8). The mRNA abundance is expressed as ratio of target gene (HIF- 1α) over house-keeping gene (β-actin); enzyme activities are expressed as U/ g protein. Citrate synthase (CS), malic enzyme (ME), isocitrate dehydrogenase (ICDH), glucose-6-phosphate dehydrogenase (G6PDH), malate dehydrogenase (MDH), lactate dehydrogenase (LDH), hexokinase (HK), pyruvate kinase (PK), Na^+/K^+-ATPase (ATPase).

Wrasse Gill Anoxia			
	0 h	**6 h**	**12 h**
HIF-1α	1.40E-03 ± 2.06E-04	2.93E-03 ± 6.32E-04	2.84E-03 ± 2.82E-04
CS	23.74 ± 1.78	20.46 ± 1.14	25.73 ± 2.30
ME	5.34 ± 0.66	5.31 ± 2.26	5.80 ± 1.80
ICDH	15.22 ± 2.96	21.83 ± 2.39	24.40 ± 2.31
G6PDH	21.04 ± 3.44	20.05 ± 4.27	36.35 ± 6.72
MDH	287.09 ± 43.96	396.45 ± 41.84	407.19 ± 27.91
LDH	455.67 ± 72.35	513.95 ± 50.13	528.78 ± 64.50
HK	67.53 ± 8.04	55.32 ± 8.46	80.96 ± 3.67
PK	151.83 ± 9.68	145.00 ± 18.25	160.58 ± 11.27
ATPase	19.80 ± 3.02	17.46 ± 6.13	25.06 ± 6.17

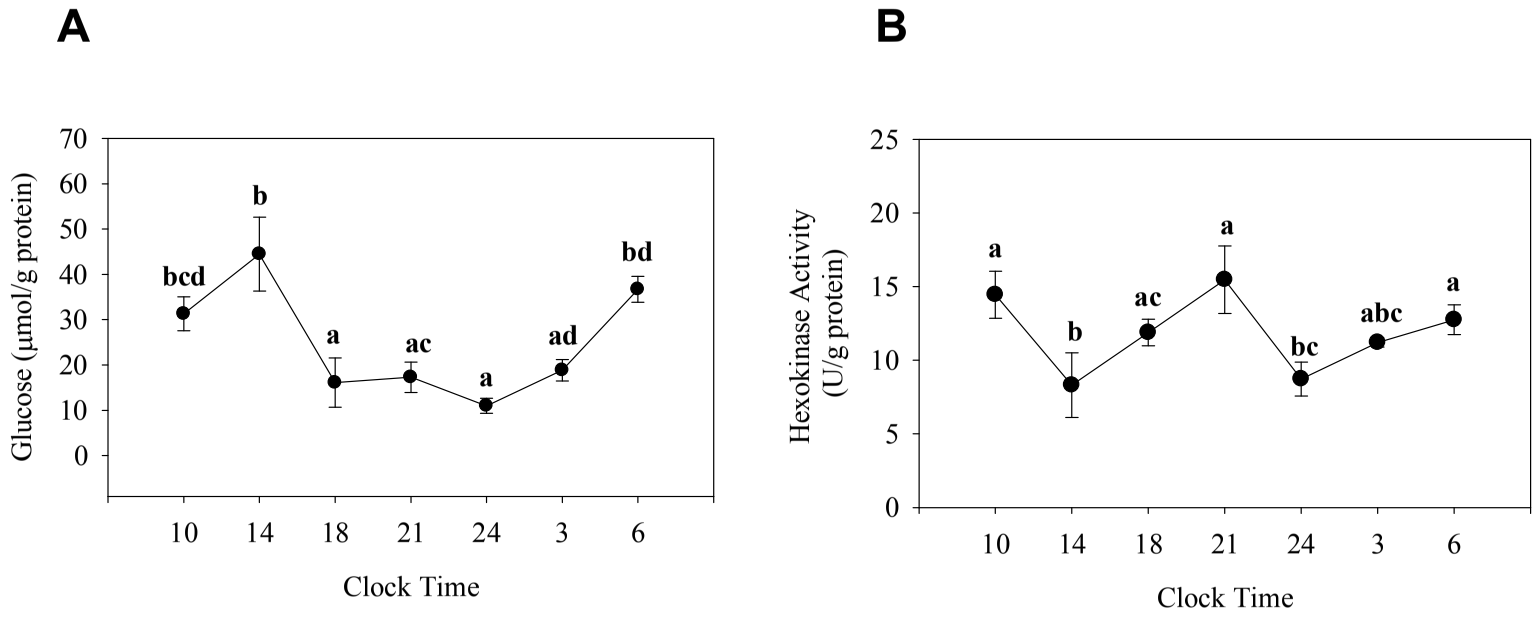
A
Glucose (µmol/g protein)
70
60
50
40
30
20
10
0
bcd
b
a
ac
a
ad
bd
10
14
18
21
24
3
6
Clock Time
B
Hexokinase Activity (U/g protein)
25
20
15
10
5
0
a
b
ac
a
bc
abc
a
10
14
18
21
24
3
6
Clock Time

Figure 5. Heart glucose and hexokinase activity during the 24 h diurnal cycle.

