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Effects of reduced water oxygen saturation on growth and plasma cortisol levels in juvenile lumpfish (*Cyclopterus lumpus* L.) in aquaculture

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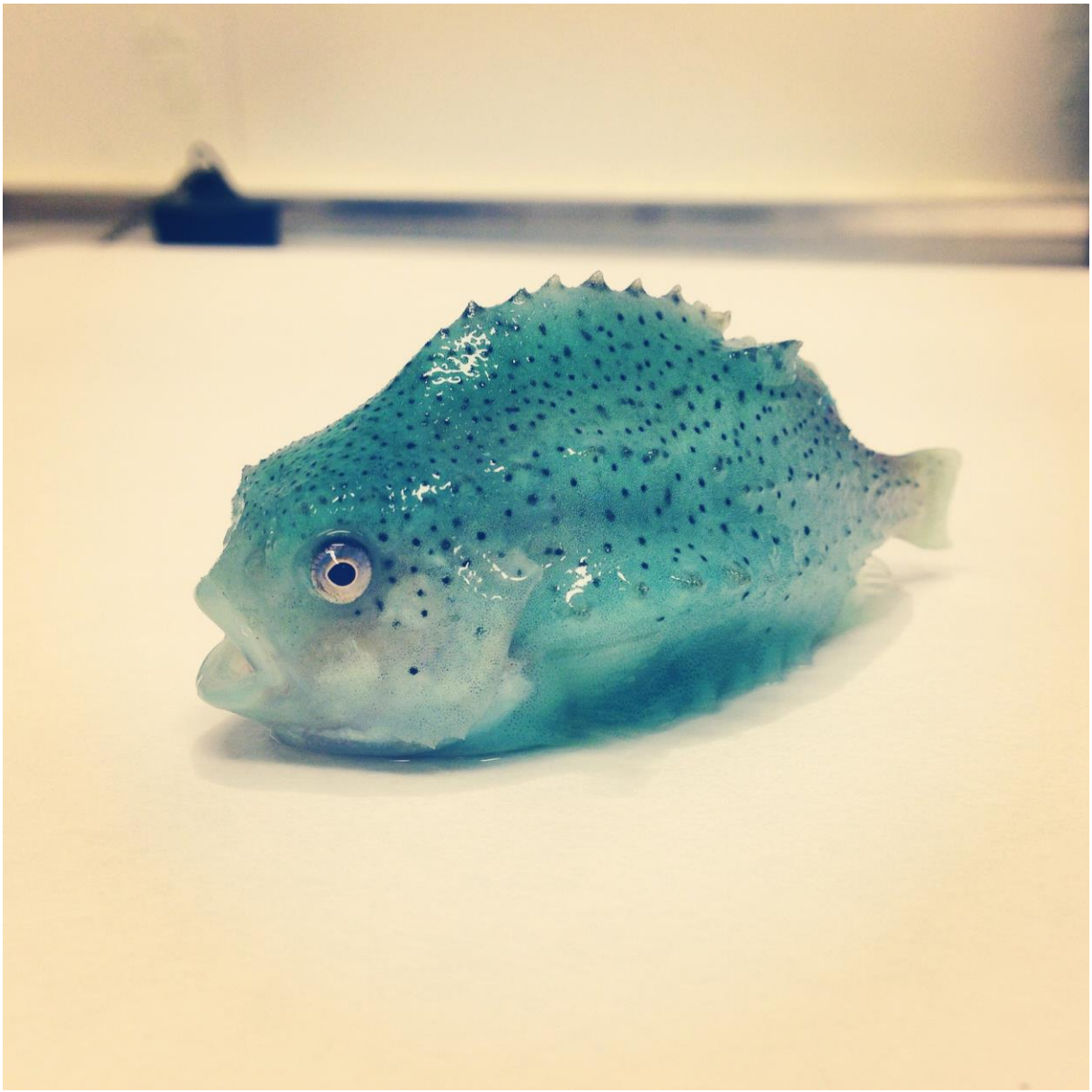

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Abstract

Lumpfish (*Cyclopterus lumpus* L.) is considered a promising species for biological removal of the parasite salmon lice (*Lepeophtherius salmonis*) on salmon in the salmon farming industry (Imsland, et al., 2014). Oxygen saturation in water is a limiting factor for fish metabolism and thereby growth and food intake (Kramer, 1987; Jobling 1994). In order to obtain and maintain optimal fish welfare, and thereby effective farming of lumpfish it is important that requirements are met and that the environment is as close to optimum as possible. There are, however, no reports on the physiological responses to reduced oxygen saturations and hypoxia done on lumpfish.

The purpose of this study of the juvenile Atlantic lumpfish is to answer the following two questions in relation to different oxygen saturation in the water:

(i) How does different oxygen saturation in the water (from normoxia to hypoxia) affect growth of the lumpfish; and

(ii) what are the effects of different oxygen saturations on blood plasma levels of the stress hormone cortisol.

Two experiments were carried out; one long-term experiment to examine the effects of reduced oxygen saturation *on growth and plasma cortisol*, and one short-term experiment of extreme hypoxia and disturbance. The experiments were conducted between October 2014 and December 2014 and included in total 178 individually tagged and 270 untagged lumpfish.

There is no current knowledge of the amount of dissolved oxygen in water (DO), which is required to meet the metabolic demand of lumpfish. As for other species, such as Atlantic salmon (*Salmo salar*), the demand will depend on a range of factors such as the metabolic rate (MR), which decreases with increasing size and increases with temperature, feeding level, swimming speed and stress (Barnes et al., 2011).

The purpose of the experiments was to adapt 4 different groups of lumpfish (in triplicates) to oxygen saturations at 55% (S.E. 1.08), 69% (S.E. 0.56), 81% (S.E. 0.47) and 96% (S.E. 0.32).

It is common knowledge that hypoxia often induces a stress response in fish, which may have severe long-term consequences if homeostasis is not re-established. It is therefore important to have knowledge about hypoxia tolerance when optimizing culture conditions for aquacultural species, such as lumpfish.

In the present study, primary responses (cortisol secretion) to long-term and short-term hypoxia are examined. For the long-term experiment groups that were exposed to lower oxygen saturation; 55%, 69% and 81% had increased plasma cortisol levels and decreased final body mass, length and overall specific growth rate compared to the control group which were exposed to 96% oxygen saturation. Highest plasma cortisol levels were displayed in lumpfish that were exposed to acute, extreme hypoxia and handling disturbance at ~47ng/ml after 120 minutes.

To conclude; juvenile lumpfish is highly sensitive to reduced oxygen saturations and negative effects in terms of growth are already evident for lumpfish reared at 81% oxygen saturation. For lumpfish reared at 55% oxygen saturation, the welfare was so poor that the replicates were terminated approximately after one month. Increase in plasma cortisol levels as an effect of reduced oxygen saturation were expressed in twofold of control (pre-stress) levels for fish held at 55% and 69%, however compared to active pelagic fish species the levels were low.

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Introduction - content

Salmon lice – an on-going battle

Since 2005, the production costs for salmon farming in Norway has increased with approximately 40%, nominally adjusted for inflation. Costs related to salmon lice are a biological cost that Nofima includes under the cost parameter "other operating costs". Other operating costs increased by 77%, and accounted for an average of 21% of the total operating costs in 2014 for the salmon farming industry, compared to 17% in 2005. The increase in operating costs has over the last 5 years particularly been affected by increased costs related to salmon lice (Iversen et.al., 2015).

Salmon lice (*Lepeophtherius salmonis*) are a concern and a cost driver for the Norwegian salmon farming industry, and at the moment perhaps the greatest challenge the Norwegian salmon farming industry is facing. The diminishing effect of chemical treatment against sea lice is concerning, and salmon lice resistant to medical treatment are developing at high-speed several places along the Norwegian coastline.

Salmon lice are naturally occurring parasites of salmon in seawater, and have been known to man since mid-1700 (reviewed in; Torrissen et al., 2013). Intensive salmon farming provides unnaturally high densities of potential hosts for the salmon lice, which has led to an extra source of infection and lice production, and consequently an increased infection threat for wild salmon.

The life cycle of lice comprises non-feeding planktonic larvae (nauplii), infective planktonic copepodites, immature chalimus embedded on the host skin, mobile pre-adults and adults that move freely over the host's skin. Each of the stages is separated from the preceding stage by a molt. To complete their life cycle, the salmon lice are dependent on a salmonid host. The host-parasite relationship often results in skin lesions on the host, which may cause osmoregulatory problems and makes the fish more vulnerable to secondary infections (Hayward et al., 2011; Torrissen et al., 2013).

The salmon lice have been a serious problem for the Atlantic salmon farming industry since the 1970s and have a greater economic impact than any other parasite (reviewed in Torrissen et al., 2013). Because the salmon lice are a naturally occurring parasite where aquaculture production

of salmon in open-cage systems takes place, the management of salmon lice infestations will remain an on-going battle.

Traditionally, salmon lice infestations have been treated with chemotherapeutics, however a continuous development of resistance to delousing agents such as pyrethroids (Alphamax®, Betamax® & Excis®), organophosphates (Salmosan®), avermectins (SLICE®) (Burridge et al., 2010), and hydrogen peroxide (Treasurer et al., 2000), has forced the industry to develop and use non-chemical methods such as cleaner fish, laser (Stingray®), lice skirt (Calanus®), flusher etc. Effects from delousing agents on no-target organisms such as benthic invertebrates and crustaceans have been reported by a number of authors (reviewed in Page & Burridge, 2014). This gives rise to bad publicity for the salmon farming industry, providing yet another incentive to combat salmon lice with non-chemical methods.

Cleaner fish in aquaculture

In cleaning symbiosis, one species (the cleaner) feed parasites from another species (the host) (Feder 1966, reviewed in Bjordal, 1991). Biological control of salmon lice in open net-cages is a common method to sustain low salmon lice infestations. Lusedata.no reports that in week 40, 2015, approximately 64% of Norwegian salmon farmers utilize cleaner fish as a part of their strategy to keep salmon lice infestations below the national max level, which is set at 0.5 mature female salmon lice per fish (statistikk.lusedata.no) (lovdata.no/dokument/SF/forskrift/2012-12-05-1140).

Several species of wrasse (*Labridus*) has until recent years been the only cleaner fish used in the Norwegian salmon farming industry. Along the Norwegian coastline, there are five common wrasse species, including; rock-cook (*Centrolabrus exoletus*), goldsinny (*Ctenolabrus rupestris*), corkwing (*Symphodus melops*), ballan wrasse (*Labrus bergylta*), blue steel wrasse (*Labrus bimaculatus*), which mostly resides along the south and west coast, seldom further north than Lofoten (68^{o}) (imr.no, 2010). Wrasse has been reported to feed actively on salmon lice at approximately 12^{o}C by (Skiftesvik et al., 2013). However, when temperature is low, wrasse becomes very passive (a median temperature of 7.7^{o}C has by Lein et al. (2013) been reported as a temperature were wrasse will not graze on salmon lice), and it is, therefore, a need for a specie that will maintain grazing of salmon lice at low temperatures. The aquaculture industry has therefore initiated the use of lumpfish (*Cyclopterus lumpus*) as a cleaner fish on

account of this species naturally habits the northern part of Norway and is consequently not restrained by low temperatures in these areas (Blacker, 1983; Holst, 1993).

Lumpfish - general

Davenport (1985) describes the lumpfish as a unique species, well established on morphological grounds with no close relatives. It is the only species of the genus *Cyclopterus*, and easily recognizable as a short, thick and scale-less fish with a high dorsal crest that covers the first dorsal fin entirely. Where other teleost normally have paired pelvic fins, the lumpfish has a ventral suction disc that allows them to rest and hide on vegetation such as sessile and floating seaweeds (Davenport, 1985). Lumpfish is distributed on both sides of the North Atlantic, from Spitsbergen in the north to Portugal in the south (Blacker, 1983). Lumpfish is common along the whole Norwegian coastline, however, the main part of the population spawns along the coast of Nordland, Troms, and Finnmark (Holst, 1993). The semi-pelagic teleost spends most of its life pelagic in offshore waters, usually in the upper 50-60 meters, and often over abyssal depths (Blacker, 1983; Holst, 1993). Lumpfish spawn in the sub-littoral zone on rocky substrate and conducts a migration from offshore to where they were conceived, to spawn. The lumpfish's first year is spent near shore along the coastline, more often nearby or attached (with their unique suction disc) to floating seaweed, for shelter and protection, but also because seaweed shelters the preferred prey specie *harpacticoids* (Ingólfsson & Kristjánsson, 2002). After approximately one year, when the juveniles have reached a size of 4-7 cm, seaweeds can no longer function as protection, and they are forced to leave the coastline and migrate to deeper water to avoid predation.

The lumpfish as cleaner fish

Lumpfish were tested and reported to be an efficient delouse agent already in 2001 when there was found over 100 salmon lice in one lumpfish (Willumsen, 2001). Imsland et al. (2014) reported that in cages where there was a lumpfish density of 10-15% of the salmon number, lice infestation levels where significantly lower, especially for the salmon lice stages pre-adult and mature males and females. This indicates that the lumpfish is a suitable species for biological delousing of Atlantic salmon (Imsland, et al., 2014).

The demand for cleaner fish is high, and the demand for lumpfish cannot be covered by wild-catch of lumpfish. It will, therefore, be necessary to facilitate commercial farming of lumpfish to meet the increasing demand. There are already several commercial operators that breed lumpfish for the salmon farming industry, including Norsk Oppdrettsservice. Even though commercial operators already exist, there is little knowledge of the lumpfish's requirements and tolerance to different conditions in aquaculture, such as for example the oxygen saturation in the tank.

