Temperature and size-dependency of lumpfish (*Cyclopterus lumpus*) oxygen requirement and tolerance

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Abstract

Lumpfish are currently produced and utilizes as cleaner fish to control sea lice infestation rates in salmon net pens, but information on environmental requirements is still limited. This study aimed to determine the zone of environmental hypoxia for two relevant fish sizes (15 and 60 g) and temperatures (5 and 12°C), using intermittent flow respirometry (referred to as 15:5, 15:12, 60:5, 60:12), and to investigate parameters of stress in response to acute changes in dissolved oxygen (DO, % air saturation) from normoxia to 47, 63, 98 (control), 148 and 194 % O_2 at 10 °C. The standard and maximal metabolic rates (SMR and MMR) were measured in normoxia (n=8), and MMR was measured at 5 – 6 DO levels ranging from 20 – 160 % O_2 (n=8 per DO) to define the upper and lower boundaries of the hypoxic zone (DO_{lim} and DO_{crit}). SMR, MMR and the aerobic metabolic scope (AS) increased with temperature and decreased with fish size. Similar effects of temperature and size were found on DO_{crit} – DO_{lim} ranges: 20 – 55 (15:5), 35 – 147 (15:12), 21 – 53 (60:5) and 22 – 89 (60:12) % O_2 air saturation. Results from acute exposure tests resulted in elevated cortisol levels at 63 and 47% O_2, although not statistically significant at 47% O_2. Other parameters of hypoxic or hyperoxic stress (lactate, pH, osmolality, lipid peroxidation rates, catalase activity) were not affected. Results from the present study suggest that lumpfish may experience oxygen levels in sea cages that restricts metabolism, performance and induce hypoxic stress.

1. Introduction

Control over sea lice (*Lepeophtheirus salmonis* and *Caligus* sp.) infestation rates has become an increasing challenge for salmon aquaculture worldwide. Cleaner represent a biological means of control, and lumpfish (*Cyclopterus lumpus*) is widely used due to its tolerance for low temperatures, the successful up-scaling of juvenile production, and reports of lice gracing (Imsland et al., 2014 a,b,c; 2015 a,b; 2016). The commercial production in Norway has grown fast, reaching 38 million individuals in 2019 (Norwegian Directorate of Fisheries, 2020). A challenge for the production and use of lumpfish is that mortality rates after transfer to salmon sea cages is high (Powell et al., 2018). Infectious diseases, skin lesions caused by handling, sea lice (*Caligus* sp.), sub-optimal feeds, husbandry practices and sea cage environment are suggested as factors thought to affect the mortality rates (Norwegian Veterinary Institute, 2020).

A prerequisite for lumpfish health and function, is that environmental factors are within suitable ranges for this species. This study focuses on environmental oxygen (DO; dissolved oxygen), the most important limiting factor of fish metabolism (Fry 1947;1971). Studies have shown that this environmental factor may vary substantially in salmon sea cages (Oppedal et al. 2011; Stien et al. 2012; Oldham et al. 2018) and in lumpfish transport (Remen and Jonassen2017). Reduced DO in salmon net pens surrounded by lice skirts may reduce welfare status and subsequently, the delousing potential of cleaner fish (Bui et al., 2020). Information on suitable DO levels for lumpfish is however limited. A study by Jørgensen et al. (2017) suggests that lumpfish require at least 80 % O_2 to maintain growth rates at 10°C, and observed moderate increases in plasma cortisol levels at 69 and 55% O_2.

A study performed with progressive hypoxia, registered higher plasma cortisol levels as DO was reduced to 20% O_2, but low levels of plasma lactate (Hvas and Oppedal 2019). More information on oxygen requirements and hypoxia tolerance is necessary to provide practical guidelines for aquaculturists.

The DO required to fulfil the oxygen demand of fish, depends on factors affecting aerobic metabolism, and the capacity of ventilatory and circulatory systems to provide tissues with oxygen (Pörtner 2010). It is well established that the metabolic rate (MR) of teleosts declines exponentially with increasing fish size (Brett and Groves 1979, Jobling 1994), and that temperature controls metabolism (Fry 1971; Brett and Groves, 1979; Farrell 2009). With increasing temperature, the standard metabolic rate (SMR) increases, while the maximum metabolic rate (MMR) increases and then plateaus with further increase. The temperature where the difference between MMR og SMR, termed the aerobic scope (AS), is maximized, is termed the thermal optimum (T_{opt}), while the upper and lower limits of the thermal niche are defined as temperatures where MMR=SMR and AS=0 (Fry 1947;1971; Farrell 2009).

The aerobic scope represents the capacity to perform any activity beyond basic life-supporting functions, and is widely used as a framework to assess ecological effects of environmental factors (Fry 1947;1971; Claireaux and Lefrançois 2007; Pörtner and Farrell 2008). Although the use of this framework to explain mechanisms of climate change impacts on water-breathers has been criticized (see Claireaux and Chabot 2016; Jutfelt 2021, and references therein), it is widely used to assess oxygen effects on marine teleosts (e.g., Claireaux et al. 2000; Claireaux and Lagardère 1999; Lefrancois and Claireaux 2003; Seibel and Deutch 2020). The DO range where AS progressively falls towards zero is

67 termed the zone of environmental hypoxia (Farrell and Richards 2009). The upper
68 boundary of this zone, the incipient limiting DO level (DO_{lim}), represents the DO where
69 MMR is first limited. The lower boundary, the critical DO level (DO_{crit}) represents the DO
70 where MMR is reduced to the level of SMR and AS = 0. Survival below DO_{crit} is time-
71 limited (reviewed by Claireaux and Chabot 2016). Knowledge of the zone of
72 environmental hypoxia provides a basis for defining hypoxia severity during a given drop
73 in DO (Seibel and Deutch 2020; Seibel et al. 2021).

74 In an aquaculture setting, the metabolic rate is expected to vary at an intermediate
75 level between SMR and MMR, as a result of varying activity level (termed the routine
76 metabolic rate, RMR, Neill and Bryan 1991). The DO required to support RMR is termed
77 the limiting oxygen concentration (LOC), and represents a linear (Seibel and Deutch 2020;
78 Seibel et al. 2021) or curvilinear (Claireaux and Chabot 2016) continuum, ranging from
79 DO_{lim} to DO_{crit}, depending on the level of RMR (Neill and Bryan, 1991).

80 At present, there is no consensus on the succession of effects which occurs as DO
81 declines from DO_{lim} to DO_{crit}, for a fish with a given RMR. Claireaux and Chabot (2016)
82 propose that oxyregulation (increased gill ventilation and perfusion, and increased
83 perfusion of critical tissues) upholds RMR as DO declines from DO_{lim} to LOC, and that
84 extraneous metabolic costs (feeding, swimming, digestion) are gradually suppressed with
85 reductions in DO below LOC. In a recent review, Jutfelt (2021) suggest that feed intake is
86 reduced at DO levels higher than LOC, to conserve aerobic scope for other activites than
87 food digestion and assimiliation. In post-smolt Atlantic salmon (*Salmo salar*), negative
88 effects on appetite have been observed at DO higher than LOC, and further reductions in

appetite, along with increases in plasma cortisol and lactate levels, occur at DO levels closer to the LOC (Remen et al. 2012; Remen et al. 2016).

The main aim of this study was to establish practical oxygen guidelines for this new aquaculture species, by determining metabolic oxygen requirements, the zone of environmental hypoxia, and the physiological responses to acute changes in DO. Investigations were performed with relevant fish sizes (15 and 60 g) and temperatures (5 and 12 °C) for lumpfish production and use in net pens. DO_{lim} and DO_{crit} were defined on basis of respiratory measurements of SMR and MMR, which also yields information of temperature, size and oxygen effects on the aerobic scope of this species.

2. Materials and methods

2.1. Experimental animals

Juvenile lumpfish of wild origin (wild caught broodstock caught near Hekkingen, N 69.37 E 17.48), produced at a commercial production facility (Senja Akvakultursenter, Senja, Norway) were used for the experiments. 300 juveniles (average weight 5.7 g) were transported on truck on 17 January 2018, and acclimated to 3 m^3 holding tanks at Research and Innovation Station Kraknes (Akvaplan-niva AS, Tromsø; Norway). Fish were kept at ambient temperature (4.1 – 4.6°C), 92 – 100% O_2, continuous lighting and excess, continuous feeding (Gemma Diamond, Skretting, Norway) for 13 days. After this, the temperature was gradually increased from ambient temperature to 8°C by 31 January, to increase fish growth rates, with the use of an inline heating system. Dissolved oxygen levels were between 85 and 107% of air saturation (tank outlet). This resulted in a doubling of fish size before experiment start-up on 21 February 2018.