Glucose (A) level, and hexokinase activity (B) in the heart of the three-spot wrasse. The x-axis represents the clock time, and the y-axis represents the units of measurement (glucose level in μmol/g protein; hexokinase activity in U/g protein). Values represent means ± SEM (*N*=4-8); Different letters indicate significant difference (P≤0.05, One-way ANOVA).

Table 13. Wrasse heart measurements of enzyme activities during diurnal cycle

Heart metabolites and enzyme activities at different sampling time points (clock time) over a 24 h diurnal cycle. Values represent mean ± SEM (*N*=4-8). Significant time effects are shown as superscripts ($P \leq 0.05$, one-way-ANOVA); Values with same superscripts are not significantly different. Enzyme activities are expressed as U/ g protein; metabolites are expressed as µmol/ g protein. Citrate synthase (CS), malic enzyme (ME), lactate dehydrogenase (LDH).

Wrasse Heart Measurements: Diurnal Cycle							
	10:00	**14:00**	**18:00**	**21:00**	**24:00**	**3:00**	**6:00**
CS	300.30 ± 15.83 [b]	n/a	250.88 ± 31.26[b]	n/a	238.36 ± 29.65[ab]	218.32 ± 28.65[ab]	171.76 ± 32.74[a]
ME	31.14 ± 5.88 [a]	6.58 ± 0.65 [b]	29.22 ± 6.95 [a]	35.32 ± 8.23 [a]	41.34 ± 10.12 [a]	41.93 ± 8.43 [a]	21.72 ± 1.90 [a]
LDH	2521.12 ± 46.93	2574.98 ± 74.61	2702.45 ± 197.74	2664.55 ± 200.65	3084.18 ± 349.19	2990.58 ± 276.79	2373.16 ± 145.27
Glycogen	151.20 ± 16.30	175.41 ± 40.66	150.23 ± 21.44	94.12 ± 12.59	100.37 ± 14.66	120.75 ± 15.66	119.88 ± 45.68
Lactate	10.94 ± 2.13 [ad]	7.99 ± 1.56 [cd]	3.18 ± 0.73 [b]	4.38 ± 1.39 [b]	16.43 ± 3.06 [a]	3.34 ± 0.49 [b]	6.10 ± 1.55 [bd]

Table 14. Wrasse heart measurements of enzyme activities and metabolites during night time sampling points, with and without sand

Heart enzyme activities and metabolite measurements at night time sampling points (21:00, 24:00, 3:00, 6:00 clock time) with sand (S) and without sand (WS). Values represent mean ± SEM (*N*=3-6). Significant time and sand effects and the interactions are shown (P≤0.05; two-way-ANOVA). Enzyme activities are expressed as U/ g protein; metabolite measurements are expressed as µmol/g protein.

Wrasse Heart Measurements: Night Time									P≤0.05
	21:00		24:00		3:00		6:00		
	With Sand	Without Sand	With Sand	Without Sand	With Sand	Without Sand	With Sand	Without Sand	
CS	n/a	n/a	238.36 ± 29.65	n/a	218.32 ± 28.65	254.77 ± 13.19	171.76 ± 32.74	266.39 ± 33.58	S < WS
ME	35.32 ± 8.23	34.88 ± 4.67	41.34 ± 10.12	48.99 ± 6.91	41.93 ± 8.43	41.10 ± 17.27	21.72 ± 1.90	39.24 ± 4.29	n/s
LDH	2664.55 ± 200.65	2945.84 ± 188.71	3084.18 ± 349.19	3108.80 ± 164.36	2990.58 ± 276.79	2850.32 ± 255.99	2373.16 ± 145.27	3054.31 ± 177.45	n/s
HK	15.47 ± 2.29	10.99 ± 1.65	8.72 ± 1.16	11.30 ± 1.47	11.20 ± 0.30	12.53 ± 2.02	12.74 ± 1.01	10.98 ± 3.01	n/s
Glucose	17.27 ± 3.34	10.89 ± 2.29	10.99 ± 1.69	7.08 ± 1.27	18.84 ± 2.37	9.54 ± 1.84	36.67 ± 2.87	n/a	S > WS 21,3>24
Glycogen	94.12 ± 12.59	106.37 ± 13.27	100.37 ± 14.66	100.14 ± 19.18	120.75 ± 15.66	83.36 ± 6.79	119.88 ± 45.68	n/a	n/s
Lactate	4.38 ± 1.39	8.65 ± 2.16	16.43 ± 3.06	9.67 ± 0.93	3.34 ± 0.49	4.69 ± 1.05	6.10 ± 1.55	n/a	24>21,3

Table 15. Wrasse heart measurements of enzyme activities and metabolites during anoxia exposure

Heart enzyme activities and metabolite measurements at 0, 6, and 12 h of anoxia in three-spot wrasse. Values represent mean ± SEM (*N*=2-8). Significant time effects are shown as superscripts (P≤0.05, One-way ANOVA); Values with same superscripts are not significantly different. The metabolites are expressed as µmol/ g protein; enzyme activities are expressed as U/ g protein. Citrate synthase (CS), malic enzyme (ME) lactate dehydrogenase (LDH), and hexokinase (HK).