To obtain and maintain optimal fish welfare for lumpfish, it is important that its requirements are met and that the environment is as close to optimum as possible, or, at least, well within limits of tolerance. The focus of the present study is therefore to investigate the physiological responses of the lumpfish to reduced oxygen saturation in the water in a farming situation. The hypoxia sensitivity of lumpfish is likely to reflect where they habit in nature, i.e. areas with low oxygen saturation can be avoided, and they are most likely not able to successfully acclimate to an hypoxic environment.

How does low oxygen saturation and thereby hypoxia affect fish?

General

Simply defined, hypoxia is a shortage of oxygen (O_2). Due to lower availability of oxygen in the water, oxygen is much more likely to be limiting for aquatic organisms than for terrestrial ones (Kramer, 1987). Oxygen is a limiting factor for fish metabolism, and according to Jobling (1994) the oxygen saturation is called critical if growth and food intake are negatively influenced. Thus aerobic metabolism decline, and oxygen delivery do not satisfy metabolic needs. The critical levels of oxygen saturation for fish are usually between 50% to 70% oxygen saturation, whereas salmonids are at the top of this range (Jobling, 1994). The specific growth rate (SGR) may in challenging situations, as severe hypoxia is (<50%), go from positive to negative as reported in rainbow trout (*Oncorhynchus mykiss*) at oxygen saturation at 39.6% (Wang et al., 2009).

The consequence of hypoxia is a reduction in the arterial oxygen partial pressure (PO_2), hence arterial oxygen content (Perry et al., 2009). Aquatic hypoxia is defined as dissolved oxygen concentrations below 2-3 mg O_2/L in marine and estuarine environments (Farrell & Richards, 2009). However, using an environmental concentration of O_2 is a poor way to describe hypoxia,

due to the fact that what is functionally hypoxic for one fish species is not necessarily functionally hypoxic for other fish species. Temperature is an important consideration, since it affects the fish's oxygen demand and the amount of dissolved oxygen available in the water. Fish can compensate for low oxygen saturation by increased gill ventilation, increased gill perfusion and delivery of more blood to the tissues and thereby more oxygen, increased blood hemoglobin concentration and thereby increased oxygen carrying capacity of the blood, or increased tissue oxygen extraction. Exercising/active fish are more sensitive than sluggish/resting fish to decreasing oxygen saturation and hypoxia (Kramer, 1987).

Fish welfare in hypoxia

The term welfare was probably first applied to fish by Shelbourne (1975), and the concept fish welfare was first legitimtised within the UK´s animal experimentation legislation in 1986 (Ellis, et al., 2012). According to Huntingford et.al. 2006, welfare should be used to describe the "quality of life" that the individual experiences; however, how to define and measure animal welfare is a cause for debate, particularly in fish (Huntingford, et al., 2006).

Physical health is considered as a measure of welfare and is undoubtedly a necessary requirement for good welfare, but how fish experience and feel about their "quality of life" is not possible to measure. It is however generally accepted that it can be indicated by the emotional monitoring system that have evolved to guide animals to fulfill their basic needs, in other words; to get what they need and avoid harm and danger. As an example fish will, when exposed to noxious events, respond and behave abnormally to avoid the noxious event (Braithwaite & Boulcott, 2007; Stien, et al., 2013). It is also obvious that sub-optimal performance (e.g. reduced growth) may be associated with compromised welfare. For example, prolonged activation of the stress response (which long-term exposure to hypoxic environment may cause) will lead to a tertiary response and negative effects, which are undeniable effects that will reduce the "quality of life" for fish (Huntingford, et al., 2006). Severe hypoxia not only suppress growth, but also may reduce oxygen uptake rate, as well as other fundamental needs as osmoregulation and cardiac function and thereby cause poor welfare (Stien et al., 2013).

Physiological changes in fish from stress

Overview

Stress in teleost fish is by Bonga defined as; "*a condition in which the dynamic equilibrium of animal organisms called homeostasis is threatened or disturbed as a result of the actions of intrinsic or extrinsic stimuli, commonly defined as stressors"* (Bonga, 1997). Barton & Iwama (1991) describes the effects of stress "*to be considered as a change in biological condition beyond the normal resting state that challenges homeostasis and, thus, presents a threat to the fish´s health. Stress itself can not be measured and only the responses to stimuli can be quantitatively determined to reflect the degree of severity of stress experienced"* (Barton & Iwama 1991).

A stress response is a response that manages the fish to cope with stressors by readjusting its biological activities. The acute stress response in fish includes stimulation of oxygen uptake and distribution, mobilization of energy substrates and reallocation of energy away from non-vital functions such as reproduction, growth, and immune functions. In a longer term scenario, stress may result in inhibition of growth, reproductive failure and reduced resistance to pathogens (Bonga, 1997). The stress response in fish, as in mammals (Seley, 1936) consists of primary, secondary and tertiary responses (Mazeaud et al., 1977; Barton & Iwama, 1991; Bonga, 1997).

Plasma cortisol is the most widely used indicator of stressed fish and is considered useful to determine the severity of the stressors (Barton & Iwama, 1991; Bonga, 1997). The basal level of cortisol is low (ideally <5 ng/ml, however up to 30-40 ng/ml is recognized as unstressed levels of circulating corticosteroids), and increases rapidly when fish is exposed to stressors (Barton & Iwama, 1991). Cortisol is recognized as a good indicator of stress, because once levels are elevated it takes at least one hour before returning to normal levels, as opposed to catecholamines, which have a very short half-life in blood. For many fish species exposed to prolonged, and thereby chronic stressors, cortisol levels may remain high, but under peak levels. Barton & Iwama (1991) reported that fish exposed to acute stressors may experience cortisol levels 10 or even 100 times higher than basal levels, and 40-200 ng/ml is normal elevations when it comes to peak post-stress levels.

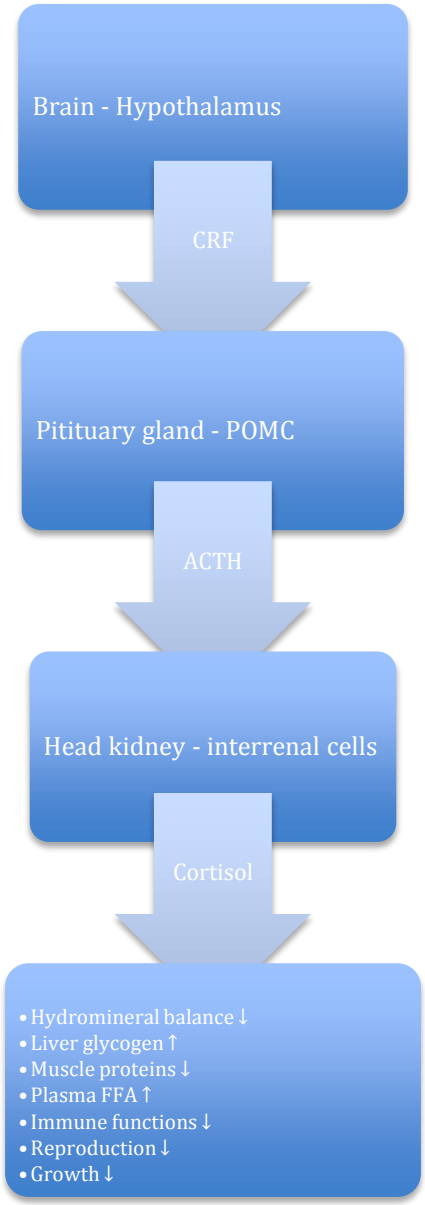

Figure 1: A schematic and simplified illustration of the HPI – axis in teleosts that describes the processes that lead to cortisol release and biological responses to cortisol. Based on Bonga 1997. Please note that the last box is effects and processes stimulated by the HPI-axis, and not a part of the HPI-axis itself. ↑ indicates that the process is stimulated, and ↓ indicates that a process is inhibited.

HPI axis

The primary phase is a neuroendocrine response that includes the release of the hormones catecholamines (CAs) and the corticosteroid cortisol. Upon stressor exposure, an endocrine axis called the hypothalamus – pituitary – interrenal (HPI) axis is mobilized (figure 1). Activation of this axis terminate in the release of catecholamine's (CAs) and cortisol.

CAs, primarily epinephrine (adrenaline) is a rapid first response to stressors, and 1-3 minutes

after exposure to an acute stressor a CA peak can be observed, however due to short circulating half-life the levels drops fast and may reach pre-stress levels less than 10 minutes after exposure to the stressor. CAs is released from the chromaffin cells located in the head kidneys.

Stressors also stimulate the hypothalamus to produce "corticotropin releasing factor" (CRF) which through neurons will find its way to the pituitary gland, and there stimulate the production of "pro-opiomelanocortin" (POMC). POMC is a peptide that will split and be the origin of "adrenocorticotropic hormone" (ACTH). ACTH is released from the pituitary and subsequently transported with the blood to the head kidney where it will bind to melanocortin receptors in the interrenal tissue. This binding is stimulating cortisol production in the interrenal cells. Due to the long signalling pathway from the hypothalamus to the head kidney by hormone transportation, and as cortisol needs to be produced upon stimulation by ACTH, blood cortisol levels typically takes a few minutes to rise in response to acute stress, and will return to pre-stress levels first many hours later (Pickering & Pottinger 1989, reviewed in Huntingford, et al., 2006). Due to the slow response of cortisol to a stressor, compared to CAs, cortisol is the most used indicator of stress responses in fish (Barton & Iwama, 1991).

Secondary responses are mainly responses to the hormones produced in the HPI axis and include increases in cardiac output, oxygen uptake, and distribution, mobilization of energy substrates, but also disturbance of the hydromineral balance (Barton & Iwama, 1991; Bonga, 1997).

Tertiary responses (figure 1) may occur if the stressors are long-lasting and the fish is not able to restore homeostasis (chronic stress). Effects of the tertiary response include long-term effects as reduced tolerance to hypoxia, reduced growth, reduced capacity to reproduce, and suppressed immune system (Barton & Iwama, 1991; Bonga, 1997).

Due to long-term effects of stress it is in the fish farmer's best interest to avoid stress levels and thereby keep the fish alive and healthy. Cultural practices to control and minimize the fish exposure to stressors are therefore recognized as crucial for success in aquaculture (Barton & Iwama, 1991).

Stress responses to hypoxia

"Lifestyle" will affect how different fish species will cope with hypoxic conditions. A general consensus is that fast swimming/active fish have low tolerance to hypoxic conditions, due to

high energy expenditure and thereby absence of sufficient adaption possibilities to oxygen deficit. Moderately and low mobile (sedentary) fish species have better compensatory abilities to overcome stress, and is thereby more tolerant to hypoxic exposure (Silkin & Silkina, 2005). Lumpfish is a semi-pelagic fish, and can therefore not be classified as a fully sedentary fish. Either way, we have no knowledge of their tolerance to a hypoxic environment, however the semi-pelagic nature suggests somewhere between how pelagic and benthic fish species respond.

Aim of study

Oxygen availability is a limiting factor for growth for fish, and low oxygen saturations have caused reduced growth for several fish species including juvenile white sturgeon and striped bass (Cech Jr. et al., 1984), rainbow trout (Pedersen, 1987), Atlantic cod (Chabot & Dutil, 1999), juvenile turbot (Pichavant, et al., 2000), European sea bass (Pichavant et al., 2001) and Atlantic salmon (Remen, 2012). In addition, low oxygen availability must be considered to compromise the welfare of fish. On this background, the present study was set out to determine the influence reduced oxygen saturations might have on overall growth rate, oxygen expenditure and a welfare indicator (plasma cortisol concentrations) in juvenile lumpfish.

Materials and methods

Fish and research facilities

The experiments were carried out at the Nofima´s Center for Marine Aquaculture (CMA) in Tromsø (69^{o}N) and were approved by the Norwegian Committee on Ethics in Animal Experimentation (Id 6872). The lumpfish were from different families, and details on families and hatching dates is listed in Appendix IV. The tanks used in the long-term experiment were circular 190 L filled to a constant level of 133 L with different inlet water flow rates (Appendix I, Table II & III), adjusted to the oxygen saturation that was to be achieved. Before entering the tanks, the water had been through a cleanse process: Raw water (pumped in from the ocean) → drum filter 90 µm → protein skimmer → heating to 10^{o}C → UV → vacuum degasser. The tanks were exposed to constant light during the experiment.

Experimental design

Long-term exposure to reduced water oxygen saturations

Introduction

The experiment started on October 14th 2014, and in total 396 (+ 48 for acute stress) juvenile lumpfish was included in the experiment. There were 4 different treatment groups, all of them in triplicates with 37 lumpfish in each replicate. The groups where reared at 4 different oxygen saturations originally planned to be 40%, 60%, 80% and 100% saturation. An accurate regulation of oxygen saturation over time appeared to be difficult, and the real oxygen saturations in the tanks are presented in Table 1.

Table 1: Oxygen saturation (%) in the 4 treatment groups. Water flow (L/min), actual obtained mean of treatment and temperature (°C) ± S.D. for each replicate.