2.2 Experimental set-up and protocol

The experiment started on 21 February 2018 (day 1). On this day, fish were lightly sedated (Benzoak, 20 mg/l), individually weighed (average 13 g) and split into three 3000 l holding tanks with temperature 8.6 – 9.6°C. Every 14 – 21 days, 50 fish per tank were lightly sedated and individually weighed, to estimate fish growth rates and average weights. SMR and MMR measurements were planned when the development in average weights showed that fish were approaching the desired sizes (15 and 60 g). SMR and MMR

122 measurements with 15 g fish were performed 10 – 22 days after experiment start-up, and
123 SMR and MMR measurements with 60 g fish were performed 55 – 71 days after
124 experiment start-up. This difference in sojourn time between size groups is a potential
125 confounding factor, which is difficult to avoid when testing different fish of different sizes.
126 The effect was minimized by maintaining stable conditions in the holding tank, and
127 allowing for complete temperature acclimation in all tanks (8 – 9.6 °C for a minimum of 4
128 weeks; Clark et al. 2013). For each combination of temperature and fish size, a total of 48
129 fish were used for SMR and MMR tests, and subsequently killed, with n = 8 for SMR and
130 n = 40 for MMR (n = 8 per level of DO). When fish approached the desired size, 48
131 individuals with weights close to 15 or 60 g were chosen from the three holding tanks
132 (16+16+16 fish), and transferred to a fourth, similar tank, where temperature was gradually
133 changed (1 – 2 °C day^{-1}), to achieve at least 7 days of acclimation at a temperature close to
134 the desired experimental temperatures (5 and 12 °C). After this, SMR and MMR were
135 measured (see 2.3).
136 Ideally, fish should be split into two acclimation tanks to incorporate a possible tank
137 effect in statistical analyses. There was only one tank available for thermal acclimation,
138 and as all treatment groups were of same origin, came from the same holding tanks, and
139 were acclimated in the same holding tank (at different time), we considered this an
140 acceptable limitation of the experimental design. Unfortunately, the inline heating system
141 was not able to maintain acclimation tank temperatures higher than 9 – 9.5 °C at this time
142 of year, during acclimation of the 12 ºC groups. This was however possible in the smaller
143 tank housing the four respirometers, resulting in a temperature increase of ~3 °C at transfer
144 to the respirometers. Potential implications of this are discussed in the Discussion section.

145 At the end of the respiratory experiments (day 70), 50 of the remaining fish (~60 g) were
146 transferred to 10 smaller tanks (245 l), with no feeding, continuous lighting in the lid and
147 9.6 °C water temperature. After 36 – 42 hours acclimation to the tanks, fish were subjected
148 to 5 different, acute changes in DO (47, 63, 98, 148 and 194 % O_2), with two replicate
149 tanks per DO treatment, to measure parameters of hypoxic and hyperoxic stress (see 2.4).

150 **2.3. Respirometry**

151 To achieve an estimate of DO_{crit} and DO_{lim}, SMR was measured in 8 individuals at DO
152 close to air saturation (average 104% O_2), and MMR was measured in 8 individuals at each
153 level of DO: on average 20, 44, 68, 100 and 142% of air saturation (see Table 1). The
154 procedure was repeated 4 times, one per combination of fish size (15 and 60 g) and
155 temperature (5 and 12 °C). In the following, these combinations are referred to as 15:5,
156 15:12, 60:5 and 60:12 (Table 1).

157 *2.3.1. Respirometry set-up*

158 SMR and MMR were measured in four individuals simultaneously, placed in four
159 similar, custom-made intermittent-flow respirometers, submerged in a common holding
160 tank (110 l), according to Rosewarne et al. (2016). Each respirometer was a plexiglass
161 cylindrical chamber (ID 9.1 cm, internal length 18 cm) with lid, 2 inlets and 2 outlets
162 (produced by Plexon, Skallestad, Norway), one flush pump and one recirculation pump
163 (Eheim, 5 l min^{-1}, Loligo Systems, Viborg, Denmark), toxic-free PVC tubes (ID 13 mm,
164 Loligo Systems, Viborg, Denmark), a fiber-optic oxygen sensing probe (OXROB3-CL4,
165 coupled to FireStingO2, Pyro Science GmbH, Aachen, Germany) installed in the
166 recirculation loop using a t-connector, a backwater valve on the flushing outlet to avoid
167 water exchange during closed respirometry, and an external temperature probe placed in

168 the holding tank (TSUB21, Pyro Science GmbH, Aachen, Germany connected to the
169 FireSting oxygen meter). The chambers had a total volume of 1.25 l, including tubing in
170 the recirculation loop. In both ends of the chamber, a perforated (3 mm, Ø 7.5 cm) and a
171 non-perforated (1.5 mm, Ø 5 cm) plate was installed in the center to break down the water
172 current within the chamber, and to allow for lumpfish attachment without blocking inlets
173 or outlets. Flush pumps were automatically controlled, using PC-controlled mains switches
174 (USB-Swicth 3, Cleware GmbH, Hollingstedt, Germany). All DO and temperature
175 measurements were logged using the FireStingO2 software, and MO_2 was calculated as
176 described below (2.3.4). The four intermittent flow respirometers were submerged in a tank
177 (110 l) with UV-treated inlet water, and controlled temperature and DO. Temperature was
178 controlled automatically, using an inline heating/cooling system installed in the header
179 tank. Desired DO was achieved by manually controlled addition of O_2 or N_2 using ceramic
180 diffusors in the header tank. DO measurements in the tank housing the respirometers were
181 performed with Oxyguard Handy Polaris TGP. The flow through rate in the respirometer
182 holding tank was 20 l/min, maintaining optimal water quality throughout SMR
183 measurements (DO > 100% of air saturation). Possible leaks in the respirometer chambers
184 were tested with 14% O_2 saturation in chambers submerged in 100% O_2 water for 18 hours,
185 during which DO did not increase, but rather declined slightly due to background
186 respiration. Background respiration was measured during 2 hours at 5 and 12°C, and
187 considered negligible for measurements with 60 g fish (< 1% of total O_2 consumption), but
188 large enough to affect results for 15 g fish (2 – 7% of total O_2 consumption, see 2.3.4.).
189
190 *2.3.2. SMR measurement*

SMR was measured in 8 individuals per combination of temperature and fish size. 36 hours before respirometry, 4 fish of desired size were netted out of the holding tank and transferred to a smaller tank (245 l) with similar temperature, lid, no lighting or feeding. This, and the following procedure, was repeated consecutively, to achieve 8 individuals per combination of temperature and fish size. After 36 hours, fish were placed in the four intermittent flow- respirometers at either 5 or 12 °C. The lights in the room were turned off, and the fish were left undisturbed for 65 – 72 hours. During this period, cycles of flushing and closed respirometry were set up as follows: 10/40 min (15:5 and 15:12), 20/40 min (60:5) and 20/10 min (60:12). The duration of the measurement phase was adjusted according to fish size and temperature, and was long enough to ensure $R^2 > 0,9$, and short enough to avoid drops in DO below 80% of air saturation. During the period of closed respirometry, the drop in % oxygen saturation was on average 3.9 percentage points (15:5), 9.6 points (15:12), 13.1 points (60:5) and 4.3 points (60:12), and minimum DO was 81 – 105 % O_2. The set-up allowed for 68 – 141 measurement periods per individual. At end of measurements, fish were taken out of the chamber, euthanized, and weights and lengths were recorded. Oxygen and temperature data from the FireStingO2 were downloaded for MO_2 calculation. Unfortunately, data from one measurement series is missing due to technical failure, thus only 4 individuals were measured for 60:12.

2.3.3. MMR measurement at variable DO

MMR was measured in 8 individuals for each combination of size, temperature and oxygen level (~ 145, 100, 70, 45, 20% O_2). The probe sampling rate was 0,2 measurements/s. Maximum metabolic rate was induced by 2-minute manual chasing of

214 individual fish (Ern et al. 2016), after which the fish were immediately transferred to the
215 respirometer for closed respirometry. The measurement period was set to 5 minutes to
216 enable sufficient O_2 reduction and $R^2 > 0,9$ at both temperatures and for both fish sizes.
217 For 60:5 and 60:12 measurements, 2 minutes measurements were achievable ($R^2 > 0,9$),
218 and compared to 5 minutes measurements. MMR was 18 % higher with 2 minutes
219 measurements. See Discussion section for further detail and discussion on MMR
220 methodology.