Wrasse Heart Anoxia			
	0 h	**6 h**	**12 h**
CS	300.30 ± 15.83 [a]	273.47 ± 19.58 [a]	198.38 ± 20.95 [b]
ME	31.14 ± 5.88 [a]	9.60 ± 1.11 [b]	16.84 ± 5.93 [ab]
LDH	2521.12 ± 46.93 [a]	2144.38 ± 114.75 [b]	2401.70 ± 156.94 [ab]
HK	14.45 ± 1.59 [a]	8.71 ± 1.74 [b]	17.86 ± 4.14 [a]
Glucose	31.28 ± 3.73 [a]	73.09 ± 10.37 [b]	86.42 ± 6.88 [b]
Glycogen	151.20 ± 16.30	112.21 ± 22.25	136.16 ± 28.48
Lactate	10.94 ± 2.13 [a]	35.73 ± 2.86 [b]	47.70 ± 6.12 [b]

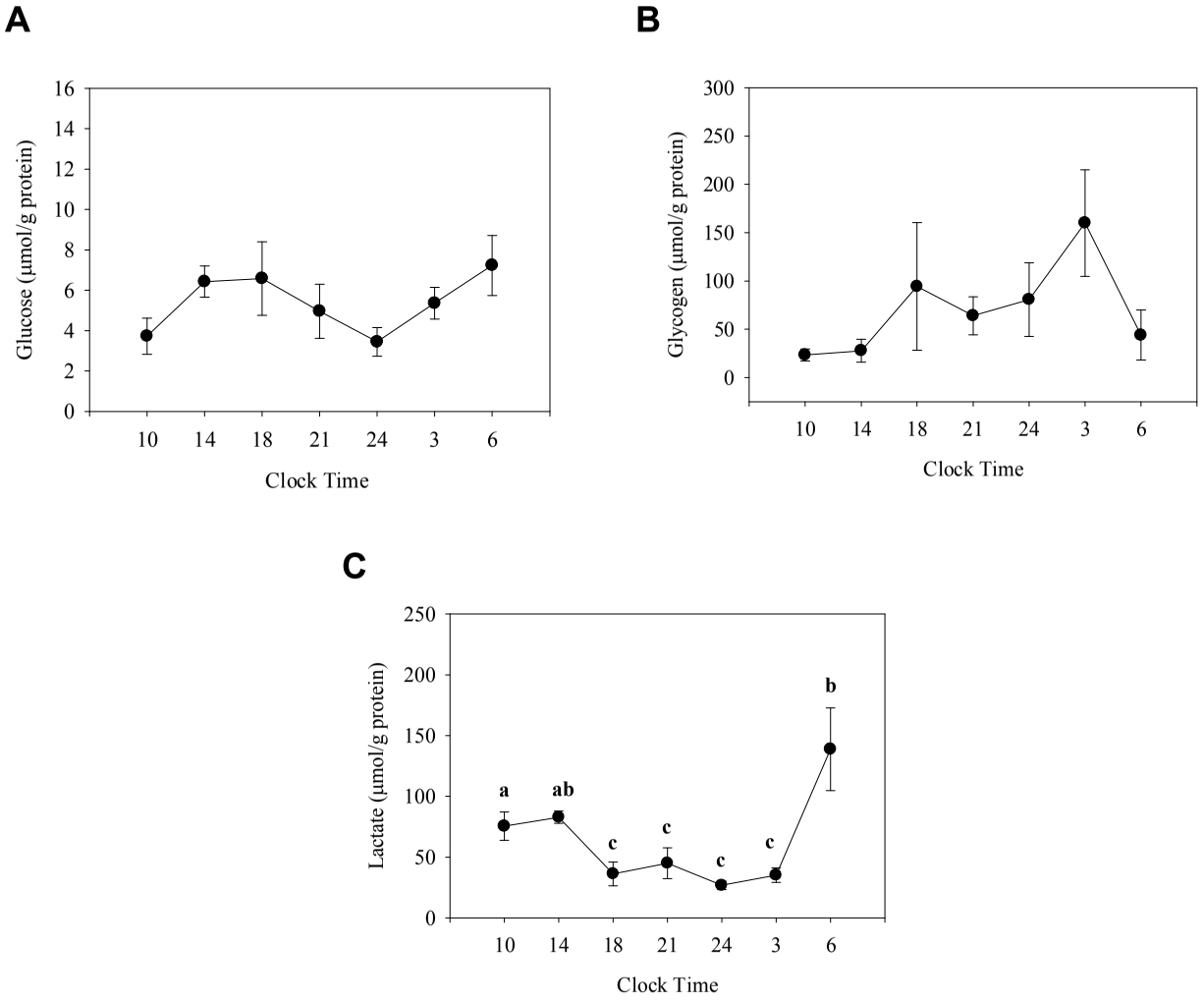
A
Glucose (µmol/g protein)
16
14
12
10
8
6
4
2
0
10 14 18 21 24 3 6
Clock Time
B
Glycogen (µmol/g protein)
300
250
200
150
100
50
0
10 14 18 21 24 3 6
Clock Time
C
Lactate (µmol/g protein)
250
200
150
100
50
0
a
ab
c
c
c
c
b
10 14 18 21 24 3 6
Clock Time

Figure 6. Muscle glucose, glycogen and lactate content during the 24 h diurnal cycle.