Group name / treatment	Water flow L/min	Treatment (mean) for each replicate	Temperature	
			Mean	**± S.D.**
55%	0.7	54.50 %	10.28	0.19
55%	0.7	53.40 %	10.28	0.19
55%	0.7	55.90 %	10.28	0.19
69%	1.2	68.50 %	10.19	0.34
69%	1.2	68.30 %	10.19	0.34
69%	1.2	70.60 %	10.19	0.34
81%	1.9	80 %	10.11	0.34

81%	1.9	80.70 %	10.11	0.34
81%	1.9	83.30 %	10.11	0.34
96%	6	94.90 %	10.09	0.33
96%	6	95.70 %	10.09	0.33
96%	6	95.90 %	10.09	0.33

Although the aim was to keep oxygen saturation constant over time within a tank, fluctuations occurred (Table 1). The fluctuations were however limited, and the oxygen saturation differed continuously between treatment groups. Oxygen saturation (%) was used as an indicator of oxygen availability since saturation tells us what the fish is offered without the need to take into account temperature and salinity (Stien, et al., 2013). The inlet water was approximately 10ºC and salinity 334 ppm when delivered to the tanks through the entire experiment of 57 days. Measurements of temperature and oxygen saturation were conducted daily throughout the experiment. All tanks were fed automatically with a robot once per hour, around the clock, trough the entire experiment. The amount of feed was based on tables established at CMA and the groups 81% and 96% were fed with a 3% feed of body weight/day. Group 69% and 55% were fed with 2% and 1% body weight/day, respectively (Appendix IV). Visual inspection showed that all treatment groups were fed in excess. Initial size of fish were similar among all treatment groups, having a mean body mass of 24.3 g (S.D. 4.08), 24.3 g (S.D. 3.9), 24.5 g (S.D. 4.7), 23.0 g (S.D. 3.6) and length of 7.2 cm (S.D. 0.5), 7.2 cm (S.D. 0.4), 7.2 cm (S.D. 0.5), 7.1 cm (S.D. 0.5) in the 55%, 69%, 81% and 96% oxygen saturation groups, respectively.

Tagging of fish and size measurements

On October 14th 2014 15 out of a total of 37 fish in each of the 12 groups (except for treatment group D2 where only 13 out of totally 35 fish were pit-tagged) were anesthetized with FINQUEL ca. 0.1g/1^{-1}) and pit-tagged intraperitoneally with Trovan ® giving each of them a unique identity-mark. The pit-tagged individuals were then marked with an injection of fluorescent paint as shown in figure 2 so that they would be easier to detect in the tank. These fish were used for body mass and length measurement that were performed at approximately 4 weeks intervals on the following dates: October 14th 2014, November 6th 2014, December 10th 2014. At all recording dates, fish were scanned for tags and then body mass measurement were recorded to the nearest 0.01 gram and length to the nearest 0.1 cm. Initial biomass in the tanks at the start of the experiment, including both tagged and untagged individuals (n=37), varied between 754 – 884 gram, representing a biomass per volume of water of 5.6 – 6.6 kg m^3 water.

The 3 replicates reared at 55% were terminated after 23 days at November 6th 2014 due to low overall growth and observations indicating poor fish welfare. The remaining part of the experiment terminated 57 days after start on December 10th 2014.

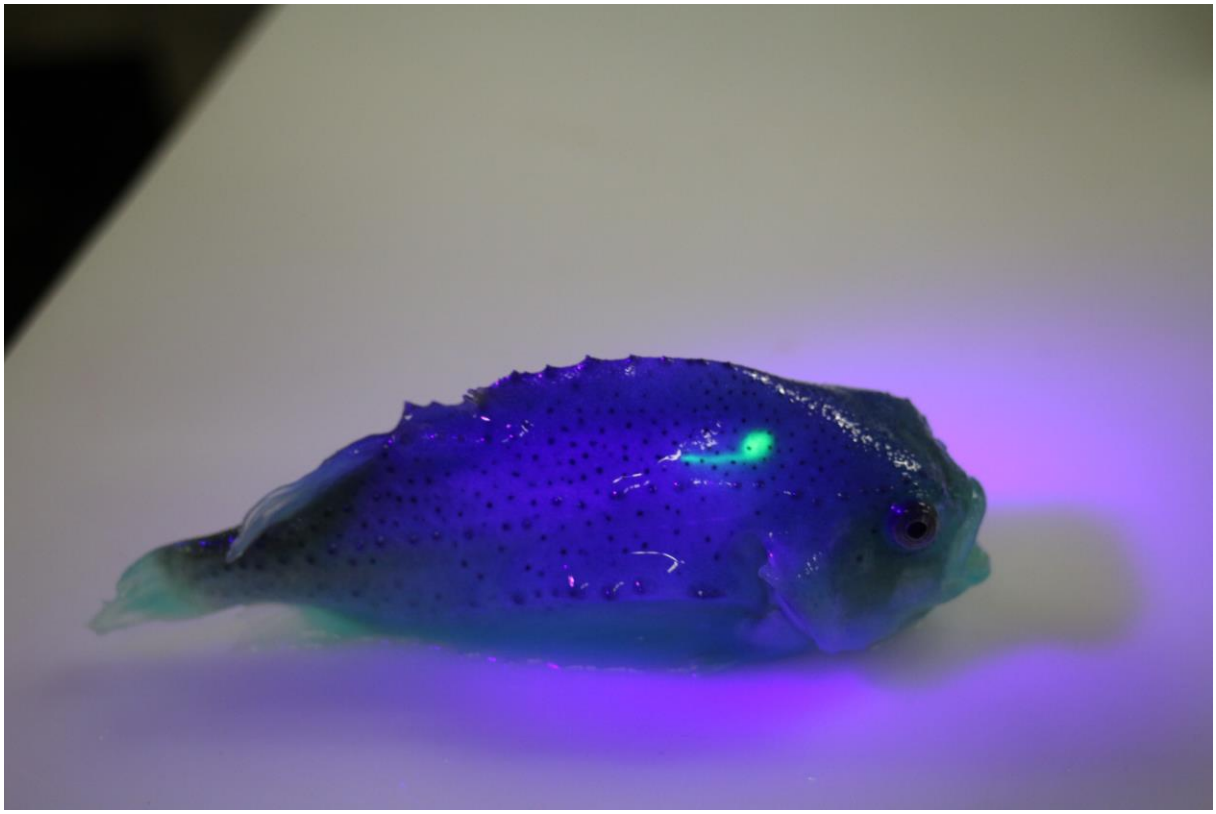

Figure 2: Juvenile lumpfish injected with fluorescent paint making it easily visible in the tank.

Blood sampling

On November 6th and December 10th, 5 fish were randomly selected for blood sampling from the untagged individuals in each of the 12 tanks. Before sampling all fish were immediately after removal from the tanks anesthetized with BENZOCAINE (60ppm). Blood samples were taken with 2 ml lithium heparin tubes ((34 IU) Vacutainer®-tubes) injected into the caudal vein, which is located under the spinal chord.

After blood sampling, the collected fish were exposed to a lethal dose of Benzocaine (200ppm). All blood samples were immediately transmitted to Eppendorf tubes and centrifuged (HECO, Z 326 K. Maxdrehzahl; 1800 r/min.) for 10 minutes to separate blood plasma from blood cells. Blood plasma was pipetted out and transmitted to new 0.5 ml Eppendorf tubes and stored at -20°C until analysing.

Water inflow rates

Reduced oxygen saturation in the tanks was obtained by reducing the water inflow (without reducing water volume in the tanks) to a level where oxygen saturation was as close as possible to intended one. Inflow water volume in the four different treatments was set to try to keep the oxygen saturation constant. However, some adjustments were made due to higher oxygen saturations than expected in inlet water (Table III, Appendix I).

Ventilation frequency

Ventilation frequency is commonly used as an indicator of a response to stress in fish (Kramer, 1987; van Rooij & Videler, 1996). For lumpfish, the gill ventilation frequencies were measured by counting how many times the gill cover (operculum) was opened (beats) during one minute. 5 fish from each of the 12 tanks were observed and counted at the following dates: September 24th, November 6th, and December 10th (only for the groups 69%, 81% and 96%, since the 55% group had been terminated in November due to low fish welfare) (see Appendix I, Table VIII).

Oxygen consumption

Calculations on oxygen consumption are based on measurements on oxygen saturation of water entering and leaving the tanks, water flow-through rate, total biomass in the tank, salinity and temperature. It was assumed that intake water had an oxygen saturation at 100% which at ~10^{o}C is 9.18 mg/L. Calculations for November 6th and December 10th is listed in Table IX & X, Appendix I. For November mean oxygen saturation between October 14th and November 6th, were used, and for December 10th mean oxygen saturation from November 6th and December 10th were used.

Specific growth rate (SGR) was calculated as:

$$SGR = (O_2\ Ci - O_2\ Co) * v / Mb * min$$

where O_2 C*i* is oxygen saturation initially, and O_2 C*o* is oxygen saturation at the outlet, v is volume and Mb is total biomass.

Short-term acute hypoxia and handling experiment

The short-term acute stress experiment was carried out on November 19th 2014 and included 24 random selected lumpfish at approximately the same size (43.7 g ±S.D. 4.4) (Appendix II, Table XIV). The experiment started by killing 6 randomly selected fish as an initial control-group (pre-hypoxia treatment) with a lethal dose of benzocaine (200ppm) before blood were sampled, and length and body mass were measured. The rest of the group were then placed in

a small tank of 12 L and disturbed with handling (oxygen saturation at 30-50% were intended and measured frequently). After 30, 60 and 120 minutes 6 fish were selected killed as before after which measurements of body mass and length including blood sampling were conducted.

Analyses

Blood plasma were analysed for cortisol at the University of Calgary (2500 University Dr NW, Calgary, AB T2N 1N4, Canada) in Professor Matt Vijayan's lab facilities. All analyses were conducted with guidance from his Ph.D. student, Erin Faught and their experienced lab technician. The analyses were conducted after a protocol prepared by Erin Faught, which was based on information given in (Yeh et al., 2013) see Appendix III.

Competitive ELISA (enzyme-linked immunosorbent assay)

Like for any other ELISA test, the goal with competitive ELISA is to measure the amount of a specific substance of interest present in the given sample. Competitive ELISA stands out from the other ELISAs (direct, indirect, sandwich) because they always involve a step where the sample containing the antigen of interest is first mixed with antigen-specific antibody before, or while they are added to the ELISA plate. The ELISA plate is coated with a competing antigen, which binds free antibodies in the first mix. In this manner, a huge amount of antigen in the sample results in few free antibodies and vice versa. A second antibody that is conveniently conjugated to an enzyme is then added. The second antibody will bind to the first antibody, before the well is washed again to remove all unbound enzyme conjugated antibodies. Finally, a solution of a colour-genic enzyme substrate is added, the interaction of the substrate with the enzyme on the second antibody generates visible colour. The amount of colour change produced is inversely related to the amount of substance of interest in the sample. In other words, samples with a high concentration of the protein of interest will produce less colour change because very little enzyme conjugate will be able to bind to the ELISA plate. Samples with very little protein of interest will produce a lot of colour because a lot of the enzyme conjugate will be able to bind to the ELISA plate. The development of colour in the wells with the specific antibody is quantified with an electronic plate reader. A dilution of the sample may sometimes be done, if the amount of the antigen is very high.

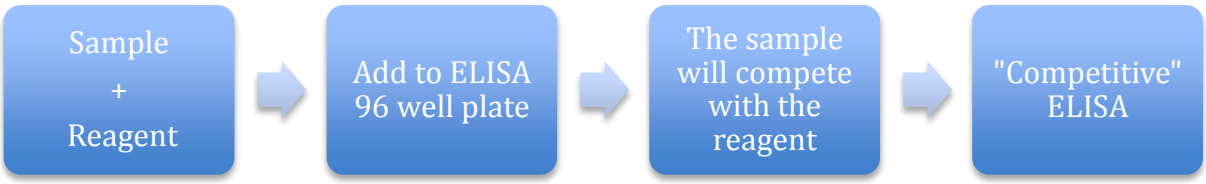

Figure 3: Competitive ELISA (simplified).

Cortisol levels were measured using a **competitive** enzyme-linked immunosorbent assay (ELISA) (fig. 3) based on the protocol of (Yeh et al., 2013). 4 out of the 5 samples from each of the group and each date were analysed from the long-term experiment, including the 24 samples from the acute stress and disturbance experiment, altogether 108 samples. All samples were analysed 2 x dilution and in replicates of 2.

High binding 96 well plates (Immulon HB, VWR) were coated with 100 µl of cortisol monoclonal antibody (1.6 µg/ml; East Coast Bio, ME, USA) in phosphate buffered saline (1 x PBS; 10 x stock: 1.37M NaCl; 27M KCl, 18 mM KH_2PO_4), for 16 hours at 4°C. The plate was then washed with PBS with 0.05% Tween 20 (TPBS; 300 µl/well) and blocked with 0.1% bovine serum albumin (300 µl/well; BSA; Sigma) for 1 hour at room temperature. Standards comprised of cortisol (Sigma) serial diluted (0 ng/ml – 25 ng/ml) in PBS and 50 µl of either standards or samples were added to the wells in duplicate. Cortisol conjugated to horseradish peroxidase (1:160 dilution; East Coast Bio, ME, USA) diluted in PBS was added to each well. Plates were incubated for 2 hours, shaking, at room temperature. The plate was washed as described above, and the detection reagent was added (41 mM TMB and 8 mM TBABH in 200 mM potassium citrate, pH 4). After 1 hour, the reaction was stopped with 1 M sulphuric acid (stop solution). Wells were read at 450 nm using a microplate reader (VersaMax, Molecular Devices, CA, USA).

The standard curve (fig. 4) was made with a serial dilution of one sample (blood plasma from lumpfish) to get absorbance reading for known values. The standard curve is linear with a high correlation coefficient (r= 0.948), meaning 94.8% of the data is supporting that the standard curve is linear. The standard curve can be used to compare the known values to unknown values, here cortisol in blood plasma from lumpfish.

See Appendix III for more detailed descriptions of the analyses of cortisol, solution recipes, and construction of the standard curve.