221 The estimate of normoxic MMR was based on data from 8 fish studied at DO between
222 102 – 162% (8 highest DO levels). These DO levels were higher than DO_{lim} for all groups
223 expect for the 15:12 group, where 4 measurements were performed at DO below DO_{lim}
224 (123 – 135% O_2, DO_{lim} =147% O_2). By excluding these 4 measurements, the MMR-
225 estimate was only slightly lower (0,1 mg O_2/kg/min eq. to 2 %). The effect of including
226 measurements at 123–135% O_2 was therefore considered negligible, and all results (n=8)
227 were included in figures and statistical tests.

228

229 *2.3.4. Calculations*

230 The oxygen consumption rate (MO_2, mg O_2 kg^{-1} min^{-1}) for each measurement period
231 was calculated based on the slope (K) of the linear decrease in DO (mg l^{-1}) over time (min)
232 within the chamber during closed respirometry, which occurs after the initial wait phase
233 (Rosewarne et al., 2016). The slope (K) was determined using simple regression analysis
234 (TIBCO Statsoft Statistica). Measurements were only considered valid if R^2 was higher
235 than 0,9. The MO_2 was calculated according to the equation

236

$$MO_2 = KVM^{-1} \quad (1)$$

where MO_2 is the oxygen consumption rate (mg O_2 kg^{-1} min^{-1}), K is the rate of DO decline (mg O_2 min^{-1}), V is the volume of the respirometer corrected for the volume of fish (l), and M is the body mass of the animal (kg; Svendsen et al., 2015).

Background respiration rates were considered negligible for measurements with 60 g fish (< 1% of total O_2 consumption), but large enough to affect results for 15 g fish (3,3 $\times 10^{-4}$ mg O_2 min^{-1} at 5 °C and 6,7 $\times 10^{-4}$ mg O_2 min^{-1} at 12°C). Rates were corrected for, using the equation

$$MO_{2\,\text{corrected}} = (K_1V_1 - K_2V_2)\, M^{-1} \quad (2)$$

where $MO_{2\,\text{corrected}}$ is fish MO_2 corrected for background respiration, K_1 and K_2 are the rates of decline (mg O_2 min^{-1}) in oxygen content over time in the respirometer during the measurement phase when the animal is present and absent, respectively, V_1 and V_2 are the respirometer volumes (l) when the animal is present and absent, respectively, and M is the body mass of the animal (kg).

For calculation of SMR per individual, the mean of measurements below the 20% quantile (n = 13 – 27) was used (Chabot et al. 2016).

DO_{lim} was determined using the "segmented" package in R 3.1.2 (The R Foundation for Statistical Computing© 2011, www.r-project.org, Muggeo et al. 2008). This method simultaneously estimates slope parameters and the turning point(s) within a standard linear model framework:

$$\psi = \alpha + \beta_1 x_i + \beta_2 (x_i - \psi)_+, \quad (3)$$

where ψ is the breakpoint DO (DO_{lim}), α is the intercept (MO_2, mg kg^{-1} min^{-1}), β_1 is the left slope, β_2 is the difference-in-slopes, x_i is DO (% of air saturation) and $(x_i - \psi)_+ = (x_i - \psi) \times I(x_i > \psi)$ and $I(\cdot)$ is the indicator function equal to one when the statement is true (Muggeo 2008). The model and the estimated DO_{lim} were only considered valid if the test for difference in regression line slopes for DO levels above and below the DO_{lim} returned p-values lower than 0,05 (Davies test; Muggeo, 2008).

DO_{crit} was defined as the DO where MMR was reduced to a level corresponding to the measured SMR for the given combination of temperature and fish size. This DO level was determined with the use of coefficients (α and β_1) from the break-point analysis (formula 3) and the SMR estimate, with the use of the following formula:

$$\theta \pm \delta\theta = \frac{\gamma - \alpha}{\beta 1} \pm \frac{\delta\gamma - \delta\alpha}{\beta 1} \quad (4)$$

where $\theta \pm \delta\theta$ is DO_{crit} (% air saturation) ± SE, γ is SMR (mg kg^{-1} min^{-1}), α is the intercept, β_1 is the slope, and $\delta\gamma$ and $\delta\alpha$ are the standard errors of SMR and intercept estimates, respectively.

The aerobic metabolic scope (AS, mg O_2 kg^{-1} min^{-1}) ± SE was calculated as follows:

$$AS \pm \delta AS = \varphi - \gamma \pm \sqrt{\delta\varphi^2 + \delta\gamma^2}, \quad (5)$$

where φ is MMR, γ is SMR, and $\delta\varphi$ are $\delta\gamma$ the standard errors of φ and γ, respectively.

283 The rate of change in SMR with increasing temperature, the temperature quotient (Q_{10}) was
284 calculated as:
285
286 $$Q_{10} = (R_2 / R_1)^{10/T2 - T1} \quad (6)$$
287
288 where R is SMR (mg kg^{-1} min^{-1}) and T is temperature (^{o}C).
289 **2.4 Acute hypoxia and hyperoxia experiment**
290 This experiment was performed at end of the study (day 70), using fish from the same
291 group (mean weights 51 – 65 g, see Table 2). After 36 – 42 hours acclimation to
292 experimental tanks (245 l tanks with lid, 9.6 °C, no feeding, continuous lighting), fish were
293 subjected to 5 different, acute changes in DO (47, 63, 98 [control], 148 and 194 % O_2), to
294 measure parameters of hypoxic and hyperoxic stress. There were two replicate tanks per
295 DO level, with 5 fish per tank.
296 The DO change was achieved by switching the tank inlet source from oxygen air-
297 saturated water, to DO manipulated inlet water. DO manipulation was performed in two
298 adjacent tanks, where DO was either reduced or increased by the addition of N_2 or O_2,
299 using ceramic diffusors, to achieve a stable, new level of DO with 30 l/min water flow.
300 Additional tubes and valves were set up to allow for switching between main inlet water
301 (air saturation) and water from the DO manipulation tanks. The switch resulted in a rapid
302 increase or decrease in DO (within 30 min) followed by a period with stable, new DO levels
303 in the tanks (49 – 66 min, see Table 2), after which fish were netted out for blood and liver
304 sampling. Exposure and sampling were performed sequentially in the different tanks over
305 a total period of 6 hours (98→47→148→63→194 % O_2). Care was to taken to achieve

similar exposure duration, and to avoid disturbance of fish within the tanks during the exposure and sampling period. For instance, lights in the room were off, lids were on until fish were sampled, and human activities were reduced to a minimum. At the end of each tank exposure period, 5 fish per tank were rapidly netted out, anesthetized with metomidate (15 mg l^{-1}) and blood was withdrawn from the heart using heparinized syringes. Fish were then killed by Finquel overdose (60 mg l^{-1}), weights and lengths were measured, and the liver was dissected out and weighed. A subsample was cut out, weighed and immediately frozen in liquid N_2 for later analysis of parameters of oxidative stress: catalase activity and lipid peroxidation rate (see 2.4.1). Blood samples were centrifuged (6 min, 6000 rpm), plasma was collected, immediately frozen in liquid N_2, and stored at -70°C for later analysis of parameters of hypoxic stress: plasma cortisol, osmolality, lactate and pH.

2.4.1 Plasma and liver analyses

Plasma samples were analyzed for cortisol concentration using ELISA kit (DEH 3388, Demeditec Diagnostics GmbH, Kiel, Germany), osmolality using 210 Micro Osmometer (Fiske® Assosicates, Massachusets, USA), and lactate and pH using ABL90 Flex analyser (Radiometer Medical ApS, Brønshøj, Denmark).

Liver samples were analysed for parameters of oxidative stress. Catalase activity was quantified using spectrophotometer, based on the measurement of the disappearance of hydrogen peroxyde with time at 240 nm (Livingstone et al., 1992). Lipid peroxidation rates were evaluated by measuring the amount of malondialdehyde (MDA) released (Buege and Aust, 1978). Liver samples were homogenized in phosphate buffer (50 mM, pH 7.0) in the ratio 1/5 of weight/volume and centrifuged at 12000 rpm for 15 minutes at 4°C. The

supernatants were gently pipetted out, transferred into two clean Eppendorf tubes and kept in -80°C until further analyses.. Total protein concentrations were determined using the method of Bradford (1976) with bovine serum albumin as a standard and Brilliant Blue G 250 as a reactant. The reaction was measured spectrophotometrically at 570 nm in microplate. For the catalase activity quantification, supernatants were diluted 5 times in ice cold phosphate buffer (50 mM and pH 7), before transfer to a quartz cuvette. H_2O_2 was added as a substrate immediately before reading with a spectrophotometer at 240 nm continuously for 1 min. For the lipid peroxidation bioassay, 200 µL sample was transferred into a glass reagent tube with 800 µL of the 15 % trichloroacetic / thiobarbituric acid buffer. The tubes were incubated in water bath at 100 C° for 15 minutes and the reaction stopped when the tubes were transferred on ice and maintained there to cool down. The mixture was transferred into Eppendorf tubes and centrifuged at 2500 rpm for 10 minutes at 4 C°. The supernatants were used for the bioassay, transferred into a cuvette and read with a UV/VIS spectrophotometer at 535 nm.