Glucose (A), glycogen (B) and lactate (C) levels in muscle of the three-spot wrasse. The x-axis represents the clock time, and the y-axis represents the units of measurement (µmol/g protein). Values represent means ± SEM (*N*=5-7); Different letters indicate significant difference (P≤0.05, one-way ANOVA). Citrate synthase (CS), malic enzyme (ME), lactate dehydrogenase (LDH), hexokinase (HK). n/s indicates no significant treatment effect.

Table 16. Wrasse muscle measurements of metabolites during night time sampling points, with and without sand

Muscle metabolite measurements at night time sampling points (21:00, 24:00, 3:00, 6:00 clock time) with sand (S) and without sand (WS). Values represent mean ± SEM (*N*=2-6). Significant sand effects and the interaction are shown (P≤0.05; two-way-ANOVA). Metabolite measurements are expressed as µmol/g protein. n/s indicates no significant treatment effect.

Wrasse Muscle Measurements: Night Time									P≤0.05
	21:00		24:00		3:00		6:00		
	With Sand	Without Sand	With Sand	Without Sand	With Sand	Without Sand	With Sand	Without Sand	
Glucose	4.96 ± 1.34	3.30 ± 0.85	3.44 ± 0.71	3.77 ± 0.64	5.36 ± 0.79	3.87 ± 1.02	7.23 ± 1.48	3.64 ± 1.40	n/s
Glycogen	64.02 ± 19.66	71.98 ± 20.56	80.85 ± 38.04	54.92 ± 20.26	160.05 ± 55.23	62.95 ± 9.16	44.04 ± 25.89	11.07 ± 5.65	n/s
Lactate	45.03 ± 12.75	31.44 ± 5.28	26.85 ± 3.49	43.18 ± 9.70	35.22 ± 5.90	47.53 ± 16.32	138.86 ± 34.00	58.04 ± 14.25	6>21,24,3

Table 17. Wrasse muscle measurements of metabolites during anoxia exposure

Muscle enzyme activities and metabolite measurements at 0, 6, and 12 h of anoxia in three-spot wrasse. Values represent mean ± SEM (*N*= 4-7). Significant time effects are shown as superscripts (P≤0.05, One-way-ANOVA); Values with same superscripts are not significantly different. The metabolites are expressed as µmol/g protein.

Muscle Anoxia			
	0 h	**6 h**	**12 h**
Glucose	3.73 ± 0.90 [a]	10.26 ± 2.84 [ab]	18.52 ± 9.24 [b]
Glycogen	23.52 ± 6.13	139.88 ± 56.63	64.64 ± 46.70
Lactate	75.47 ± 11.70 [a]	131.03 ± 31.43 [ab]	179.78 ± 34.24 [b]

4 Discussion

The three-spot wrasse (*Halichoeres trimaculatus*) is a coral reef fish that burrows into the sand and remains hidden at night. The objective of the study was to examine the diurnal changes in the expression of genes (hypoxia-inducible factor-1α and neuroglobin) and activation of biochemical pathways (by measuring the activities of some of the glycolytic and TCA cycle enzymes) in response to this life-style. The biochemical strategy associated with sand-dwelling was investigated by comparing a group of fish that were maintained in tanks with no sand at night. Overall, the results demonstrate a tissue-specific metabolic adjustment, including enhanced glycolytic capacity in the brain, while the muscle, liver and gills showed a decline in metabolic capacity in the sand at night.

4.1 Tissue-specific molecular response in wrasse over a 24 h period

HIF-1α mRNA abundance was measured in the brain, liver and gill of the three-spot wrasse during the 24 h diurnal cycle. The brain HIF-1α mRNA level was the highest of the three tissues measured; almost 20 and 40 fold higher than liver and gills, respectively, suggesting tissue-specific response of HIF-1α gene expression. The higher levels in the brain compared to other tissues suggest that HIF-1α plays an important neuroprotective role in response to oxygen fluctuations (Semenza, 2000). Diurnal changes in these transcript levels showing an increasing trend during night time in the brain and liver suggest a role for HIF-1α in cellular and systemic oxygen homeostasis. It is well established that HIF-1α maintains oxygen homeostasis by regulating the expression of genes involved in several key physiological processes, including erythropoiesis and glycolysis (Nikinmaa, 2002; Nikinmaa and Rees, 2005). However, the magnitude of change in the wrasse tissues is minimal during the 24 h period reflecting either the tissues are not oxygen deprived or the transcript changes are not a good measure of hypoxia exposure in the wrasse. Indeed, studies have reported that despite the DNA binding activity of

HIF-1α and other oxygen-regulated genes, mRNA levels remained unaffected by hypoxia exposure in cell lines, pointing to regulation at the protein level (Wenger *et al*., 1997; Wiesener *et al*., 1998). In addition to transcriptional regulation, post-transcriptional regulation of HIF-1α in response to hypoxia exposure is well documented (Hochachka and Lutz, 2001; Kassahn *et al*., 2007). We hypothesize that similar regulatory mechanisms may exist in three-spot wrasse and further studies, including determination of HIF-1α protein content, are warranted to reveal the role of this key protein in oxygen sensing in this species.