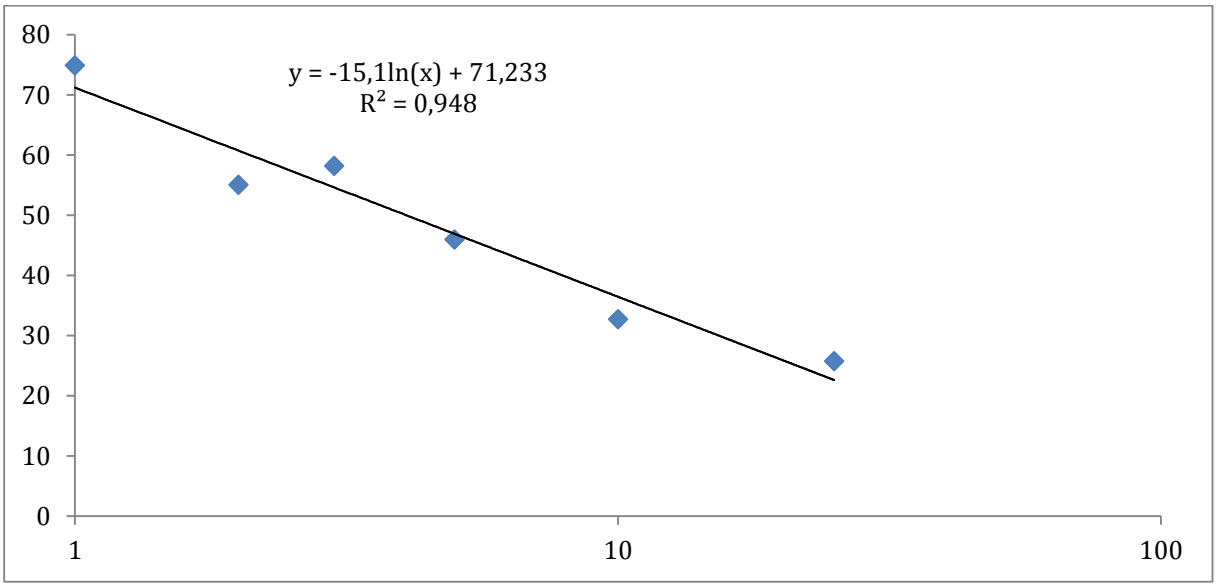

Figure 4: Standard curve for cortisol results.

Calculations & statistical methods

All data is presented as mean ± standard error of mean (SEM). Specific growth rate (SGR) were calculated by the following formula $(\ln W_2 - \ln W_2)\,(t_2 - t_1)^{-1}$, where W_2 and W_1 are body mass at date 2 and date 1, respectively and $t_2 - t_1$ is the number of days between measurements.

One-way ANOVA

All statistical analyses in this study were done in STATISTICA 11.0 (Statsoft, Inc,. 2013).

Analysis of variance or ANOVA is a parametric method appropriate for comparing the means for 2 or more independent populations. ANOVA assess whether the variance in the different populations is significantly different from each other, thus determine if the independent variable has an effect. The effect of oxygen saturation on body mass, specific growth rate and plasma cortisol levels was tested using a one-way ANOVA.

Based on the standard curve, were R^2 is close to 1, cortisol levels can highly be correlated with reduced oxygen saturations.

The outcome of an ANOVA test was considered statistically significant for $p < 0.05$. For results where $p < 0.05$ a Tukey´s Honestly - Significant - Difference - Test were conducted, which revealed if there were a significant difference between groups (here; for growth or cortisol for either time or oxygen saturation).

Results

The oxygen saturation in the 4 different treatment groups was as described in table 3. The actual obtained oxygen saturation in each of the treatment groups deviated from planned saturation. Water flow for each treatment group is listed in Appendix I, Table III.

Table 2. Average oxygen saturation for each treatment group (more details in Appendix I). Overall treatment is in thesis used as name of each of the groups.

Overall / mean treatment (oxygen saturation)	S.E.	Oct - Nov	Nov – Dec
55%	1.08	54.6%	-
69%	0.56	69.8 %	68.7 %
81%	0.47	83.5 %	79.8 %
96%	0.32	96.4 %	94.9 %

Mortality

No mortality was observed during the experiment. However, 2 lumpfish from tank 238 (81% saturation) had fin rot at their tailfin and, therefore, euthanised (neither of them were pit-tagged). The replicates reared at 55% oxygen saturation were struggling with the hypoxic conditions almost from the beginning of the experiment. The fish ate almost nothing of what they was fed, the ventilation rate was high, and due to low water exchange the water quickly got turbid, although cleaning was frequent to avoid this. After slightly less than one month in unhealthy conditions, it was therefore decided to terminate this group from the experiment.

Experiment 1 – Long-term exposure to reduced oxygen saturations

Effects of oxygen saturation on growth (body mass and length)

There was no significant difference in mean body mass between groups (one-way ANOVA; $p = 0.274$) (Fig. 5, Table XIII, Appendix I) at the start of the experiment. From then on, oxygen saturation in water affected the growth of juvenile lumpfish (Fig. 5, Table XIV, XV Appendix I). In November, there were a significant overall effect of oxygen saturation on body mass (one-way ANOVA; $p = 0.00$). The pairwise test (Tukey´s Honestly-Significant-Difference Test) revealed that the 55% saturation group had a lower body mass than the 69% group ($p = 0.00$) and that the 69% group had a lower body mass than the 96% saturation group ($p = 0.03$). Body mass of the 81% group was intermediate, and not significantly different from the 69% and 96% groups ($p > 0.05$).

Also in December there were an overall significant effect of oxygen saturation on body mass ($p = 0.00$) (Table XV, Appendix I), and the pairwise test revealed that the 69% group had a

lower body mass than the 81% group (p = 0.00), which had a lower body mass than the 95% saturation group (p = 0.00).

For group 55% there was a significant increase in body mass from October to November (p = 0.016). The body mass of the other groups (69%, 81% and 96% saturation) also changed significantly during the experiment (overall ANOVA result: $p < 0.05$) and the pairwise test revealed significant increases in body mass from October to November, and from November to December (p = 0.00). From October to November the four treatment groups 55%, 69%, 81% and 96% increased their body mass with 8.8%, 41.9%, 54.6% and 70.3% respectively. From October to December the three treatment groups 69%, 81% and 96% increased their body mass with 74.8%, 123.2% and 243.3%, respectively.

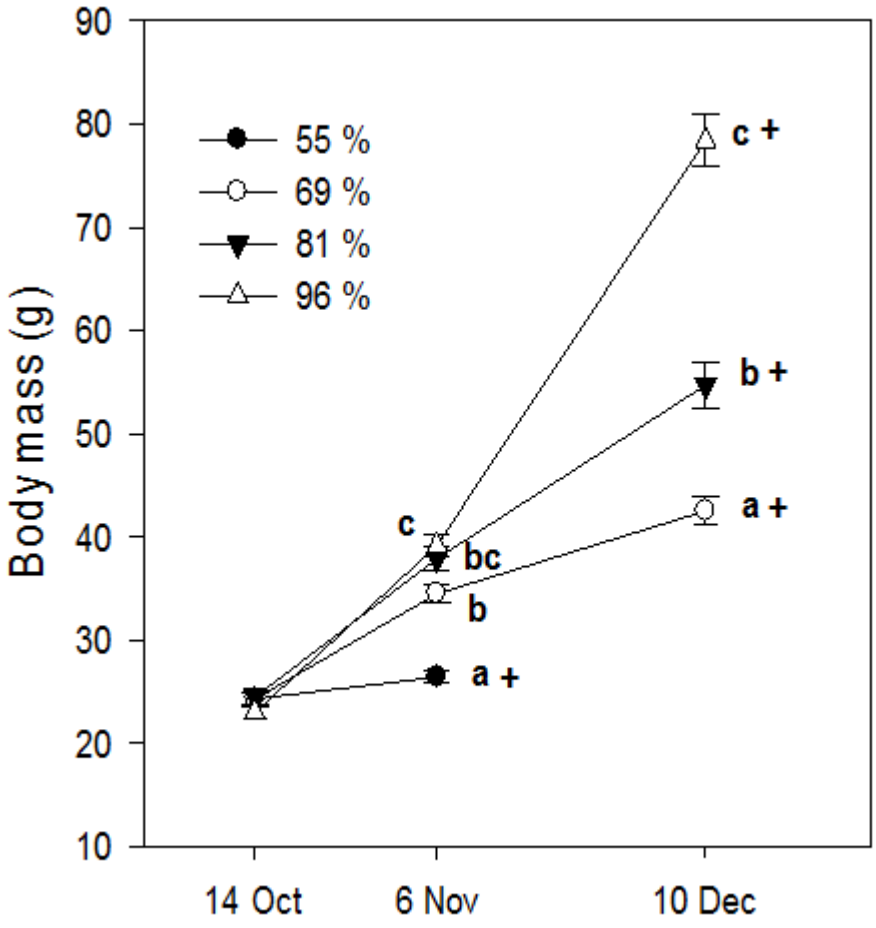

Figure 5: Mean body mass (± SEM) for individually tagged juvenile lumpfish during the two experimental periods. Different letters denote significant differences between groups and the +-sign indicate that there has been a significant increase in body mass during the course of the experiment.

With regards to length (fig. 6, Table V, Appendix I), there was initially no significant difference in length between the 4 treatment groups ($p = 1.00$). The pairwise test revealed that in November group 96% did not display significantly higher growth in length compared to group 69% ($p = 0.657$), neither did group 96% and 81% ($p = 0.996$) nor 69% and 81% ($p = 0.996$) differ. However group 69%, 81% and 96% all had significantly higher length than group 55% ($p = 0.00$). In December there were significant differences in length between the 3 remaining groups ($p < 0.05$).

From October to November the 4 treatment groups 55%, 69%, 81% and 96%, increased their length with 8.9%, 19.2%, 21.1% and 24.1% respectively. The 3 treatment groups 69%, 81% and 96% increased their length with respectively 33.1%, 40.7% and 59.1% from October to December.

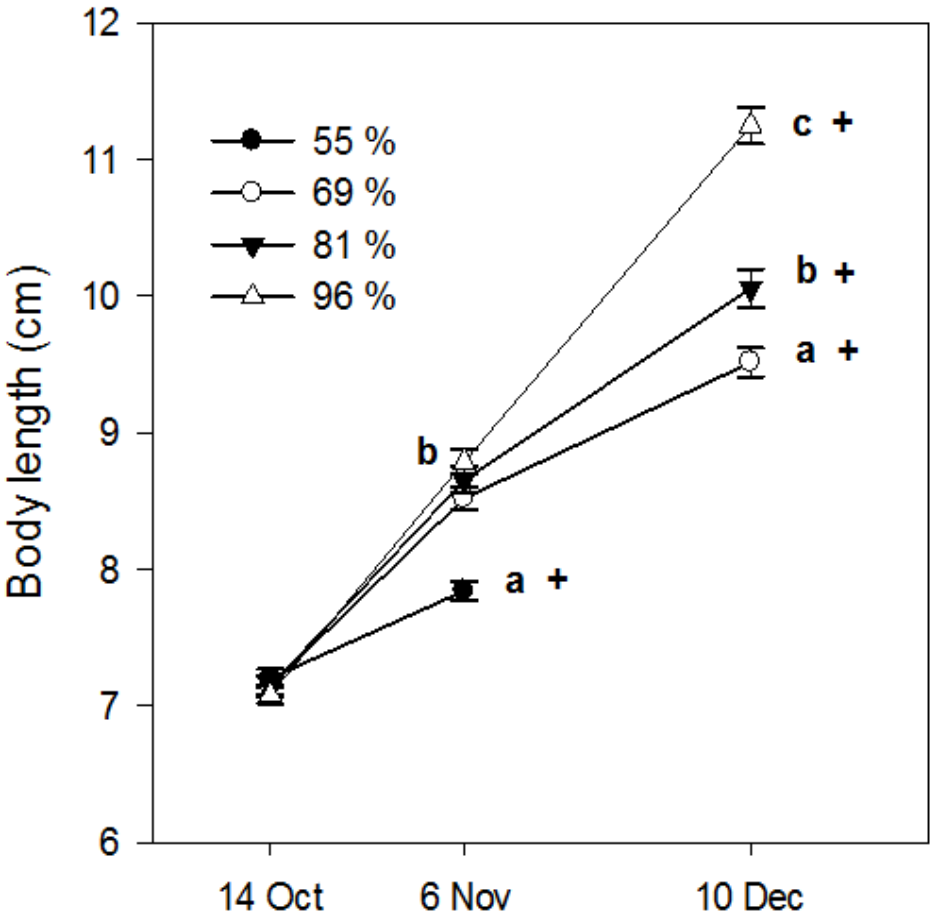

Figure 6: Mean body length (± SEM) for individually tagged juvenile lumpfish during the two experimental periods. Different letters denote significant differences between groups and the +-sign indicate that there has been a significant increase in body length during the course of the experiment.

In accordance with the body mass data, the data on SGR in the different treatment groups showed that all groups had a positive growth (fig. 7; Table VI, & VII in Appendix I). Further, there was a gradual, and significant increase in SGR with increasing water oxygen saturation during the first part of the experiment between October and November, and there were significant differences between all groups ($p < 0.05$). During the last part of the experiment (Nov-Dec), there was observed reduced SGR for groups reared at 69 % ($p = 0.0$) and 81 % ($p = 0.00$) oxygen saturation compared to these groups during the first part of the experiment. A small decline in SGR was also seen in the 96 % group, but this difference was not significant ($p = 0.153$).