2.5. Statistical analyses

All statistical analyses were performed using TIBCO Statistica® 13.3.0. The interactive effects of temperature and fish size on lumpfish SMR and MMR were tested using full factorial ANOVA, with separate tests for the two different variables. For AS, DO_{lim} and DO_{crit}, such test were not possible (n=1). In this case, 95% confidence intervals were used to estimate statistically significant differences between groups (non-overlapping 95% CI). The effect of DO on plasma and liver parameters of hypoxic and hyperoxic stress were tested using one-way ANOVA, except plasma osmolality. For osmolality, the assumption

of homogeneity of variances was violated, also after log-transformation, and the non-parametric Kruskal-Wallis test was used to investigate the effect of DO. Plasma cortisol values were log-transformed to achieve normality and homogeneity of variances before the one-way ANOVA test. For all ANOVA test, assumptions of normality and homogeneity of variances were checked using p-plots and Levene's test (Brown and Forsythe 1974), respectively. Post-hoc analyses were performed using Newman Keuls test. The significance level (α) was 0,05.

3. Results

3.1 Effects of temperature and fish size on metabolic rates and metabolic scope

Statistically significant effects of size, temperature and their interaction were found for both SMR and MMR (Fig. 1A). SMR and MMR were higher in 15 g fish than in 60 g fish at both temperatures, but significantly different at 12°C only. With temperature increasing from 5 to 12°C, the increase in SMR was 2.4- fold for 15 g fish, and 2.0- fold for 60 g fish, equivalent to 3.4 and 2.8 in terms of Q_{10}, respectively. For all combinations of temperature and fish sizes, the MMR values were 3.3 – 3.8 times higher than the measured SMR. The aerobic scope (AS) reflects these effects of temperature and size on SMR and MMR: AS increased with temperature, and decreased with fish size, but was only significantly different between sizes at 12 °C (Fig. 1B).

3.2. Effects of temperature and fish size on estimates of DO_{lim} and DO_{crit}

Results from respirometry trials shows the limiting effect of environmental DO on lumpfish MO_2 (Fig. 2A – D). For all combinations of temperature and fish size, MMR was reduced to levels similar to, or lower than, SMR at the lowest oxygen saturation (approximately 20% O_2).

DO_{crit} estimates were within the same range (20 – 22 % O_2) for 15:5, 60:5 and 60:12 groups, and higher (35% O_2) in the 15:12 group (Fig. 3A). The difference was only significant between 15:12 and 60:5, based on estimates of statistical significance (non-overlapping 95% CI).

For three of the four tested combinations of temperature and fish size (15:5, 60:5 and 60:12), MMR was relatively stable with lowered DO until a breakpoint (DO_{lim}), below which MMR steadily decreased with further DO reductions (Fig. 2A, C, D). For these three groups, DO_{lim} estimates (mean ± SE) were similar at 5 °C (55±5 and 53±5 % O_2 for 15:5 and 60:5) and higher at 12 °C (89±6 % O_2 for 60:12). For the fourth group (15:12), MMR was more variable at DO > air saturation (Fig. 2B), and started to decline at a considerably higher DO: 147±7 % O_2 (Fig. 3B).

3.4 Effects of acute DO change on parameters of hypoxic and hyperoxic stress

Results from analyses of parameters of hypoxic and hyperoxic stress are shown in Fig. 4A-F. A significant effect of DO on plasma cortisol was observed (MS = 0.53, F = 4.16, d.f. = 4, $P = 0.006$), in terms of a 2.4 - fold increase in plasma cortisol concentration at 63% O_2 compared to the control (98% O_2). The 1.7 - fold increase observed at the lower DO level (47% O_2) was not statistically significant ($P = 0.16$). Osmolality was lower at 63% O_2 compared to the control but was not significantly different from any of the other groups. No effects of DO reduction or increase were found on plasma lactate concentrations ($F_{4, 45} = 0.99$, $P = 0.42$), plasma pH ($F_{4, 45} = 1.1$, $P = 0.38$), liver catalase activities ($F_{4, 45} = 1.85$, $P = 0.14$) or liver lipid peroxidation rates ($F_{1, 8} = 0.48$, $P = 0.51$).

Discussion

The present study provides new information on metabolic rates (SMR and MMR), the zone of environmental hypoxia, and the effects of acute changes in DO on hypoxic and

hyperoxic stress parameters for fish sizes and temperatures relevant for lumpish production and use in net pens.

4.1 Methodological considerations

Due to inadequate water heating, the acclimation tank water was 2.5 – 3.0 °C lower than the 12 °C respirometer test temperature. The acute increase in temperature at transfer to the respirometers makes insufficient thermal acclimation a possible confounding factor in the present experiment. Acclimation is a class of phenotypic plasticity which includes reversible changes in physiological phenotypes as a result of environmental exposures in the time range of days to months (reviewed by Schulte et al. 2011). As a result of acclimation, the effect of temperature change on metabolism can be reduced, but not completely abolished (reviewed by Jutfelt 2020). An acclimation period of 1-3 weeks is considered appropriate for studies of thermal effects (Clark et al. 2013). Acclimation to higher temperature generally results in lowered SMR, while MMR is less altered (Sandblom et al. 2016, Jutfelt et al. 2021).

There is no available information on the capacity and rate of acclimation of lumpfish specifically, but these generalizations suggest that the SMR presented for 12 °C is somewhat overestimated compared to what should be expected in acclimated fish, and that MMR is less affected by the insufficient acclimation. For the purpose of this study, namely to provide oxygen guidelines for lumpfish, this error can be considered acceptable. Firstly, the temperature step is small (2.5 - 3 °C), with relatively small expected effect on SMR (~10%, Hvas et al. 2018). Secondly, the temperature range in question (9 - 12 °C) is within a range where this species grows and functions well (Nytrø et al. 2014, Hvas et al. 2018).

Finally, the implication of overestimating SMR in the present study is that DO_{crit} is also over-estimated. The effect of temperature on DO_{crit} was small, and a possible over-estimation of DO_{crit} at 12 °C would to a small degree change the practical guidelines developed by the assembled findings in Fig. 5A-B. For direct comparison with other studies of the metabolism of fully acclimated lumpfish, the potential effect of insufficient acclimatization should be considered.

As discussed below, the method for measuring MMR in lumpfish, should be evaluated based on the collected findings in the present study, as well as studies performed by Hvas et al. (2018) and Ern et al. (2016).

4.2 Metabolic rates and metabolic scope

SMR and MMR were higher at 12 °C compared to 5 °C, and significantly higher in 15 g fish than in 60 g fish at 12 °C. These general effects of temperature and fish size on SMR and MMR are in accordance with established models of fish metabolism (Fry 1971; Brett and Groves 1979; Jobling 1994; Farrell 2009) and previous studies of lumpfish metabolism (Killen et al. 2007; Ern et al. 2016; Hvas et al. 2018).

In spite of the potential confounding effect of insufficient acclimation (discussed above), the SMR values were in accordance with results from Ern et al. (2016). They performed SMR measurements with comparable methodology to the present study. Ern et al. reported SMR values of 80 and 114 mg O_2 kg^{-1} h^{-1} for 22 g lumpfish at 10 and 16 °C, compared to 103 mg O_2 kg^{-1} h^{-1} for 15 g fish at 12 °C in the present study. In contrast to hypothesized effects of size on metabolism, the SMR values presented by Hvas et al. (2018) for ~300 g fish (~110 mg O_2 kg^{-1} h^{-1} estimated for 12 °C) were higher than observed for