Recent studies have implicated neuroglobin involvement in neuronal oxygen homeostasis by promoting survival of neuronal cells during hypoxia (Sun *et al*, 2001, 2003; Pesche *et al*, 2002; Schmidt *et al*, 2003). Indeed, neuroglobin transcripts were elevated under oxygen limitation in brain tissues of mouse and freshwater turtle (*Trachemys scripta)* (Sun *et al*., 2001; Milton *et al*., 2006). However, we did not observe any significant changes in the wrasse neuroglobin mRNA abundance over the 24 h diurnal period. While the reason for this is unknown, one proposal is that the brain may be supplied with oxygen even while the fish are under the sand at night. For instance, studies have shown that in hypoxia or anoxia tolerant species, the brain oxygen supply is maintained by increasing cerebral blood flow and/or vasodilation (Soderstrom *et al*., 1999). Consequently, one hypothesis is that wrasse brain does not experience oxygen limitation at night while under the sand. However, one cannot discount the possibility that the neuroglobin response may be species-, cell- and oxygen tension-specific as reported in mammalian studies. For instance, mammalian neuronal cell lines showed significant increase in neuroglobin levels under severe hypoxia (0.3 % O_2) (Sun *et al*., 2001; Fordel *et al*., 2004), while whole brain tissue failed to show this response to hypoxia (10 % O_2) (Mammen *et al*., 2002; Schmidt-Kastner; 2006). Taken together, this study demonstrates a lack of diurnal rhythm in brain neuroglobin expression in the three-spot wrasse, while the factors modulating neuroglobin expression in fish remain to be determined.

4.2 Tissue-specific biochemical response in wrasse over a 24 h period

Glucose is one of the most important fuels for the maintenance of energy homeostasis. During periods of oxygen limitation, glucose demand increases as it is the only cellular fuel that can be utilized by the animal (Nilsson and Renshaw, 2004). During hypoxia, the energy demands required for maintenance of proper cellular functions are primarily met by anaerobic glycolysis. Consequently, circulating glucose and/or liver glycogen reserves are an important source of fuel for ATP production during hypoxia. Indeed, the rate of glucose delivery is increased in a tissue-specific fashion due to a combination of elevated glucose level in blood as well as increased blood flow (Nilsson *et al*., 1994; Johansson *et al*., 1995; Lutz and Nilsson, 1997).

4.2.1 Plasma metabolites

One of the characteristics of hypoxia and anoxia tolerant species is the rise in plasma glucose concentration, usually followed by a noticeable increase in plasma lactate level as an indication of anaerobic metabolism (Penney, 1974; Shoubridge and Hochachka, 1980). This increase in blood glucose and lactate has been well documented in many hypoxia tolerant species, including common carp (*Cyprinus carpio*, L), crucian carp (*Carassius carassius*), tambaqui (*Colossoma macropomum*), cichlid (*Astronotus ocellatus*), gulf killifish (*Fundulus grandis)*, sole (*Solea solea*) (Dalla via *et al*., 1994; Van Raaij *et al*., 1996; Muusze *et al*., 1998; Virani and Reese, 2000; Affonso *et al*., 2002; Nilsson and Renshaw, 2004). However, this biochemical adaptation to oxygen limitation was not seen in the wrasse while in the sand. Instead, plasma glucose level decreased and remained low at night. This may reflect a low glucose production capacity at night and this is supported by the reduced liver gluconeogenic capacity in the wrasse along with an absence in glycogen breakdown. The plasma lactate level also decreased and remained low at night and agrees with the lack of muscle glycogen breakdown, a main contributor to lactate production during oxygen limitation, at night when the fish are under the sand. This absence of a muscle glycolytic response and lactate build up at night appears to be an

important biochemical adaptation for sand dwelling in the wrasse. This is in contrast to other hypoxia-tolerant species that usually accumulate lactate due to anaerobic respiration. While some fish, including goldfish (*Carassius auratus)*, avoid lactate buildup by producing ethanol as the major glycolytic end-product and releasing it at the gills, many hypoxia intolerant species are unable to avoid the problem of acidosis caused by high lactate buildup (Lutz and Nilsson, 1997). The lack of lactate production in the wrasse may be either due to the availability of oxygen to the tissue and/or suppression in tissue metabolic capacity. The tissue-specific metabolic response (see below) supports a reduced metabolic capacity in the muscle and this may be related to the sand dwelling life-style of the animal.

4.2.2 Biochemical responses in the muscle

During hypoxia, the lactate accumulation in the plasma is mostly due to tissue anaerobic glycolysis and subsequent release of the metabolite into the circulation (Dunn and Hochachka, 1986). Of these tissues, muscle glycogen contributes mostly to the increase in plasma lactate concentration (Van Raaij *et al*., 1996; Kam and Miligan, 2006). The plasma lactate accumulation was not seen in the wrasse and may be due to the lack of muscle locomotor activity while under the sand at night. This lack of movement reduces the energy demand, thereby limiting glycogen breakdown and lactate buildup, which can be detrimental to the fish. Indeed, metabolic suppression is a key biochemical adaptation to hypoxia survival in animals, including reduction in locomotor activity of fish under hypoxic conditions (Dalla Via *et al*., 1994; Hochachka *et al*., 1996; Virani and Reese, 2000). Hypoxia intolerant species lack this key strategy, leading to lactate accumulation in the body (a Pasteur effect) from white muscle glycogen breakdown (Dunn and Hochachka, 1986). The sand-dwelling life-style of the wrasse reduces the muscle energy demand associated with posture and locomotor activity, thereby avoiding metabolic acidosis. This is supported by the lack of muscle glycogen breakdown at night while under the sand, suggesting metabolic suppression in wrasse muscle. This would limit

lactate accumulation in muscle leading to a lower plasma lactate level at night in the wrasse while under the sand.