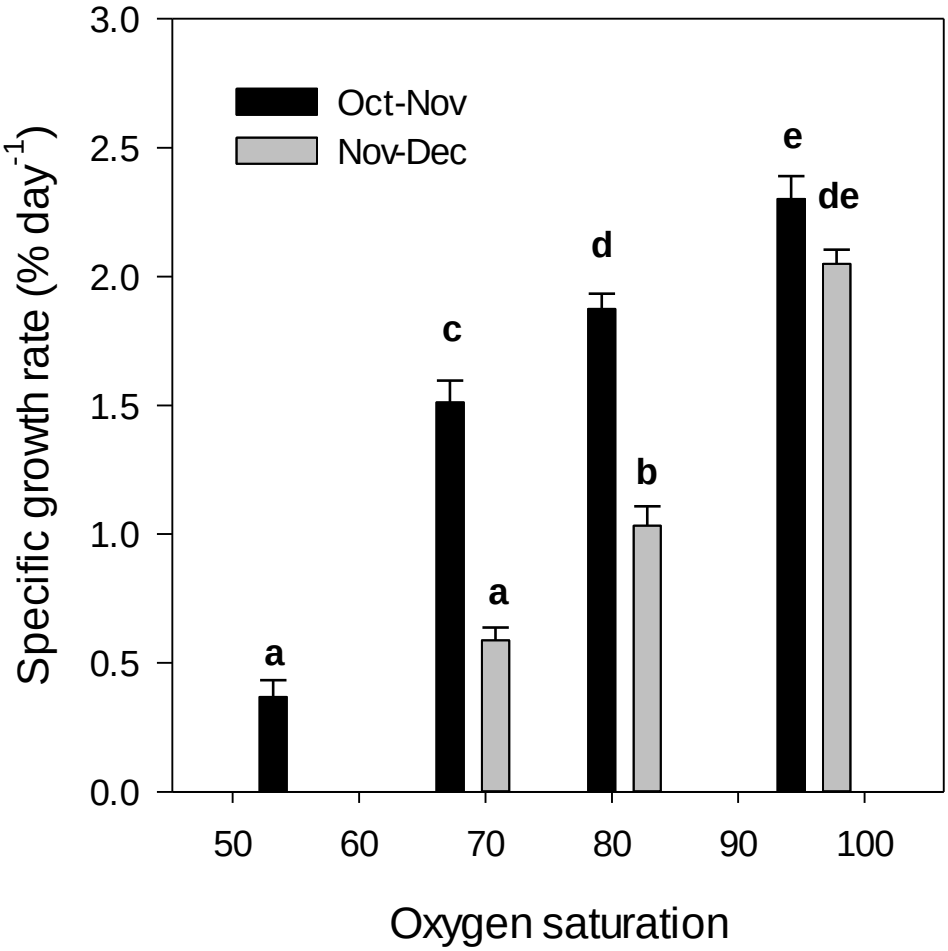

Figure 7: Specific growth rates (% day^{-1}, ± SEM) for individually tagged juvenile lumpfish during the two experimental periods. Different letters indicate significant differences between and within treatments (Tukey´s Honestly-Significant-Difference-Test; $p < 0.05$).

Cortisol

There were significantly higher plasma cortisol levels (fig. 8) in November in the treatment groups 69% and 55% than in the 96% group ($p = 0.018$; 0.007). Cortisol levels did not differ between treatment groups 69% and 55% ($p > 0.05$), neither between the groups 96% and 81% ($p = 0.831$) nor 81% and 69% ($p = 0.408$) at sampling in November. In December, there were no significant differences in plasma cortisol levels between the treatment groups 69% and 81% ($p = 0.155$), 69% and 96% ($p = 0.823$) nor 81% and 96% ($p = 0.881$), but plasma cortisol level in the 81% saturation group appeared to be lower than in the 69% group ($p = 0.404$). There were no significant differences between November and December within the groups ($p > 0.05$). See Table XI & XII in Appendix I for descriptive statistics for cortisol levels during the long-term experiment.

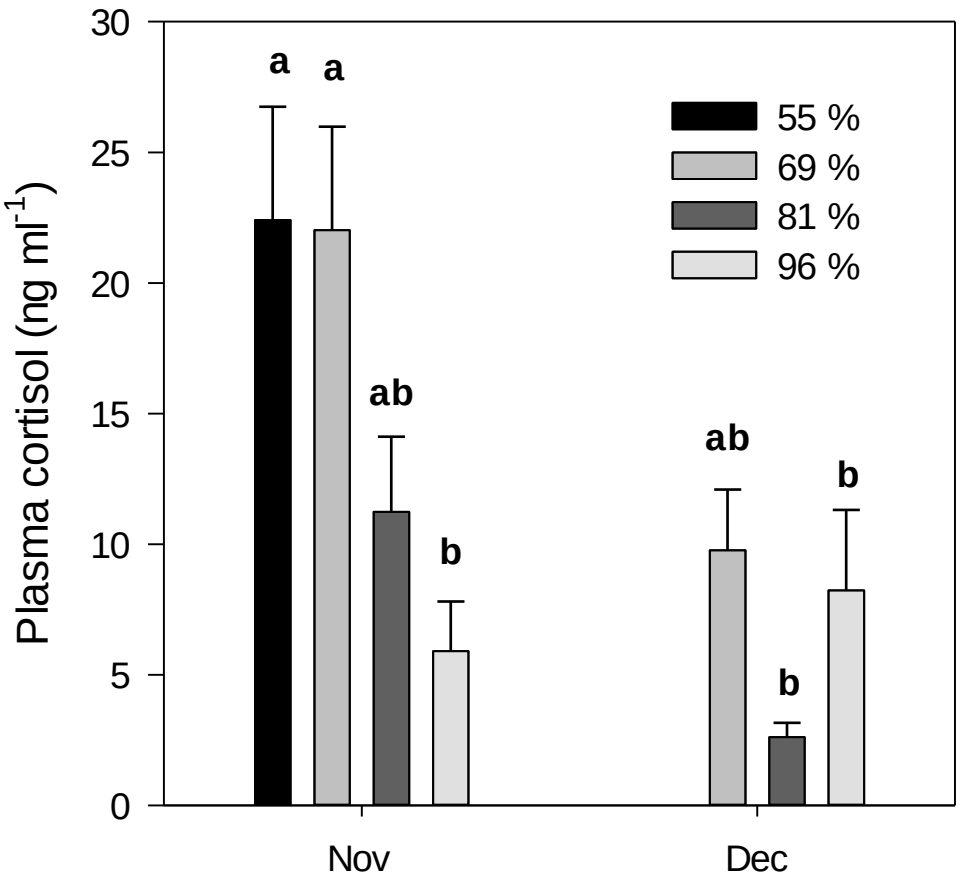

Figure 8: Mean plasma cortisol concentration (± SEM) in the fish from the different saturation groups sampled in November and December. Different letters denote significant difference between groups at each sampling date and within groups between sampling dates.

Food intake, ventilation rate, oxygen consumption and behaviour

Despite no exact numbers, observation during the experiment indicates that lumpfish reared at 55% and 69% oxygen saturation had a lower feed intake (appetite) than fish reared at 81% and 96% oxygen saturation (see information in the M&M section).

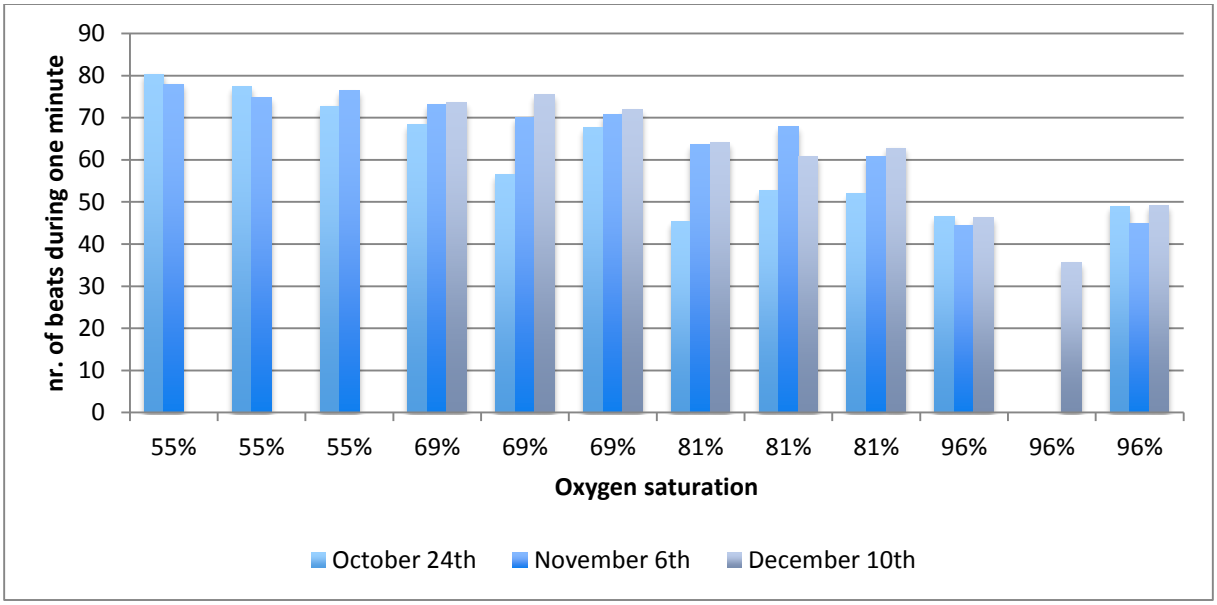

Figure 9: Mean ventilation rate during one minute for October, November and December for the groups at different oxygen saturations.

Lumpfish reared at 55% oxygen saturation had 1.6 times / 60.4% higher ventilation rate than lumpfish reared at 96% at the first measure conducted 10 days after the start of the experiment, on October 24th (fig. 9, Table VIII, Appendix I). During the experiment ventilation rate for fish reared at 55% and 96% were virtually stable. However, for lumpfish reared at 69% there was a 15.6% increase in mean ventilation rate from October 24th to December 10th. As for lumpfish reared at 81%, the same pattern was displayed with a 26% increase in ventilation rate. Except for high ventilation rate especially in 55% groups, but also in the 69% groups, the lumpfish were relatively calm. No panic or aggression was expressed.

Oxygen consumption were measured and calculated for whole groups of lumpfish, and there is therefore not conducted any statistics on these results. There is in November nearly twofold higher specific oxygen consumption for lumpfish reared at 55% compared to lumpfish reared at 96% oxygen saturation (fig 10, Table IX, Appendix I).

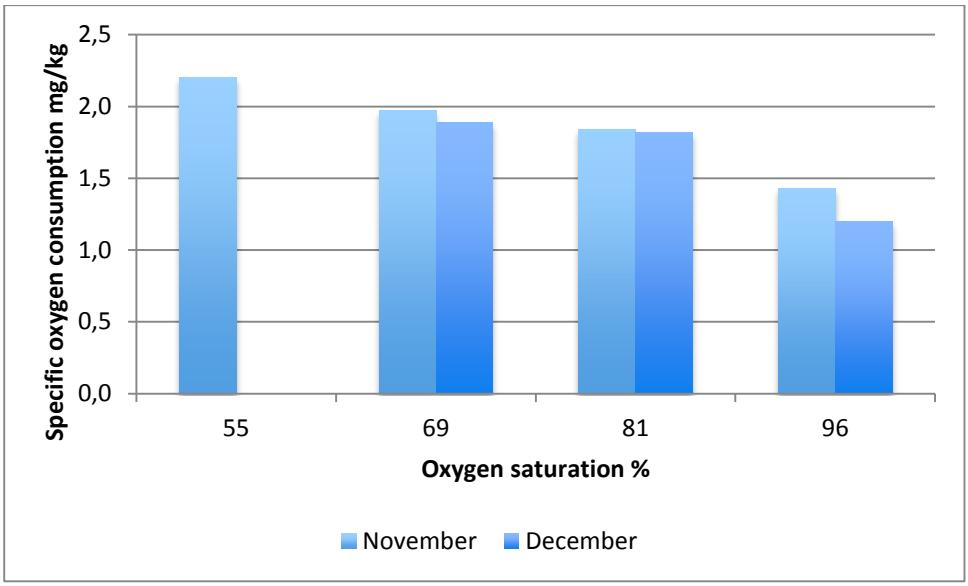

Figure 10: Mean specific oxygen consumption for November and December for the groups at different oxygen saturation.

Experiment 2: Short-term acute, severe hypoxia and disturbance

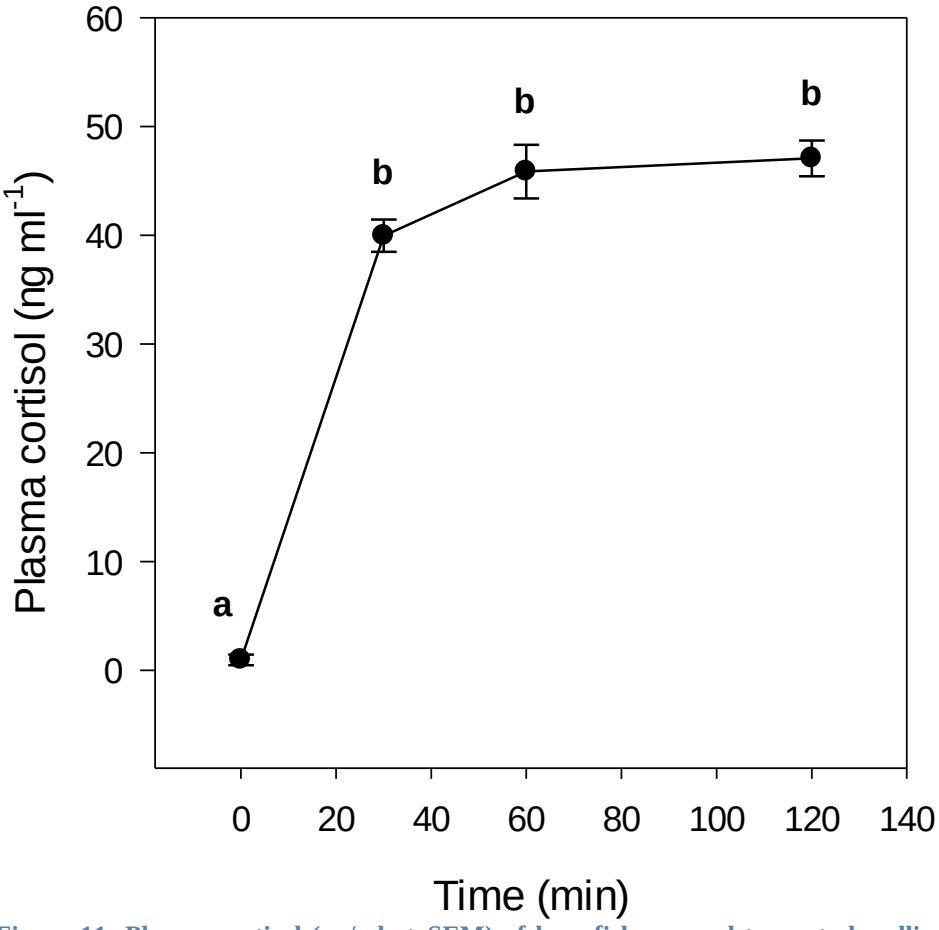

Figure 11: Plasma cortisol (ng/ml; ± SEM) of lumpfish exposed to acute handling and disturbance, and increasing hypoxia. Different letters denote significant differences between groups.