451 ~60 g fish at 12 °C in the present experiment (60 mg O_2 kg^{-1} h^{-1}). This may be related to
452 measurement period. In the experiment performed by Hvas et al. (2018) the acclimation
453 period may have been too short (18 h) to achieve resting state (Chabot et al. 2016).
454 The MMR measured in 15 g fish at 12 °C in the present study was higher (338 mg O_2
455 kg^{-1} h^{-1}) than what was previously observed in lumpfish of similar size (22 g) at comparable
456 temperatures (~ 230 and 300 mg O_2 kg^{-1} h^{-1} 10 and 16 °C), using similar methodology, by
457 Ern et al. (2016). Considering the high DO_{lim} observed at 12 °C for 15 g fish in the present
458 experiment, it is possible that respirometer DO (92 – 94% of air saturation) was too low to
459 allow for maximal metabolic rates in the study by Ern et al. (2016). Compared to Hvas et
460 al. (2018), the MMR observed in 60 g fish at 12 °C in the present study (216 mg O_2 kg^{-1} h^{-1})
461 was lower than what was estimated for 300 g lumpfish at 12 °C in their study (~260 mg
462 O_2 kg^{-1} h^{-1}). This was not expected, based on size differences. It is possible that higher
463 metabolic rates are achievable if lumpfish are forced to swim at maximal speed, such as
464 performed by Hvas et al. (2018). Based on data from 60 g fish in the present experiment,
465 a shorter measurement period after chasing could also produce higher MMR. In this group,
466 2 minute measurements resulted in 18 % higher MMR, than what was found with the 5
467 minutes measurements. A protocol for MMR measurement for this species has not yet
468 been described, and further investigations are warranted to investigate the effect of
469 different protocols on the resulting MMR.
470 The differences in SMR and MMR between the present and previous studies, also
471 resulted in differences in aerobic and factorial aerobic scopes (AS and FAS). The FAS was
472 higher than previously reported: 3,1 – 3,7 *vs*. 1,5 – 3,0 in Killen et al. (2007), Ern et al.
473 (2016) and Hvas et al. (2018). As discussed above, the present methodology may have

allowed for detection of lower SMR and/or higher MMR, and suggest that the metabolic scope of this species may be higher than previously suggested (Hvas et al. 2018). The metabolic scope found for the smallest size group (15 g) at the highest temperature (12 °C), may contribute to explain the high growth rates (6 – 7% day $^{-1}$) which has been observed in young lumpfish (< 11 g; Nytrø et al. 2014).

4.3 The zone of environmental hypoxia

The effects of temperature and fish size on DO_{lim} and DO_{crit} were similar to what was found for SMR and MMR. For 15 g fish, the DO_{crit} – DO_{lim} ranges were 20 – 55 (5 °C), and 35 – 147% O_2 (12 °C), and for 60 g fish, the DO_{crit} – DO_{lim} ranges were 21 – 53 (5 °C) and 22 – 89% O_2 (12 °C). These ranges represent the boundaries of the zone of environmental hypoxia for the tested temperature and fish sizes, and can be used to estimate hypoxia severity (e.g., Seibel et al. 2021).

This study is the first to report DO_{lim} values for lumpfish, which represents a DO below which oxygen becomes a limiting factor for the aerobic metabolic scope (Fry 1971). DO_{lim} is expected to vary with factors known to affect the metabolic rate, such as temperature or life stage (Fry 1971; Neill and Bryan 1991). Results from the present study is in line with this and suggest that both temperature and size influence this threshold. Observed values were in the range of 53 – 55% O_2 at 5 °C, and substantially higher at 12 °C: 89 – 147 % O_2. The DO_{lim} found for 15 g lumpfish at 12 °C is high (147% O_2), considering the hypothesis that the cardiorespiratory system of fish has evolved to maximize MO_2 at air saturation (Claireaux and Chabot 2016). This hypothesis is however contradicted in more recent reviews, which summarizes recent findings in teleosts subjected to high

497 temperatures (above T_{opt}). In these studies, AS was substantially increased in hyperoxia
498 compared to normoxia (reviewed by McArley et al. 2020 and Jutfelt et al. 2021). Results
499 from studies investigating metabolism and growth of lumpfish propose that 12 °C is likely
500 below T_{opt} for 15 g fish (Nytrø et al. 2014; Hvas et al. 2018), suggesting that hyperoxia
501 may also increase AS at lower temperatures than T_{opt} in young lumpfish.

502 DO_{crit} represents a threshold below which AS is zero, and where further reduction in
503 DO compromises survival in resting fish (reviewed by Clarieaux and Chabot 2016). The
504 only other study providing DO_{crit} values for lumpfish, showed results for 22 g lumpfish at
505 10 and 16 °C (34 and 41% O_2; Ern et al., 2016) which were in good consistency with the
506 value obtained for 15:12 (35% O_2). For the remaining groups, their DO_{crit} values (20 – 22%
507 O_2) were comparable to what has been observed in other marine species such as cod, sea
508 bass, sole and turbot (~20% O_2; reviewed by Chabot and Claireaux, 2008). No mortalities
509 were observed during respirometer trials with ~20% O_2 for ~10 minutes. This severity ×
510 duration was therefore not lethal for the tested temperatures. Observed respiratory
511 challenges (unrhythmic, slow and pronounced opercular movements/ gaping) as well as
512 minimal locomotory response to handling at end of 12 °C and 20% O_2 exposure, does
513 however indicate that these fish were approaching exhaustion and loss of equilibrium at
514 this level of DO.

515

516 **4.4 Responses to acute changes in DO**

517 In acute hypoxia tests performed with 60 g fish at 10 °C, we observed elevated plasma
518 cortisol levels at 47 and 63% O_2, although not significantly increased at 47% O_2 compared
519 to the control (98% O_2). Increased DO levels (148 and 194% O_2) did not result in significant

change in hypoxic or hyperoxic stress parameters. The elevated cortisol levels at 47 and 63% O_2 is in accordance with Jørgensen et al. (2017), who observed increased plasma cortisol levels at 55 and 69% O_2 in ~ 40 g lumpfish at 10°C. The levels were however low in both studies (~20 – 50 ng ml^{-1}), compared to what has been observed in 185 g lumpfish subjected to lower DO levels (~20% O_2) at a similar temperature of 9 °C (~180 ng ml^{-1}, Hvas and Oppedal2019). The difference in plasma cortisol concentrations is likely an effect of hypoxia severity, as fish in the study by Hvas and Oppedal (2019) were subjected to oxygen levels close to what has been defined as DO_{crit} in the present study.

The results from the present study confirms previous observations of low lactic acid levels in plasma of lumpfish subjected to hypoxia or high current velocity in swimming tests (Jørgensen et al. 2017, Hvas et al.2018; Hvas and Oppedal 2019). It is possible that the use of their ventral suction disc to attach to surfaces and reduce locomotion may serve to lower energy demand in face of limiting DO levels.

The lack of effect of high DO levels (148 and 194% O_2) on parameters of hypoxic or hyperoxic stress is in accordance with conclusions in a review by Dong et al. (2011). Here, they conclude that teleosts are generally able to tolerate DO levels up to 200% O_2 without adverse effected on physiology or behaviour.

4.5 Practical oxygen guidelines for lumpfish

The main aim of this study was to provide practical oxygen guidelines for production and use of lumpfish. Results from the present study has therefore been combined with previous findings and other guidelines to form advice for the aquaculture industry in Fig. 5A-B. In these two figures, linear relationships between temperature and the two DO

thresholds (DO_{lim} and DO_{crit}) have been included, to enable advice for temperatures between the two test temperatures used in the present experiment (5 and 12 °C). These linear relationships are not necessarily correct representations of the relationships between temperature and DO_{crit}/DO_{lim}, but it can be argued that they are reasonable as guidelines for aquaculture: The increase in SMR and MMR is close to linear within temperature ranges above T_{min} and below T_{opt} in marine teleosts (e.g., Claireaux et al. 2000; Claireaux and Lagardère 1999; Lefrancois and Claireaux 2003; Hvas et al. 2018). A temperature range of 5 - 12 °C is likely within this segment of the thermal niche for 15 - 60 g lumpfish (Hvas et al. 2018; Nytrø et al. 2014). A close correlation between SMR/MMR and DO_{crit}/DO_{lim} was found across temperatures and fish sizes in the present study (not shown).

Appetite has been found to be a sensitive indicator of hypoxia, and is possibly the first detectable effect of environmental hypoxia (Remen et al. 2012; Jørgensen et al. 2017; review by Jutfelt 2021). Appetite was not measured in the present study, but reductions are expected to occur at DO levels higher than levels inducing increased plasma cortisol (Remen et al. 2012; Jørgensen et al. 2017). In the present experiment, plasma cortisol was increased to moderate levels (35-50 ng ml^{-1}) within the middle third of the hypoxic zone (represented as stars in Fig. 5B), suggesting that appetite is first reduced at DO higher than this, i.e., within the upper third of the hypoxic zone.

Within the middle third of the hypoxic zone, a further reduction in appetite and growth is expected, combined with increased levels of plasma cortisol, based on results presented by Jørgensen et al. (2017). In the lower third of the hypoxic zone, increasing levels of hypoxic stress, towards levels observed at ~DO_{crit} is expected (~180 ng ml^{-1}; Hvas and

565 Oppedal 2019). Below this level, survival is time-limited (reviewed by Claireaux and
566 Chabot 2016).

567 In summary, it is suggested that the temperature- and size dependent boundaries of the
568 zone of environmental hypoxia (DO_{lim} and DO_{crit}) can be used as guidelines to determine
569 suitable oxygen levels, as well as effects of hypoxia severity for lumpfish. The zone of
570 environmental hypoxia can be divided into three, in which the onset of reduced appetite is
571 expected within the upper third, the onset of hypoxic stress is expected within the middle
572 third, and ceased growth and severe hypoxic stress is expected in the lower third. Below
573 DO_{crit}, survival is compromised.