Interestingly, a significant increase in plasma lactate and glucose levels along with a decrease in muscle glycogen content at dawn suggests transient elevation in glycolytic capacity in the wrasse. The increase in plasma lactate level may be the result of lactate release from the muscle as other tissues, including liver and heart, did not show any significant changes in their tissue glycolytic capacity. The higher muscle glycolytic capacity and plasma lactate levels at dawn is an indication of locomotor activity in the sand, which precedes the emergence of the fish from the sand.

4.2.3 Biochemical responses in the liver

Liver glycogen storage plays an important role in supporting whole animal metabolism during hypoxia exposure. Indeed, the liver stores of glycogen in the anoxia-tolerant *Carassius* species and the freshwater turtles are among the largest of any vertebrates studied (Hyvarinen *et al*., 1985; Nilsson and Renshaw, 2004). Liver glycogenolysis contributes primarily to the rise in blood glucose observed with oxygen limitation (Johnston and Moon, 1979; Van Raaij *et al*., 1996), while gluconeogenesis is of minor importance (Dunn and Hochachka, 1986; Van Raaij *et al*., 1996). However, the liver glycogen content in wrasse did not vary with the diurnal cycle, and the lactate accumulation was minimal. Also, the activity of the key gluconeogenic enzyme, phosphoenolpyruvate carboxykinase (PEPCK), showed very little change over the diurnal period suggesting that the gluconeogenic capacity was not altered in the wrasse over the 24 h diurnal cycle. Interestingly, in the absence of sand at night, the liver metabolic capacity increased as indicated by higher activities of enzymes involved in intermediary metabolism compared to the sand group. This supports the notion that sand dwelling limits the energy expenditure in the liver at night.

4.2.4 Biochemical responses in the gills

The gill Na^+/K^+-ATPase activity, a key indicator of energy demand, showed a decreasing trend at 3:00 and 6:00 supporting a reduction in energy metabolism (Hochachka and Lutz, 2001). Studies in anoxia-tolerant freshwater turtle hepatocytes showed that one of the largest energy sinks under normoxic conditions is Na^+/K^+ pumping and this is drastically reduced during anoxia (Buck and Hochachka, 1993; Hochachka *et al*., 1996). This decrease in sodium pump activity was also shown in the turtle brain, along with a depression in ATP demand (Hyland *et al*., 1997). Another indication of metabolic suppression in the gill is the significant decrease in glucose-6-phosphate dehydrogenase (G6PDH) activity, an enzyme involved in providing NADPH for lipid biosynthesis. The decrease in these oxidative enzyme activities at night may also suggest a reduction in protein turnover, which is one of the major ATP sinks that is shown to be suppressed during hypoxia (Buck *et al*., 1993; Hochachka and Lutz, 2001). Together, the results point to a metabolic suppression in the gills of wrasse associated with the sand-dwelling life-style.

4.2.5 Biochemical responses in the heart

In response to hypoxic exposure, studies in fish and mammals have indicated high anaerobic glycolytic activities in the heart, generating most of the required ATP and leading to lactate accumulation (Allen *et al*., 1985; Dunn and Hochachka, 1986; Arthur *et al*., 1992). However, wrasse heart showed a reduced glycolytic capacity at night while hiding in the sand and this is also reflected in the lower lactate levels. The glucose level in the heart was also lower at night suggesting that the exogenous glucose delivery to the heart is unchanged between the day and night. The lack of change in heart hexokinase activity supports this argument. Also, glycogen content and some of the glycolytic enzymes remained unchanged during the night, pointing to a lack of change in glycolytic capacity at night. The decrease in both glucose and lactate in the heart at night may be related also to their use as fuels in fish heart. Indeed, glucose and lactate are

preferentially oxidized for energy production in the heart of fish (Lanctin *et al*., 1980; Milligan and Farrell, 1991). While the heart showed the highest CS activity compared to all the other tissues measured, this aerobic enzyme activity decreased significantly at night compared to the day. This supports a reduction in the oxidative metabolic capacity of the heart while the fish is hiding in the sand. The reduction in mitochondrial enzyme activities, including citrate synthase activities, is routinely used as an indicator of metabolic suppression in hibernating and hypoxia exposed animals (Stuart *et al*., 1998a,b; St-Pierre *et al*., 2000; Bishop *et al*., 2002; Lai *et al*., 2003; Chippari-Gomes *et al*., 2005). Indeed, in the absence of sand, the CS activity was higher than in the sand group supporting a reduction in oxidative metabolic capacity at night in the wrasse heart while under the sand.