During severe hypoxia in this experiment (water oxygen saturation 30%), the fish had increased ventilation rate, and some agitation was observed, but mostly they were calm at the bottom of the small tank (12L). Cortisol levels had increased during the first 30 minutes, from a pre-stress level of ~0.97 to a significant different post-stress level of ~33.3 ng/ml ($p = 0.00$) (fig. 11, Table XVII, Appendix II). From then on, there was not a significant change in plasma cortisol levels until the end of the experiment at 120 min where mean peak plasma cortisol levels was at ~ 47 ng/ml ($p > 0.05$).

Discussion

The results from the present study showed that decreasing oxygen saturation increasingly compromised the growth performance of lumpfish. This was evident even at a reduction from 96% to 81% oxygen saturation (figures 5,6,7). The study also showed that low oxygen saturation not only reduced growth, but also elicited a physiological stress response displayed as initiating the HPI-axis, measured as plasma cortisol release.

The effect of reduced oxygen saturation in water on overall growth

At what water oxygen saturation level growth is affected, varies among species and is likely to depend on the individual species ability to compensate physiologically for the reduction in available oxygen (Wang et al., 2009). Lumpfish is a semi-pelagic specie (Blacker, 1983; Holst, 1993), which habits normoxic environment in nature. It is reasonable to assume that it in its natural environment easily can avoid areas with reduced oxygen saturations. Based on that the expectations for lumpfish was that it would not be able to successfully acclimate to reduced oxygen saturations, but rather be seen as hypoxia-sensitive specie. For hypoxia-sensitive species, the SGR will decline with decreasing feeding levels, and not return until exposure to hypoxia ceases (Wang et al., 2009).

Reduced oxygen saturations were reflected on growth rate, depending on severity and time course of the reduced oxygen saturation. As seen on lumpfish held at 55% and 69% oxygen saturation, the overall growth rate is poor compared to lumpfish held at 81% and 96%. Although all treatment groups had a positive growth rate (for body mass and length, fig. 5 & 6) during the entire experiment, the replicates reared at 96% oxygen saturation stood out from the other treatment groups with a much higher increase in overall growth the entire period, with a total 243% increase in body mass (Table VI & VII, Appendix I).

Since reduced oxygen saturation decline food intake and leaves less energy available for growth due to increased energetic costs for ventilation and increasing metabolic costs of digestion (Kramer, 1987; Jobling 1994; Wang et al., 2009), it is not surprising that fish reared at the lowest oxygen saturation (55%) had the lowest SGR (Appendix I, Table VI & VII). Fish will need to reallocate the use of energy from growth (and other non-vital activities) to activities that allow the fish to cope with stress when exposed to stressors (Barton & Iwama, 1991). Since growth is suppressed, it is reasonable to assume that lumpfish perceive reduced oxygen

saturation as a stressor, and therefore reallocate energy from feeding and growth to ventilatory response to be able to cope with the reduced oxygen saturations.

Feed intake has for several fish species been reported to decline and this seem to be the main reason for reduced growth during exposure to reduced oxygen saturations and hypoxia (Kramer, 1987; Wang et al., 2009). Brett & Groves (1979) reported that digestion could five-fold the oxygen consumption, and that a return to the oxygen demand at pre-feeding levels could take up to several hours. It is therefore advantageous to reduce food intake to lessen the metabolic burden associated with digestion during long-term hypoxia, thus save most of the aerobic scope for physical activity (reviewed in Chabot & Dutil, 1999). Since growth of fish is largely dependent on food consumption (Jobling, 1994) growth rate will decrease with decreasing food intake. As seen among other fish species (Jobling 1994; Chabot & Dutil, 1999; Pichavant et al., 2001; Foss et al., 2002; Wang et al., 2009, Remen 2012) lumpfish also reduced their food intake during exposure to reduced oxygen saturations. Reduced feed intake (for both hand- and robot feeding) was observed among the lumpfish reared at 55% and 69% oxygen saturation. The observations are consistent with low SGR for treatment groups 55% and 69%, compared to treatment groups 81% and 96% were SGR were significantly higher (figure 7).

Feeding were reduced (Appendix IV) one week in to experiment for lumpfish reared at 69% and 55% due to observations clearly stating that the lumpfish in these groups did not eat all they were fed. Excess feed accumulated and caused turbidity, which is a factor that can cause stress for fish (Bonga, 1997), and could therefore have disturbed the experiment, by for example reduced growth further. Although feeding was reduced observations conducted made it clear that they were still fed to satiation and reduced feeding was probably an effect of lack of appetite when exposed to reduced oxygen saturations. Reduced food intake is consistent with what is reported for sea bass and turbot fed to satiation where exposure to hypoxia led to decrease in food intake, respectively 1.5 – 1.7 and 1.7 – 1.8 times lower (Pichavant et al., 2001). This is also consistent with what Foss et. al., (2002) reported on juvenile spotted wolffish (*Anarhichas minor*) that were exposed to hypoxia; reduced food consumption and thereby suppressed growth performance. For salmon the depression in feed intake started when oxygen was reduced to levels of about 70% oxygen saturation at 16^{o}C, and from there decreased gradually with declining oxygen saturation (Remen, 2012). Although there are no numbers from this study to confirm the observations, it is reasonable to indicate that feeding rates and total feed consumption for lumpfish was suppressed during exposure to reduced oxygen saturations and negatively affected the overall growth rate. Since farming of lumpfish is in an initial phase

observations are an important parts of learning how to best produce and thereby develop protocols for production (Brown et al.,1997).

Reduced growth is consistent with what Chabot & Dutil (1999) reported for Atlantic cod (*Gadus morhua*) reared at approximately the same temperature (10^{o}C) and oxygen saturations as in this study. SGR was, although some difference in size (Atlantic cod in this study were ca. 700 g), comparable with SGR for lumpfish, at 0.5, 0.6, 0.8, 0.7 and 0.9 % day^{-1} at respectively 45, 56, 65, 75 and 84% oxygen saturation, respectively (Chabot & Dutil, 1999). Plante et al. (1998) reported that neither temperature nor fish size had significantly detectable effect on hypoxia tolerance for Atlantic cod, the results may therefore be comparable.

At the time of writing, no other reports have been made on lumpfish and oxygen saturation, and therefore it is not possible to compare the results within the specie. However, it is by Nytrø (2013) reported that growth in terms of body mass, length and growth rate for juvenile lumpfish, is significantly influenced by temperature. Juvenile lumpfish reared at 10 – 13^{o}C gives 31 – 35% higher overall growth rates, and 48 – 53% higher final body mass compared to juvenile lumpfish reared at 4^{o}C (Nytrø, 2013). The temperature lumpfish were reared under in this study is therefore highly relevant for commercial farming of lumpfish.
SGR decreases with increasing body mass for several fish species (Jobling, 1994) and it is therefore reasonable to assume that this also applies for lumpfish (Nytrø 2013). Decreasing growth rate with increasing fish size was also pronounced in this study, illustrated by reduced SGR the second period (November – December) compared to the first period (October – November). The decline in SGR for all treatment groups in the second period, compared to SGR for the first period is probably not only caused not by increased fish size, but also by reduced overall oxygen saturation (Table 2, results) for all remaining treatment groups.

In general, fish have two options when exposed to hypoxia or reduced oxygen saturation. The first option, oxyconforming, is an acclimation to reduced oxygen saturation initiated by reducing oxygen demand. The second option oxyregulation, maintaining the same blood oxygen level as before reduced oxygen saturations occurred, usually seen as increased ventilation and perfusion of the gills to increase the gradient and area for oxygen uptake (Barnes et al., 2011).
A strong ventilatory response is according to Kramer (1987) the best-documented activity change in response to reduced oxygen saturation. For juvenile lumpfish a strong increase in the

ventilation activity, especially for lumpfish reared at 55% oxygen saturation, but also to some extent for lumpfish reared at 69% oxygen saturation was observed. This is consistent with what is reported for Atlantic cod by Plante et al. (1998) reared at 38% oxygen saturation. The same goes for Atlantic salmon, which also expressed a hypoxic ventilator response and therefore is defined as an oxyregulator (Perry et al., 2009). Observations from this study on lumpfish held at 55% and 69% oxygen saturation has made it clear that lumpfish will try to remain their oxygen uptake as before reduced oxygen saturations occurred, by increased ventilation and perfusion of the gills, seen as hyperventilation, and lumpfish can therefore be characterised as an oxyregulator. The purpose of oxyregulation is to increase ventilation volume and by that raise blood to water PO_2 gradient and thereby increase arterial PO_2, hence delay onset of transition from aerobic to anaerobic metabolism (Farrell & Richards, 2009). This increased ventilation comes with a cost, which eventually exceeds the benefit of the oxygen obtained due to increased ventilation (Perry et al., 2009). The extra cost of the ventilatory response leaves less energy for voluntary activities such as feeding and thereby growth (Kramer, 1987; Perry et al., 2009). The almost twofold in ventilation observed for lumpfish reared at 55% compeard to those reared at 96% have undoubtedly increased the energy level, and by that increased the need for oxygen as well. Higher oxygen consumption for lumpfish reared at lower oxygen saturation (Table IX & X, Appendix I) indicates clearly that lumpfish have higher energetic costs and thereby oxygen consumption with increasing ventilatory response. Increased oxygen consumption are not unexpected for stressed fish, as Barton & Schreck (1987) reported that stressed juvenile rainbow trout had a oxygen consumption rate more than twice compared to unstressed fish. The increased cost to oxyregulate has presumably affected the decrease in growth for juvenile lumpfish reared at reduced oxygen saturations.

Since the oxygen consumption rate in this study is measured at fish groups it provides no information about variation between individuals. The variation can be substantial as reported by Barnes et al. (2011) on Atlantic salmon (S.D. ±13-19%). In contrast to what was observed for lumpfish, white sturgeon (*Acipenser transmontanus*) overall energy expenditures decreases, and oxygen consumption rates were depressed (57% mean reduction) during exposure to hypoxia (Crocker & Cech Jr., 1997). Crocker & Cech Jr. (1997) reported that white sturgeon, as opposed to lumpfish, have the ability to decrease overall metabolism and thereby oxygen consumption to acclimate to reduced oxygen saturation and hypoxia. Although reduced feeding activity also were expressed for lumpfish, the energetic costs increased. This supports the theory that lumpfish are not able to acclimate to reduced oxygen saturations.

It is worth noting that lumpfish in tank 240, which held one of the three replicates of the 96% oxygen saturation had a higher activity level through the entire experiment than seen in all other tanks. This has influenced the mean overall growth rate and plasma cortisol levels for the 3 replicates held at 96% (Appendix I, Table IV, V, VI, VII, VIII, IX, X, XI, XII). The activity level was so high that it was not possible to measure ventilation rate October 24th and November 6th. Why the activity level where so high in this replicate is unclear.

Effect of reduced oxygen saturation on plasma cortisol levels

Whether the stress response is activated or not, and how heavily the response is, is largely influenced by individuals' ability and capacity to adapt and cope with the hypoxic environment (Perry et al., 2009). Prolonged secretion of corticosteroids, including cortisol, during chronic stress is considered to have maladaptive effect on fish due to the secondary and tertiary responses that are induced by increased cortisol levels over time (fig. 1, HPI-axis). Lumpfish have a response to stressors (here; reduced oxygen saturation) pertaining to cortisol as expected, however higher levels after reduced oxygen as a stressor were expected. The release of the stress hormone suggests that a general stress response was induced (Barton & Iwama 1991; Bonga 1997), and lumpfish reared at oxygen saturations below 69% tertiary stress response did occur. This indicates that the hypoxic environment is challenging for lumpfish, and in lack of ability to cope with the environment and restore the homeostasis the fish experience chronic stress.