574 Repeated or chronic stress is known to suppress immune responses of teleosts (reviewed
575 by Tort 2011) and has been found to alter ion balance and reduce growth in lumpfish
576 (Hanssen 2016). Drops in DO within the upper third can therefore be considered
577 acceptable, yet suboptimal with respect to lice gracing efficiency, while drops in DO within
578 the lower two thirds increasingly reduces performance, welfare and health as a function of
579 hypoxia severity, duration and frequency.

580

581 **4.6 Conclusions and relevance for the aquaculture industry**

582 The present study has provided lumpfish SMR, MMR and AS for temperatures and sizes
583 relevant for lumpfish production and use in sea cages. This is relevant for dimensioning
584 and design of lumpfish production systems, as well as oxygenation systems in lumpfish
585 transport or in Atlantic salmon sea cages. Temperature and fish size significantly
586 influenced these variables, as well as the boundaries of the zone of environmental hypoxia
587 (DO_{lim} and DO_{crit}). For 15 g fish, DO_{lim} (the upper boundary) was 55 and 147% O_2 at 5 °C

588 and 12 °C, and for 60 g fish, DO_{lim} was 53 and 89% O_2 at the same temperatures.
589 Reductions in DO to levels within the hypoxic zone may occur in salmon net pens, in
590 particular if used in combination with lice skirts (Stien et al. 2012; Bui et al. 2020). Based
591 on present and previous findings, the zone of environmental hypoxia was divided into three
592 to provide guidelines with respect to hypoxia severity: Drops within the upper third of this
593 zone induce negative effects on appetite, while lower DO levels induce hypoxic stress,
594 which increases with hypoxia severity, as DO approaches the DO_{crit} (Fig. 5). Hypoxia
595 severity, frequency and duration will determine to what degree DO drops within the
596 hypoxic zone is acceptable with regard to fish performance, health and welfare.
597 Present results suggest that short- term increases in DO up to 194% O_2 can be used
598 without negative effects, if oxygen demand is temporarily increased, such as during
599 crowding, transfer or transport at high temperatures.
600

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608 **Author statement**

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779 **Figure legends**

780

781 Fig. 1 A – C. The effect of temperature (5 and 12°C) and fish size (15 and 60 g) on average
782 (± 95% CI) A) SMR, B) MMR) and C) the aerobic scope (AS) of lumpfish. Different letters
783 denote statistically significant differences between group means (Factorial ANOVA for
784 SMR/MMR and non-overlapping 95% CI for AS).

785

786 Fig. 2A – D. Measured maximum metabolic rates (MMR, mg O_2 kg^{-1} min^{-1}) in individual
787 lumpfish subjected to different levels of O_2 (%), grouped by size and temperature in A –
788 D. Group codes = Size (g): Temperature (°C). Broken line regressions (broken lines) were
789 fitted to the data, and black, filled arrows represent statistically significant DO breakpoint
790 estimates (i.e., DO_{lim}). Mean normoxic SMR is illustrated by grey, horizontal lines, and the
791 breakpoint DO where MMR is reduced to the level of SMR (i.e., DO_{crit}) is represented by
792 open, black arrows. Dark grey, horizontal lines on the arrows represent standard errors.

793

794 Fig. 3A – B. Incipient limiting DO levels (DO_{lim}) and critical DO levels (DO_{crit}) determined
795 for 15 and 60 g lumpfish at temperatures of 5 and 12 °C. Values represent means ± 95%
796 confidence intervals, and different letters denote statistically significant differences, based
797 on non-overlapping 95% CI.

798

799 Fig. 4A – F. Effects of abrupt and short-term changes in DO (% O_2) on lumpfish plasma
800 A) cortisol and B) lactate concentrations, C) plasma pH and D) osmolality, and on liver E)
801 catalase activities (CAT) and F) lipid peroxidation rates (LPO). DO was changed from air

802 saturation (98 % O_2) to either hypoxic (47 and 63% O_2) or hyperoxic (148 or 194% O_2)
803 DO levels for 1 – 1.5 h prior to blood and liver sampling. Values are means ± SEM (n =
804 10), and different letters denote statistically significant differences in group means. N.S. =
805 not significant
806
807 Fig. 5A – B. Effect of temperature on the boundaries of the zone of environmental hypoxia
808 for lumpfish of two sizes: 15 g (A) and 60 g (B), based on present results. The limit between
809 green and yellow represents DO_{lim} and the limit between orange and red represents DO_{crit}.
810 Based on present results, combined with results from Jørgensen et al. (2017) and Hvas and
811 Oppedal (2019), the gradual impact of increasing hypoxia severity is proposed, where
812 performance is gradually reduced towards zero within the upper two thirds of the hypoxic
813 zone (yellow/light orange), and where hypoxic stress gradually increases from mild to
814 severe within the lower two thirds of this zone (light orange / orange). Stars represents DO
815 levels where elevated plasma cortisol levels were observed in the present experiment.
816

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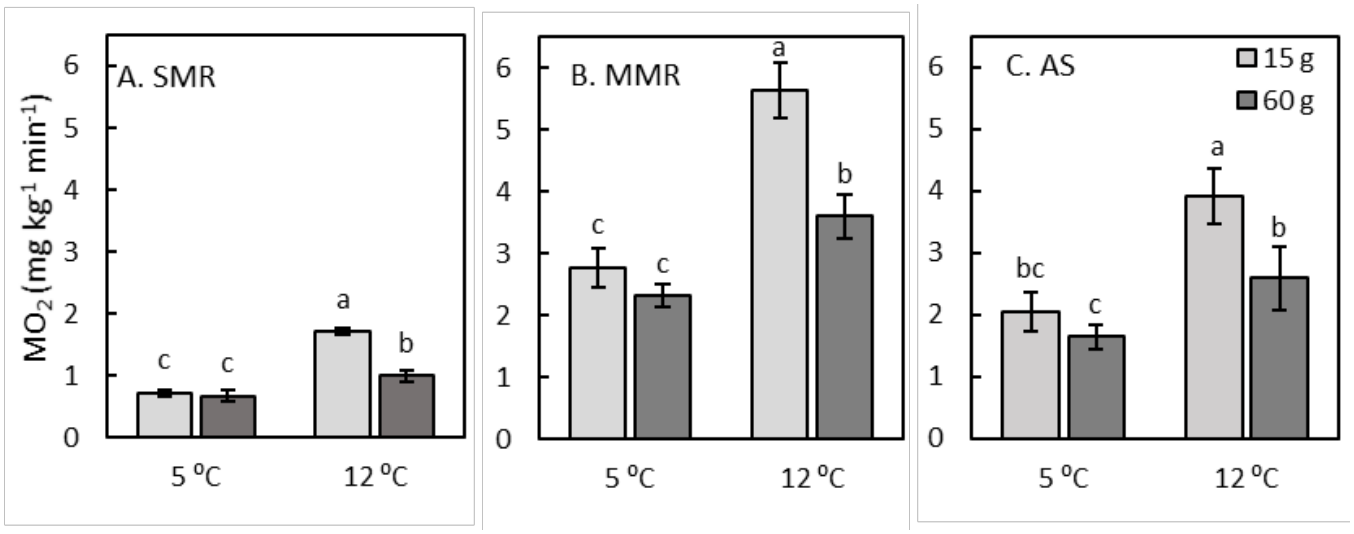

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820 Fig. 1 A-C. Remen et al.