4.2.6 Biochemical responses in the brain

Among all tissues, the brain is the most sensitive to glucose deprivation because of its high energy demands. As in mammals, glucose is preferentially used to fuel brain metabolism and the glucose utilization by rainbow trout brain was five times that of other tissues (DiAngelo and Heath, 1987; Soengas *et al*., 1998a; Washburn *et al*., 1992). Wrasse brain showed a significant increase in glucose level and maintained this high level throughout the night compared to day time. During times of oxygen limitation, ATP levels fall due to a decrease in oxidative phosphorylation, as the tissue relies exclusively on glycolysis (Soengas and Aldegunde, 2002). Since brain tissue has high energy requirements and is the most sensitive to oxygen limitation, hypoxia tolerant species have defense mechanisms to overcome the energy deficit via increasing the delivery rate of glucose to the brain (Lutz and Nilsson, 1997). The elevation in brain glucose level at night corresponded with a higher hexokinase activity, supporting an enhanced capacity for exogenous glucose uptake in the wrasse brain. Since the rate of glucose utilization in teleost brain is thought to be limited by hexokinase activity (Soengas and Aldegunde, 2002), the elevation in HK activity at night suggests enhanced circulating glucose uptake by the brain. This

would account for the lower plasma glucose levels seen in the wrasse at night while hiding in the sand.

Another important metabolite in the brain that plays an important role during hypoxia is glycogen. Brain glycogen stores are utilized for glucose production in the central nervous system during oxygen limitation (Dringen *et al*., 1993). Indeed, hypoxia-tolerant teleosts have large brain glycogen stores that are readily utilized to meet the energy demand during times of hypoglycemia (Soengas and Aldegunde, 2002). For instance, bullheads (*Ameiurus nebulosus*) and carp have brain glycogen content several fold higher than trout (a hypoxia-sensitive species) (Diangelo and Heath, 1987; Johansson *et al*., 1995). The wrasse also showed a reduction in glycogen level at night while in the sand reflecting enhanced glycogen breakdown. The lack of a concomitant increase in plasma glucose concentration, along with higher HK and PK activities at night, supports increased brain glycolytic capacity. Taken together, the wrasse utilizes both glycogen as well as exogenous glucose to fuel brain metabolism at night while hiding in the sand.

Enzymes involved in the tricarboxylic acid (TCA) cycle in the brain showed relatively small changes at night compared to daytime. Also, brain did not show any significant difference in the activities of TCA cycle enzymes at night while the fish are under the sand compared to the no sand group. These results suggest a lack of change in the brain metabolic capacity of wrasse, unlike other tissues, including liver, muscle and the gills, while under the sand at night. While the reason for this tissue-specific response is unclear, one hypothesis to explain the oxidative metabolic capacity in the brain even under the sand is that there might be a preferential blood flow to the brain. The cerebral blood flow may provide external glucose to the brain, as seen by the significant increase in the HK activity at night, as well as supplying oxygen to the brain. In support of this notion, other well characterized hypoxia tolerant animals, including the crucian carp and the freshwater turtle have been shown to increase cerebral blood flow (Nilsson and Renshaw, 2004). This increased blood flow to the brain is an adaptation to increase fuel supply to

aid in glycolytic ATP production as well as increase oxygen delivery to the brain in hypoxia tolerant animals (Soderstrom *et al*., 1999). In addition to enhanced blood flow to the brain, preferential vasodialation is also seen in hypoxia tolerant animals. For instance, epaulette sharks, which tolerate hypoxia in the coral reefs, showed constant cerebral blood flow, but the blood pressure fell 50 % during hypoxia, suggesting cerebral vasodilation (Soderstrom *et al*., 1999). Together, the results suggest a tissue-specific metabolic adjustment in the wrasse brain at night while in sand. The increased metabolic capacity of the wrasse brain at night leads to the proposal that the adaptive strategies in the three-spot wrasse include increased cerebral blood flow rate and/or cerebral vasodilation to maintain brain metabolism while the fish are under the sand.

4.3 Molecular and biochemical responses to anoxia via nitrogen bubbling

Tissue-specific metabolic suppression seen in the wrasse at night under the sand has been shown in animals undergoing anoxia. Metabolic suppression has been reported in anoxia tolerant species, including the crucian carp, goldfish and freshwater turtles, that either downregulate energy turnover (Hochachka, 1986) and/or upregulate ATP-producing pathways to overcome oxygen limitation (Buck and Hochachka, 1993). To determine whether the molecular and biochemical responses at night in the wrasse are due to a lack of oxygen under the sand, nitrogen gas was bubbled into the tanks in the absence of sand to create an anoxic environment.

Plasma glucose and lactate increased after 6 h of nitrogen bubbling, displaying a classical anoxia response (Shoubridge and Hochachka, 1980; Lutz and Nilsson, 1997). The muscle glucose level as well as lactate level significantly increased during the 12 h anoxia exposure, a response that was absent in wrasse under the sand. This supports the hypothesis that the lack of plasma lactate at night, while the fish are under the sand, is due to a reduction in energy demand associated with the absence of any locomotor activity. Therefore, based on the plasma response it appears that the fish hiding in the sand do not show an anoxic response. This

leads to the proposal that either the fish are not completely anoxic or the limited oxygen availability is sufficient to meet the reduced energy demand while under the sand at night. The reduced energy demand may be due to the lack of muscle activity while hidden in the sand, thereby limiting glycogen breakdown and the associated lactate accumulation that is seen in anoxic animals.