For lumpfish basal plasma cortisol (pre-stress) levels was generally very low (<10ng/ml), and were within the level that is generally considered representative for unstressed fish (Barton & Iwama, 1991). This was displayed in lumpfish reared at 96% oxygen saturation, and was at sampling in November 5.9ng/mL (S.E. 3.05) and December 8.24ng/mL (S.E. 4.96) (Appendix I, Table IX & X). The pre-stress levels of plasma cortisol displayed at the acute-stressor experiment are even lower (0.97ng/ml S.D. ±1.22), indicating that individual differences among lumpfish, however basal cortisol levels, are generally very low. Fish reared at 96% and 81% oxygen saturation did not display plasma cortisol levels above 10 ng/ml during the experiment, indicating that these individuals were not stressed. Plasma cortisol levels during chronic stress is expected to be well below peak levels (Bonga, 1997), which they were, approximately halved (~ 22 ng/ml) of what was displayed at severe hypoxia in experiment 2 at ~ 47 ng/ml. Lumpfish in treatment groups 55% and 69% expressed signs of chronic stress, thus higher plasma cortisol

levels were therefore expected. However, only in excess of twice as what displayed for pre-stress levels were displayed at sampling in November. Based on this it is likely that overall growth rate for lumpfish reared below 69% oxygen saturation has been affected by higher cortisol levels which might have been to suppressed circulated growth hormone (GH) which is been reported to be positive correlated with increased cortisol levels (Barton & Iwama, 1991). However the plasma cortisol levels in the replicates reared at 69% normalized during the next period (November - December) and at sampling in December plasma cortisol were reduced with 56%, from ~ 22 ng/ml to 9.8 ng/ml. Over all growth rate remained approximately the same, and it is therefore uncertainty concerning the positive correlation between a decline in circulation GH and increased plasma cortisol levels for lumpfish. Reduction in plasma cortisol levels for lumpfish reared at 69% oxygen saturation indicates acclimation to the oxygen saturation, and that they therefore were no longer stressed, or due to fatigue of the HPI-axis. Acclimation to reduced oxygen saturation over time has been reported for several species including turbot (*Scophthalmus maximus*), sea bass (*Dicentrarchus labrax*) and spotted wolffish, which all have the ability to adapt to hypoxia over time, and SGR will therefore first decline, but after adaption increase again (Pichavant et al. 2000, 2001; Foss et al., 2002; Wang et al., 2009). Based on observations and results (SGR for all groups where declining and did not increase during the experiment) from this study, juvenile lumpfish can be classified as a hypoxia-sensitive specie that do not have the ability to adapt or acclimate to prolonged hypoxia. Since cortisol levels for acute stressed fish were fairly low, only two-fold what displayed in fish reared at 55% oxygen saturation over time it is sensible to presume that it is for lumpfish as for spotted wolffish (Lays et al., 2009), the HPI-axis fatigues over time. This theory is supported by a ventilatory response, and oxygen consumption that was fairly stable for all groups throughout the experiment. The rapid increase and then waning plasma cortisol response of acute stressed lumpfish (Table XVII Appendix II) support the theory that the HPI-axis possibly fatigues over time in lumpfish. The fatigue in the HPI-axis can be as reported by Bonga (1997) due to negative feedback control of cortisol by α-MSH cells which during chronic stress in fish increase in size and activity.

Spotted wolffish displayed after short-term severe hypoxia (60 - 20% oxygen saturation for 150 minutes) and disturbance, plasma cortisol levels at what is referred to as a weak response at ~ 70ng/ml after 4-8 hours, due to a slow cortisol response (Lays et al., 2009). As opposed to the semi-pelagic lumpfish spotted wolffish is a sedentary, benthic specie. However, both of them have an apparent weak primary (cortisol) response to severe hypoxia. This reaction to stressor is not unusual for benthic species and for the pallid sturgeon (*Scaphirhynchus albus*), the plasma

cortisol levels did not exceed 30 ng/ml^{-1} when exposed to transportation for 7 hours (Barton et al., 2000). The low plasma cortisol levels at peak for lumpfish in this study are in contrasts to active pelagic species such as rainbow trout (*Oncohorhyncus mykiss*) exposed to severe hypoxia which displayed plasma cortisol levels peaking at a value of 735 ± 424 ng/ml during the last 90 minutes when the oxygen saturation only were 25% (van Raaij et al., 1996). This indicates that the semi-pelagic lumpfish is somewhere between active pelagic species and benthic sedentary species when it comes to activation of the HPI-axis and cortisol release as a response to hypoxic environment and reduced oxygen saturations.

Cortisol affects growth through the process where gluconeogenesis from proteins due to high levels of circulatory cortisol cause muscle wasting, and results in a negative nitrogen balance, and growth stops (Van Der Boon et al., 1991). Barton & Schreck (1987) reported that rainbow trout fed daily over a period of ten weeks with cortisol had suppressed growth rate. Lumpfish reared at 81% oxygen saturation increased their body mass with 123% during the experiment, this is low compared to the 243% increase lumpfish at 96% oxygen saturation experienced. The low plasma cortisol levels for the 81% groups indicate that cortisol alone probably not have influenced overall growth to a greater extent, it is more likely that reduced appetite and to some extent increased ventilator response, and oxygen consumption has affected overall growth more than plasma cortisol levels in this experiment. The uncertainty of a positive correlation between increasing cortisol levels and increased oxygen consumption for lumpfish as reported for rainbow trout by Barton & Schreck, (1987) are high, since plasma cortisol levels for fish reared at 69% more than halved from the first to the second period, however oxygen consumption remained almost equally.

How does reduced oxygen saturation affect fish welfare

Fish exposed to severe hypoxia may be unable to maintain oxygen uptake rate, as well as feed intake, and osmoregulation among other fundamental needs, which will cause reduced welfare (Stien et al., 2013). Feeding is an important welfare need of lumpfish, and has clearly been depressed for fish reared at lower oxygen saturations. This is evident when comparing results from lumpfish reared at 96% which increased their body mass with approximately 70.3% in contrast to the 8.8% increase in body mass displayed for lumpfish reared at 55% from October 14th to November 6th. Based on results and observations from this study, lumpfish should not be reared at oxygen saturation below 81% if good welfare is to be obtained. This is approximately the same level that is suggested for salmon (>80%) to avoid negative effects of

low oxygen saturation (Stien, et al., 2013). No welfare issues for lumpfish reared at oxygen saturations below 81% were observed, however below this oxygen saturation increased plasma cortisol levels and reduced feeding and thereby growth indicates reduced overall welfare.

Recommendations for lumpfish in aquaculture

The fish size, the temperature and the fish specie determine a fish´s tolerance and responses to oxygen saturation in water. Consequently, the results presented in this report are valid only for the size class and temperature tested. Reduced oxygen saturations were positively correlated with reduced growth, thus reduced growth was for lumpfish reared at 81% displayed. Therefore, if the goal is to achieve maximal growth, full oxygen saturation should be applied. Oxygen saturations below 81% reduce welfare in terms of initiation of the HPI-axis (fig 1), and by that the general stress response, thus oxygen saturations below 81% should if possible be avoided.

Conclusions

Juvenile lumpfish is very sensitive to reduced oxygen saturations and hypoxia. Compared to lumpfish held at 96% oxygen saturation negative consequences of reduced oxygen saturations in terms of significantly reduced growth and increased specific oxygen consumption are evident already at 81% oxygen saturation. The negative effects of reduced oxygen saturation increased for groups held at 69% and 55% saturation to the extent that the 55% groups had to be terminated after approximately one month.

For juvenile lumpfish oxygen saturation at 81 – 96% led to 43 – 120% higher specific growth rate and 28 - 85% higher final body mass compared to juvenile lumpfish reared at 69% oxygen saturation.

Although cortisol levels are low for all treatment groups, and at second sampling during the long-term experiment not even significantly different between fish reared at 69% and 96% oxygen saturation, standard error indicates large individual differences in each of the groups. Whether the cortisol levels alone have caused the depressed growth rate for lumpfish reared at 81% oxygen saturation is unlikely since the levels are so low. Reduced growth rate is probably an effect of a combination of the following; reduced feeding, increased costs related to ventilation response and increased cortisol levels. Due to low growth rate and higher plasma cortisol levels it seems like lumpfish is under chronic stress, and not able to restore homeostasis at oxygen saturations below 69%.

To sum up; lumpfish is highly sensitive to reduced water oxygen saturations and show low ability to acclimate to lower saturations over time (here 57 days).

Appendix I

Long-term exposure to reduced oxygen saturations

Experimental conditions

Temperature

Appendix I, table I. Descriptive statistics based on daily temperature measurements in all tanks. Mean, total number of observations (n), standard deviation (S.D.), standard error (S.E.), minimum (Min.), and maximum (Max.) are included in the table.

		Temperature	(°C)			
Treatment	**Mean**	**n**	**S.D.**	**S.E.**	**Min.**	**Max.**
55%	10.28	23	0.19	0.039	9.8	10.6
69%	10.19	57	0.34	0.045	9.2	11
81%	10.11	57	0.34	0.044	9.2	11
96%	10.09	57	0.33	0.043	9.2	10.9

Since the water in tanks with oxygen saturation at 51% and 69% spent more time in the tanks than the tanks that on average had oxygen saturation at 81% and 96%, this caused respectively 0,13°C higher temperatures but this is assumed to have little or no effects on growth or stress.

Oxygen saturation

Appendix I, table II. Descriptive statistics based on daily oxygen saturation measurements in all tanks. Mean, total number of observations (n), standard deviation (±S.D.), standard error (±S.E.), minimum (Min.), and maximum (Max.) are included in the table.

Oxygen Saturation (%)							
Replicate	**Intended Treatment**	**Actual treatment (mean)**	**n**	**S.D.**	**S.E.**	**Min.**	**Max.**
55%	40 %	54.50 %	23	5.47	1.14	45	69
55%	40 %	53.40 %	23	5.25	1.09	41	64
55%	40 %	55.90 %	23	4.86	1.01	45	66
69%	60 %	68.50 %	57	4.09	0.54	55	79.5
69%	60 %	68.30 %	57	4.33	0.57	58	80
69%	60 %	70.60 %	57	4.31	0.57	63	84
81%	80 %	80 %	57	3.94	0.52	70	87
81%	80 %	80.70 %	57	3.69	0.48	72	88.5
81%	80 %	83.30 %	57	3.16	0.41	74	90.5
96%	100 %	94.90 %	57	2.76	0.36	84	100
96%	100 %	95.70 %	57	2.46	0.32	85	100
96%3	100 %	95.90 %	57	2.06	0.27	86	101

Appendix I, table III. Water flow adjustment during the experiment. L/min.

Date	55%	55%	55%	69%	69%	69%	81%	81%	81%	96%	96%	96%
15.10.14	0.7	0.7	0.7	1.2	1.2	1.2	1.9	1.9	1.9	6	6	6
16.10.14	0.6	0.6	0.6	1.1	1.1	1.1	1.8	1.8	1.8	6	6	6
21.10.14	**0.5**	**0.5**	**0.5**	**0.9**	**0.9**	**0.9**	**1.7**	**1.7**	**1.7**	**6**	**6**	**6**

Body mass and length

In each of the tanks there were 15 lumpfish that were pit-tagged. Three times during the experiment (October 14th, November 6th, and December 10th) length and body mass were measured.

Appendix I, table IV. Descriptive statistics of body mass measurements at 14.10.2014, 06.11.2014 and 10.12.2014. Mass means (g), standard deviation (±S.D.) and standard error (±S.E.) for all groups are included in the table.

Treatment	Mass (g) October			Mass (g) November			Mass (g) December		
	Mean	**S.D.**	**S.E.**	**Mean**	**S.D.**	**S.E.**	**Mean**	**S.D.**	**S.E.**
55%	24.5	4.6	1.2	25.0	4.2	1.1	-	-	-
55%	24.5	3.4	0.9	28.4	4.0	1.0	-	-	-
55%	24.1	4.3	1.1	26.0	4.0	1.0	-	-	-
69%	24.1	4.0	1.0	32.9	4.9	1.3	40.2	7.8	2.0
69%	25.8	4.6	1.2	36.4	6.0	1.6	46.3	10.4	2.7
69%	23.2	3.0	0.8	34.2	6.3	1.6	41.2	9.3	2.4
81%	24.0	3.9	1.0	37.0	6.7	1.7	53.9	12.0	3.1
81%	24.3	5.0	1.3	37.1	8.2	2.1	51.5	14.5	3.8
81%	25.3	5.4	1.4	39.6	9.0	2.3	58.8	18.1	4.7
96%	23.5	4.3	1.1	42.5	6.9	1.8	87.9	12.0	3.1
96%	22.3	3.6	0.9	34.3	7.6	2.1	68.6	20.7	5.7
96%	23.0	2.9	0.8	40.5	4.7	1.2	79.9	12.1	3.1

Appendix I, table V. Descriptive statistics for length measurements at 14.10.2014, 06.11.2014 and 10.12.2014. Length means (cm), standard deviation (±S.D.) and standard error (±S.E.) are included in the table.

Treatment	Length (cm) October			Length (cm) November			Length (cm) December		
	Mean	S.D.	S.E.	Mean	S.D.	S.E.	Mean	S.D.	S.E.
55%	7.2	0.4	0.1	7.6	0.5	0.1			
55%	7.3	0.5	0.1	8.0	0.4	0.1			
55%	7.1	0.5	0.1	7.9	0.4	0.1			
69%	7.2	0.4	0.1	8.4	0.5	0.1	9.6	0.6	0.2
69%	7.3	0.4	0.1	8.7	0.5	0.1	9.7	0.7	0.2
69%	6.9	0.4	0.1	8.4	0.5	0.1	9.3	0.7	0.2
81%	7.0	0.4	0.1	8.7	0.5	0.1	10.2	0.7	0.2
81%	7.2	0.6	0.1	8.5	0.7	0.2	9.8	1.0	0.3
81%	7.2	0.6	0.1	8.7	0.8	0.2	10.2	1.1	0.3
96%	7.1	0.5	0.1	8.9	0.6	0.2	11.7	0.7	0.2
96%	7.1	0.5	0.1	8.6	0.8	0.2	10.8	1.3	0.3
96%	7.0	0.4	0.1	8.9	0.4	0.1	11.3	0.5	0.1

Appendix I, table VI. Descriptive statistics for SGR (% day^{-1}) for 14.10.2014 – 06.11.2014, 06.11.2014 – 10.12.2014 and overall 14.10.2014 – 10.12.2014. Mean SGR (% day^{-1})

	Mean SGR (% day^{-1})		
Oxygen saturation	Oct - Nov	Nov - Dec	Oct – Dec
55%	0.4		
69%	1.5	0.6	1.0
81%	1.9	1.1	1.4
96%	2.3	2.1	2.2

Appendix I, table VII. Descriptive statistics for overall SGR (% day^{-1}) for all replicates

Mean SGR (% day^{-1}) for all replicates		
Oxygen saturation	SGR % day-1	
55 %	0.10	* terminated in Nov
55 %	0.65	* terminated in Nov
55 %	0.33	* terminated in Nov
69 %	0.90	
69 %	1.02	
69 %	1.01	
81 %	1.42	
81 %	1.32	
81 %	1.48	
96 %	2.31	
96 %	1.97	
96 %	2.18	

Ventilation frequency

Appendix I, table VIII. Descriptive statistics for ventilation frequency, including means of 5 fish in each replicate, the number is operculum beats per minute. In tank 240, which held one of the 96% replicates there was not possible to count ventilation beats at October 24th or November 6th due to high activity level.