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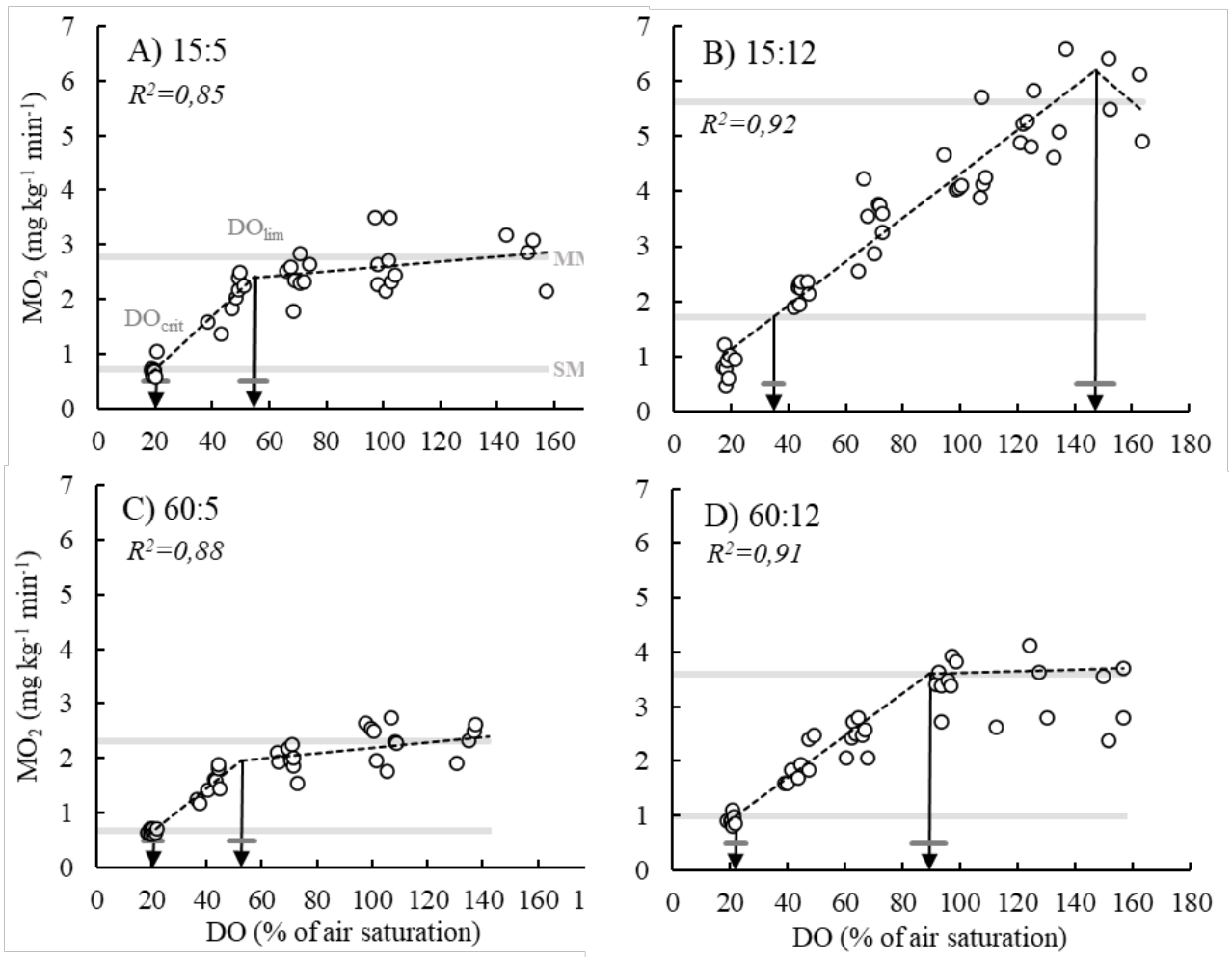

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823 Fig. 2A-D. Remen et al.

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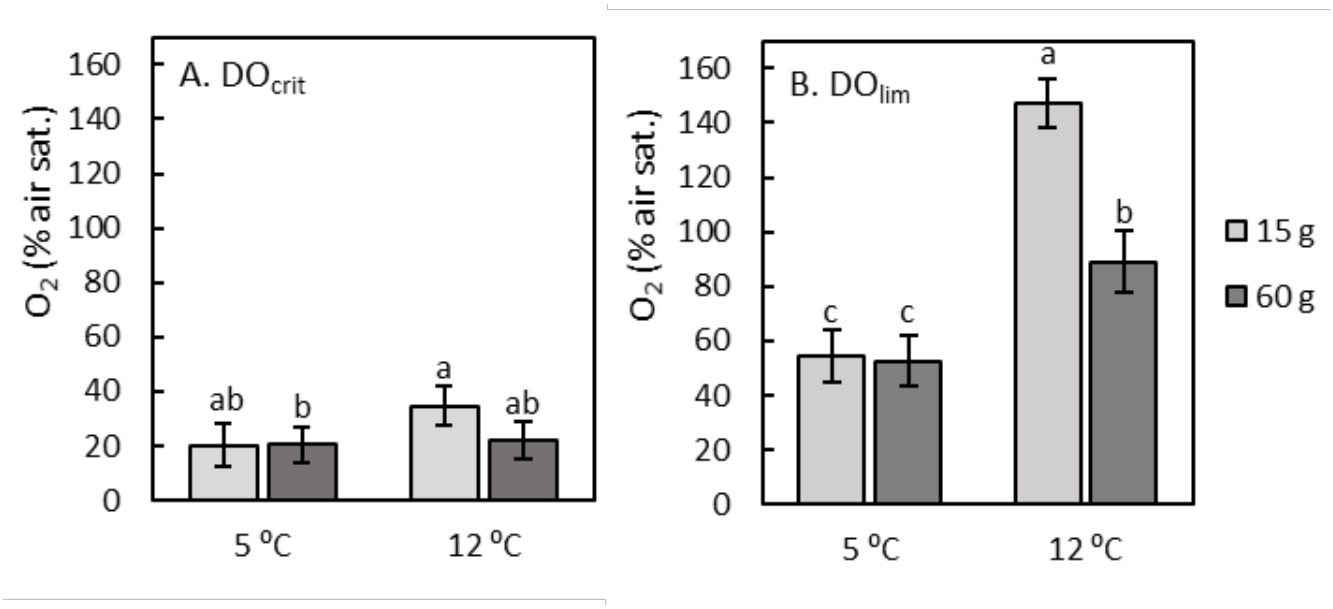

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827 Fig. 3A-B. Remen et al.

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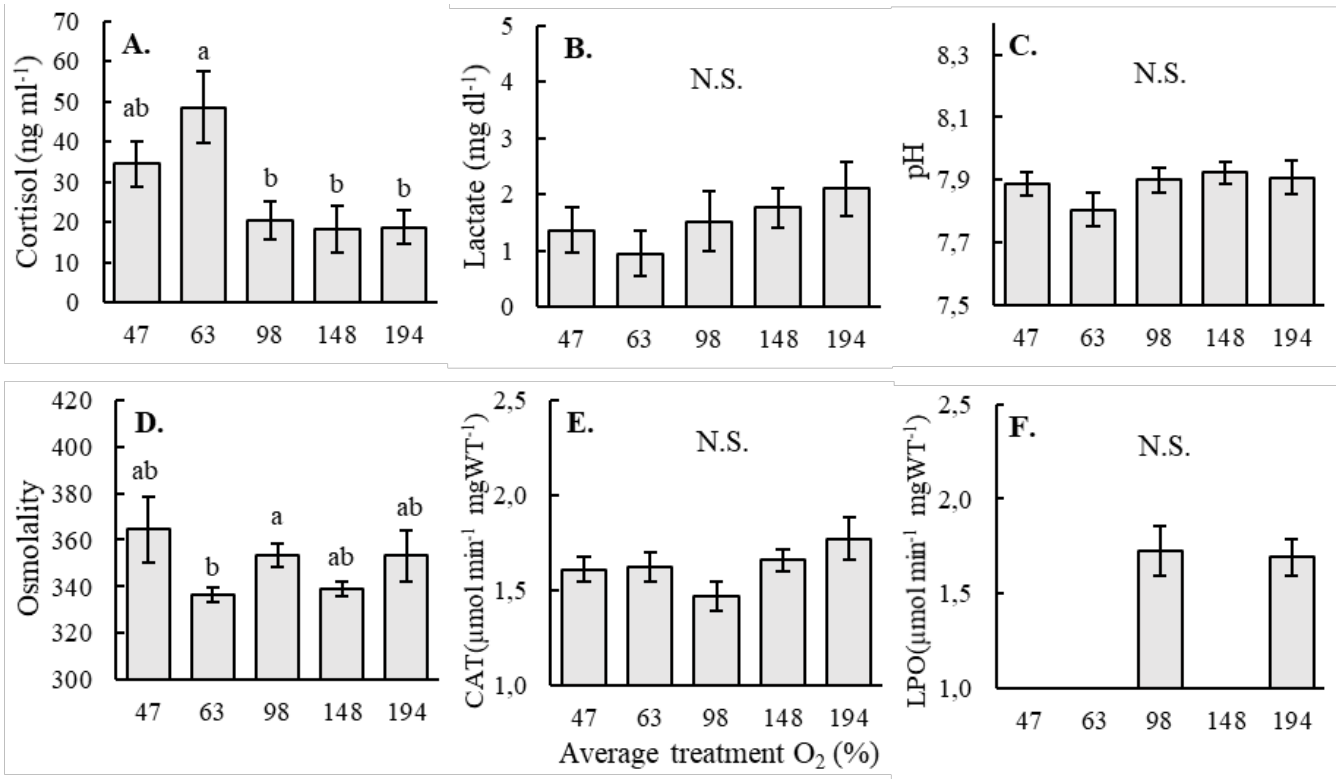
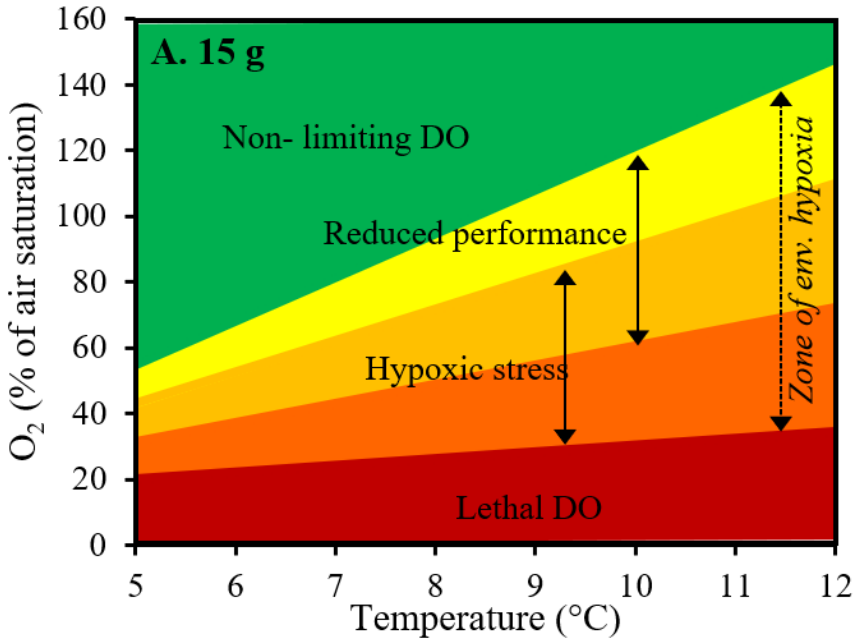