The brain showed significant increase in glucose levels throughout the 12 h anoxia period. However, it lacked changes in glycogen levels and HK activities suggesting that the brain glycolytic capacity was not affected by anoxia exposure. In fact, all the other tissues including the muscle and heart showed increase in glucose levels but glycogen levels and HK activities did not show any significant changes. These anoxia responses are different from that seen at night time while the wrasse are under the sand; brain maintained the metabolic capacity while other tissues showed metabolic suppression. The wrasse response to anoxia is surprising since many anoxia tolerant animals utilize the high glycogen storage in the liver and muscle, in addition to glucose, as energy substrates during oxygen limitation (Hochachka *et al*., 1996). One possible reason for the lack of glycolytic capacity during anoxia may be due to a different strategy in wrasse; delaying or avoiding the triggering of anaerobic glycolysis to minimize the accumulation of lactate which can lead to metabolic acidosis (Hochachka *et al*., 1996). In fact, many reports have indicated that hypoxia-tolerant animals sustain a state of reduced energy demanding instead of using anaerobic glycolysis to make up for energy deficits in a tissue-specific manner (Storey and Storey, 1990; Krumschnabel *et al*., 1994; Krumaschnabel *et al*., 1996).

In the wrasse, brain, liver, and heart citrate synthase activity was lower at 12 h, whereas isocitrate dehydrogenase activity in brain, liver, and gills remained the same over the 12 h anoxia exposure. Many animals have shown a decrease in TCA cycle enzymes including CS and ICDH activities when exposed to oxygen limitations (Lai *et al*., 2002; Chippari-Gomes *et al*., 2005; Staples *et al*., 2008). Other enzymes that are involved in energetically expensive pathways,

including gluconeogenesis (PEPCK and MDH), lipid biosynthesis (ME), as well as Na^+/K^+-ATPase activity, which is known to be a major energy sink (Hochachka *et al*., 1996), did not change over the 12 h anoxia exposure. While the reason for the lack of changes in ICDH as well as other energetically expensive pathways with during anoxia is unclear, it appears that this animal may have some unique adaptive strategy that remains to be explored.

5 General conclusions and take home messages

The hypoxia-responsive genes, including HIF-1α and neuroglobin, showed minimal changes at night, while the fish are under the sand, relative to daytime. However, clear tissue-specific differences in biochemical responses were observed in fish dwelling under the sand at night. While the brain was metabolically active, all other tissues tested, including liver, gill, muscle and heart, showed a lower metabolic capacity. This was also reflected in lowered plasma glucose and lactate levels, supporting a decrease in liver gluconeogenic and muscle glycolytic capacity when hiding under the sand. However, unlike the response to sand dwelling, significant lactate accumulation in the muscle and plasma was observed during the anoxia exposure. Consequently, the biochemical strategy for the diurnal sand dwelling at night in the wrasse does not reflect a hypoxic response. This would suggest that either the energy demand while under the sand is low enough to prevent glycolytic pathway activation or that oxygen is available for aerobic metabolism. The lack of locomotor activity while hiding in the sand would support a depressed energy demand, especially in the muscle, thereby limiting lactate accumulation and the associated acidosis.

A novel observation was the higher metabolic capacity of the brain while under the sand at night in the wrasse. This was evident from the higher glycolytic capacity coupled with an increase in the activities of TCA cycle enzymes compared to fish that were without sand at night. However, these biochemical responses were different from those seen in fish exposed to anoxia, clearly supporting an adaptive strategy that is independent of oxygen limitation. One hypothesis is that the brain may be supplied with oxygen at night while the fish are under the sand. This may involve shunting the blood flow exclusively to the brain (the most oxygen-sensitive tissue), including vasodilation of the blood vessels to the heart and brain, as reported in some of the deep sea diving mammals (Ramires *et al*., 2007). However, this may be accompanied by peripheral vasoconstriction that limits blood supply to other tissues, including the liver and muscle. Indeed,

bleeding from the caudal artery/vein in wrasse that were hidden in the sand at night was difficult relative to day time sampling, supporting the idea of peripheral vasoconstriction in this species while under the sand. Also, a recent study raises the possibility that there may be active uptake of surface water by these fish while hiding under the sand, which would be sufficient to irrigate the gills to extract oxygen (Unpublished results).

Future studies should focus on the protein characterization of the hypoxia-responsive genes, especially since these proteins are suggested to be modified and regulated post-translationally. Also, the role of HIF-1α in the regulation of neuroglobin in wrasse may be useful, as studies have reported a HIF binding site in the 5'-untranslated region of the neuroglobin gene. Some other interesting areas of research may include the role of clock genes (including *period* [per] gene) and hormones involved in circadian rhythms (including melatonin) in the regulation of hypoxia-responsive genes and other biochemical pathways involved in energy metabolism in the wrasse. Other studies may include direct measurement of metabolic rate, including oxygen consumption and energy charge ratios (ADP/ATP) to understand the energetics of sand dwelling and associated oxygen limitation in this species.

Taken together, the three-spot wrasse showed a tissue-specific difference in metabolic capacity at night while hiding under the sand. While the mechanism involved in this tissue-specific energy repartitioning at night is unclear, one hypothesis involves selective increase in blood flow to the brain while limiting peripheral circulation, as a means to maintain oxygen and glucose delivery to this critical tissue while the fish is hiding under the sand. The higher metabolic capacity of the brain, but not the other tissues, at night under the sand suggests that maintaining the brain function is essential for this diurnal lifestyle in the three-spot wrasse.

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