Treatment group	October 24th	November 6th	December 10th
55 %	80	78	terminated
55 %	77	75	terminated
55 %	73	76	terminated
69 %	68	73	74
69 %	56	70	76
69 %	68	71	72
81 %	45	64	64
81 %	53	68	61
81 %	52	61	63
96 %	47	44	46
96 %	-	-	36
96 %	49	45	49

Oxygen consumption

Appendix I, table IX. Mean oxygen consumption for each of the replicates on November 6th, for mean oxygen saturation from October 14th – November 6th. Mb is total body mass in the tank, and flow is inlet flow rate l/min.

Saturation	Flow	Mb	Specific O2 consumption
%	**l/min**	**g**	**mg/kg*min**
54.5	0.5	**913**	2.3
53.4	0.5	**995**	2.1
55.9	0.5	**934**	2.2
68.5	0.9	1214	2.1
68.3	0.9	1333	1.9
70.6	0.9	1251	1.9
80.1	1.7	1403	1.9
80.8	1.7	1369	1.9
83.3	1.7	1416	1.6
94.9	6	1521	1.4
95.7	6	1158	1.7
95.9	6	1468	1.2

Appendix I, table X. Mean oxygen consumption for each replicate on December 10th for average oxygen saturation from November 6th to December 10th. Mb is total body mass in the tank, and flow is inlet flow rate l/min.

Saturation	Flow	Mb	Specific O2 consumption
%	**l/min**	**g**	**mg/kg*min**
68.5	0.9	1313	2.0
67.6	0.9	1443	1.9
70.0	0.9	1360	1.8
78.4	1.7	1722	2.0
78.4	1.7	1668	2.0
82.0	1.7	1908	1.5
94.0	6	2631	1.3
95.3	6	1929	1.4
95.4	6	2550	1.0

Cortisol

Appendix I, table XI. Descriptive statistics for plasma cortisol levels (ng/mL) at different oxygen saturation (treatment) including mean, standard deviation (±S.D.), standard error (±S.E.), max and min.

Plasma cortisol levels in ng/mL - November 6th 2014					
Treatment	**Mean**	**S.D.**	**S.E**	**Max.**	**Min.**
55 %	7.79	2.52	1.26	10.76	5.33
55 %	28.37	11.72	5.86	38.74	13.87
55 %	31.07	15.88	7.94	52.29	13.87
69 %	20.43	16.26	7.27	38.30	1.64
69 %	15.48	14.37	7.18	30.06	2.40
69 %	30.56	9.83	4.91	42.49	20.07
81 %	9.61	11.17	6.45	22.27	1.11
81 %	9.85	7.44	3.72	20.07	2.22
81 %	9.98	11.82	5.91	26.70	0.24
96 %	2.96	3.08	1.54	7.53	0.91
96 %	8.45	10.12	5.06	23.59	2.43
96 %	6.32	5.14	2.57	13.18	0.97

Appendix I, table XII. Descriptive statistics for plasma cortisol levels (ng/mL) at different oxygen saturations (treatment) including mean, standard deviation (±S.D.), standard error (±S.E.), max and min.

Plasma cortisol levels in ng/mL – December 10th 2014					
Treatment	**Mean**	**S.D.**	**S.E**	**Max.**	**Min.**
69 %	7.52	7.30	3.65	18.32	2.42
69 %	6.41	6.86	3.43	16.67	2.36
69 %	15.37	8.56	4.28	24.30	7.85
81 %	2.81	1.92	1.11	5.02	1.51
81 %	1.15	0.33	0.17	1.55	0.80
81 %	3.94	1.76	0.88	6.35	2.48
96 %	12.63	11.12	5.56	26.08	1.44
96 %	3.07	3.99	2.00	9.04	0.66
96 %	9.01	14.65	7.33	30.92	0.18

ANOVA

Appendix I, table XIII. Test results from one – way ANOVA for individual body mass data at the first sampling in October.

One-way Analysis of Variance. Body mass, October.					
Source	**Type III SS**	**df**	**Mean Squares**	**F-Ratio**	**p-Value**
SATURAT$	66.127	3	22.042	1.306	0.274
Error	2 937.822	174	16.884		

Appendix I, table XIV. Test results from one-way ANOVA for individual body mass data at the second sampling in November.

One-way Analysis of Variance. Body mass, November.					
Source	**Type III SS**	**df**	**Mean Squares**	**F-Ratio**	**p-Value**
SATURAT$	4 420.850	3	1 473.617	35.451	0.000
Error	7 232.809	174	41.568		

Appendix I, table XV. Test results from one-way ANOVA for individual body-mass data at the third sampling in December.

One-way Analysis of Variance. Body mass, December.					
Source	**Type III SS**	**df**	**Mean Squares**	**F-Ratio**	**p-Value**
SATURAT$	30 684.616	2	15 342.308	78.856	0.000
Error	25 098.316	129	194.561		

More statistics (ANOVA & Tukey´s Honestly – Significant – Difference – Test) available at request.

Appendix II

Short-term acute stress experiment

Body mass

Appendix II, table XVI. Descriptive statistics for mean body mass including mean, standard deviation (±S.D.) and standard error (±S.E.).

Body mass (g)			
	Mean	**SD**	**SE**
0 min	43.15	3.96	1.62
30 min	46.70	5.14	2.10
60 min	42.23	4.46	1.82
120 min	42.65	3.94	1.61

Cortisol

Appendix II, table XVII. Descriptive statistics for plasma cortisol levels (ng/ml) at acute stress exposure (hypoxia 60-30% oxygen saturation) and handling including mean, standard deviation (±S.D.) and standard error (±S.E).

Plasma cortisol - ng/ml			
	Mean	**SD**	**SE**
Control (0 min)	0.97	1.22	0.50
30 min	33.31	16.58	6.77
60 min	45.87	6.04	2.47
120 min	47.08	4.05	1.65

Appendix III

Cortisol ELISA

Protocol created March 2014 by Erin Faught and is based on (Yeh, Glock, & Ryu, 2013).

1. Ensure that all buffers are made before you start

Coating the plate

2. Make the coating buffer (1.6 µg/ml of mAB). First make a 40 µg stock from the 4 µl aliquots in of mAB in the -80, then dilute to µg/ml:
   i. Thaw 4µl stock on ice (10 min)
   ii. Add 431 µl to the 4µl stock (40 µg/ml stock)
   iii. In a multichannel pipette tray add 9.6 ml of 1 x PBS
   iv. Add 400 µl of your 40 µg/ml stock to the tray (1.6 µg/ml coating). ***Mix well*** – *do this by rocking the plate back and forth several times and pipetting up and down with the multichannel pipette. If you do not mix well you will not evenly coat your plate and it will be unusable.*
3. Add 100 µl of you mAB coating into each well of a 96-Well-Plate (Immulon 2 HB), using the multichannel pipette.
4. Cover the plate with parafilm and incubate at 4^{o}C for 16 hours without shaking.

Blocking

5. Remove the coating using the multichannel pipette. Store in a 15 ml falcon tube in the – 20 for later use with the label "Cortisol mAB (East Coast Bio) - 1.6 µg/ml – your Date and Initials"
   Note: DO NOT let the plate dry out – it can wash while you put away antibody
6. Wash 3 x with 300 µl 1 x PBS-T
   Use the plate washer. Protocol: "erin"
7. Add 300 µl of blocking buffer into each well (make fresh: 0.1% BSA in 1 x PBS; see buffer recipies)
8. Incubate for 1h at room temperature without shaking
9. Wash 3 x with 300 µl 1x PBS-T

Detection

10. While blocking, prepare samples and standards, cortisol-HRP
    i. Samples: dilute in 0.1% BSA in 1 x PBS (blocking buffer)
    ii. Standards: 10µg/ml stocks (make 100 ng/ml stock and add 10 µl of 10 µg/ml to 990 1 x PBS)

iii. Cortisol-HRP (1:20): Add 3.25 µl into 61.75 µl 1 x PBS

Standard (ng/ml)	Amount of stock (µl)	1 x PBS (µl)	Stock source (ng/ml	Total (µl)
25	30	90	100	120
10	12	108	100	120
5	6	114	100	120
3	3.6	116.4	100	120
2	2.4	117.6	100	120
1	10 µl	990 µl	100	120
0.5	60	60	1	120
0	---	120	1	120

11. Add 50 µl standards and samples, in duplicate to respective wells of your coated/blocked plate.
12. Further dilute your cortisol-HRP (the competitive cortisol) stock (final in the tray 1:160):
    i. Add 62.4 µl into 4,937.6 µl of PBS
13. Add 50 µl Cortisol-HRP into each well of the plate.

Note: Steps 11-13 need to be completed as quickly as possible to avoid drying your plate.

14. Incubate for 2 hours at room temperature on a shaker.
15. Prepare detection solution 10 minutes before the end of the incubation period.
16. Wash 3 x with 300 µl 1 x PBS.
17. Add 100 µl detection solution into each well, and cover the plate with foil.
18. Incubate for 25 minutes on a shaker at room temperature.
19. Stop reaction by adding 100 µl stop solution into each well.
20. Read absorbance at 450 nm in the plate reader.

Preparation of solutions and buffers

10 x PBS (Phosphate buffered saline = antibody) (pH 7.4) – 1L		
NaCl	1.37 M	80g
KCl	27 mM	2.01g
KH_2PO_4 (Potassium phosphate, monobasic)	18 mM	2.45g
$NaHPO_4$-$12H_2O$ (Sodium phosphate, dibasic)	100 mM	35.8g

Measure out 800 ml to a breaker, add all ingredients, and stir until dissolved. Addjsut pH to 7.4 with conc. HCl. Bring to 1000 ml with analytical grade water in a graduated cylinder. Store at room temperature.

1 x PBS - 500 ml	
10 x PBS	50 ml

Bring volume with analytical grade water in a graduated cylinder. Store at room temperature.

1 x PBS- T (0.05%) - 500 ml	
10 x PBS	50 ml
Tween 20	500 µl

Bring volume to analytical grade water in a graduated cylinder. Add tween 20 and pipette up and down several times to wash out the pipette tip. Store at room temperature.

Blocking buffer (0.1% BSA) - 30ml	
BSA	30 mg

Add BSA to 30 ml of 1 x PBS. Do not stir, let it dissolve, this takes about 10 minutes.

Staining Solution A - 10ml		
TMB	41 mM	98.54 mg
TBABH	8 mM	20.58 mg

Measure TBABH in the fume hood – it is extremely dangerous! Dissolve both chemicals in 10 ml of DMA. Stor in a 15 ml falcon tube wrapped in tin foil at 4^{o}C.

Staining Solution B (pH 4.0) - 500 ml		
Potassium Citrate	250 mM	33.25 g

Measure out 350 ml to a breaker, add all ingredients, and stir until dissolved. Adjust pH to 4.0 with conc. HCl. Bring to 500 ml with analytical grade water in a graduated cylinder. Store at 4^{o}C – good for 1 month.

Stop Solution (1M Sulphuric Acid) – 500 ml		
H_2SO_4	1 M	26.65 ml

Using a glass graduated pipette, measure out the required amount of sulphuric acid. Add 473.35 ml of analytical grade water. Store in a glass bottle at room temperature.

Detection Solution

Mix 10 ml of staining solution B with 250 µl of staining solution A and 3.14 H_2O_2. Leave for at least 10 minutes before use.

Appendix IV

Lumpfish details.

Appendix IV, Table XVIII. Family name and hatching date for lumpfish included in the experiment.

Family name	n	Hatching date
R1-TMY-2014	32	May 14th
R2-TMY-2014	118	May 15th
R3-TMY-2014	99	May 14th
R8-TMY-2014	6	May 28th
R10-TMY-2014	127	May 15th

There were 62 lumpfish that were not from either of these families, and were retrieved from a surplus tank.

Feeding

The lumpfish were fed with Amber Neptune size 2.0 mm from Skretting AS.

Feed calculations were based on a daily growth rate at 3%. Due to low feed intake and quick development of turbidity in the water, feeding were reduced from 3% daily gain to 1% daily weight gain for the replicates reared at 55% oxygen saturation and from 3% to 2% daily weight gain for the replicates reared at 69% oxygen saturation one week into the experiment.

Appendix IV, Table XVX. Description of planned feeding set up by CMA.

Week	Tabell	FCR	Mean		Biomass	Feed		Biomasse	g/feed
	Growth rate (%)		Body m. (g)	n	g	g /24 h	kg/week	kg/m^3	
1	3.00	1.5	22.70	37	839.9	37.8	0.3	6.32	1.6
2	3.00	1.5	27.92	37	1033.0	46.5	0.3	7.77	1.9
3	3.00	1.5	34.34	37	1270.4	57.2	0.4	9.55	2.4
4	3.00	1.5	42,23	32	1351.3	60.8	0.4	10.16	2.5
5	3.00	1.5	51.94	32	1662.0	74.8	0.5	12.50	3.1
6	3.00	1.5	63.87	32	2044.0	92.0	0.6	15.37	3,8
7	3.00	1.5	78.56	32	2513.8	113.1	0.8	18.90	4.7
8	3.00	1.5	96.62	32	3091.7	139.1	1.0	23.25	5.8
9	3.00	1.5	118.83	32	3802.4	171.1	1.2	28.59	7.1

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