Fig. 4A-F. Remen et al.



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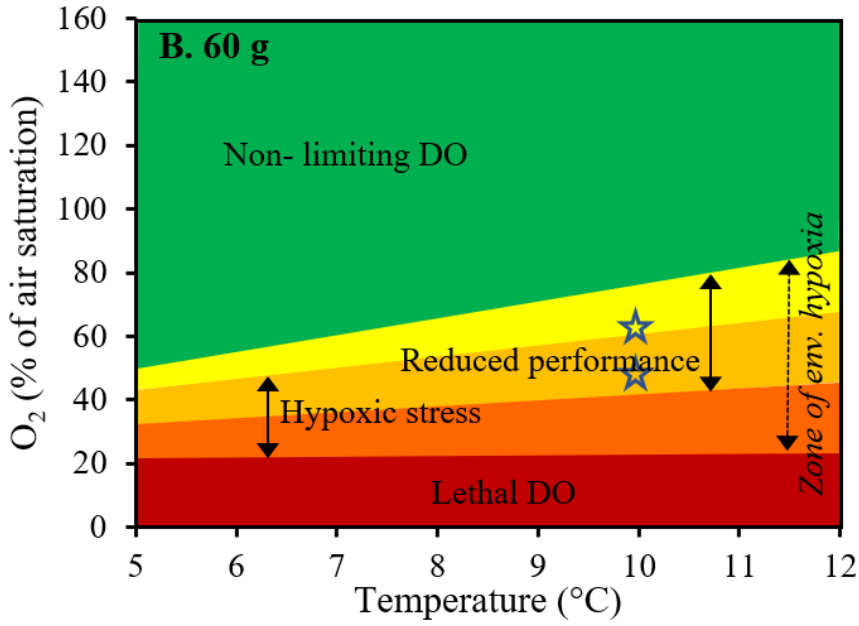

834

835 Fig. 5A-B. Remen et al.

836

837 Table 1. Average (± SD) fish weights (g) and lengths (cm, tale fin basis) per group
838 (combination of temperature, fish size and DO) in SMR and MMR measurements, mean
839 (± SD) temperature (°C), DO (% of air saturation) and number of valid (R^2 > 0,9)
840 measurements (N).

Group	Parameter	Desired DO (% O_2)	W (g)	L (cm)	T (°C)	Measured DO (% O_2)	N
15:5	SMR	100	15.1 ± 0.8	5.9 ± 0.3	5.1 ± 0.0	104 ± 2	8
	MMR	20	15.6 ± 0.2	5.9 ± 0.2	5.1±0.1	19 ± 1	8
	MMR	45	14.9 ± 0.3	6.0 ± 0.2	5.1±0.1	47 ± 4	8
	MMR	70	14.9 ± 0.2	5.7 ± 0.2	5.2±0.1	70 ± 3	8
	MMR	100	15.0 ± 0.2	5.9 ± 0.3	5.0±0.1	101 ± 3	8
	MMR	140	14.8 ± 0.2	5.9 ± 0.2	5.1±0.1	151 ± 6	4
15:12	SMR	100	15.0 ± 1.0	6.0 ± 0.3	12.5 ± 0.0	108 ± 1	8
	MMR	20	14.2 ± 1.8	6.0 ± 0.2	12.6 ± 0.1	19 ± 1	8
	MMR	45	15.1 ± 0.9	6.0 ± 0.3	12.6 ± 0.1	44 ± 2	8
	MMR	70	14.3 ± 0.9	6.0 ± 0.3	12.5 ± 0.1	70 ± 3	8
	MMR	100	14.3 ± 1.1	6.0 ± 0.2	12.5 ± 0.1	103 ± 5	8
	MMR	120	14.2 ± 1.0	6.3 ± 0.2	12.6 ± 0.1	123 ± 2	4
	MMR	140	14.6 ± 0.9	6.1 ± 0.2	12.5 ± 0.1	145 ± 14	8
60:5	SMR	100	60.0 ± 4.6	9.6 ± 0.3	5.1 ± 0.0	98 ± 3	8
	MMR	20	61.8 ± 3.7	9.8 ± 0.3	5.2 ± 0.0	20 ± 1	8
	MMR	45	63.9 ± 6.4	9.8 ± 0.3	5.2 ± 0.0	42 ± 3	8
	MMR	70	60.8 ± 5.2	9.6 ± 0.3	5.2 ± 0.0	70 ± 3	8
	MMR	100	60.2 ± 5.6	9.5 ± 0.4	5.1 ± 0.0	104 ±4	8
	MMR	140	54.5 ± 1.3	9.3 ± 0.2	5.2 ± 0.0	138 ± 4	8
60:12	SMR	100	62.0 ± 3.9	9.9 ± 0.4	12.2 ± 0.0	105 ± 1	4
	MMR	20	59.6 ± 6.9	9.5 ± 0.5	12.0 ± 0.2	22 ± 1	8
	MMR	45	59.7 ± 6.8	9.9 ± 0.5	12.0 ± 0.1	46 ± 4	8
	MMR	70	61.9 ± 8.7	9.6 ± 0.5	12.0 ± 0.1	67 ± 2	8
	MMR	100	49.4 ± 6.2	9.1 ± 0.5	11.9 ± 0.1	98 ± 2	8
	MMR	140	61.2 ± 5.9	9.3 ± 0.5	12.0 ± 0.1	143 ± 17	8

841

842 Table 2. Average (± SD) measured DO (% of air saturation) during acute change in DO in
843 all replicate tanks, exposure time (min), number of individuals sampled for blood and liver
844 (N), and average (±SD) weights and lengths of fish sampled per tank replicate.

Desired DO ($\%O_2$)	**Replicate**	**Measured DO ($\%O_2$)**	**Exposure time (min)**	**N**	**W (g)**	**L cm**
45	1	47 ± 3	57	5	64 ± 9	9.8 ± 0.4
	2	48 ± 2	60	5	61 ± 14	9.6 ± 0.7
65	1	64 ± 0	52	5	65 ± 7	9.7 ± 0.2
	2	63 ± 0	66	5	59 ± 15	9.3 ± 0.6
100	1	98 ± 0	60	5	60 ± 9	9.8 ± 0.7
	2	98 ± 0	60	5	58 ± 10	9.9 ± 0.8
150	1	149 ± 3	49	5	64 ± 9	10.0 ± 0.4
	2	148 ± 3	58	5	65 ± 4	10.0 ± 0.5
200	1	195 ± 5	62	5	51 ± 12	9.2 ± 0.3
	2	194 ± 5	66	5	52 ± 9	9.2 ± 1.2

845