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## **Hypoxia tolerance thresholds for post-smolt Atlantic salmon: Dependency of temperature and hypoxia acclimation**

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1 **Hypoxia tolerance thresholds for post-smolt Atlantic salmon: Dependency**  
2 **of temperature and hypoxia acclimation**

3

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9

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11 gill ventilation

12

13

14

15 **Abstract**

16 In order to establish hypoxia tolerance thresholds for Atlantic salmon (*Salmo salar*) in the on-  
17 growing phase, the effect of temperature (6, 12 and 18 °C) and hypoxia acclimation (33 days  
18 of hypoxic periods occurring every 6 hours at 16 °C) on the oxygen consumption rate (MO<sub>2</sub>)  
19 and limiting oxygen concentration (LOC; referred to as the hypoxia tolerance threshold) was  
20 investigated in fish were kept under production-like conditions (fed, undisturbed and freely  
21 swimming fish in tanks). Further, the effects of temperature and oxygen on the relationship  
22 between gill ventilation frequency (Vf) and MO<sub>2</sub> were studied in order to evaluate Vf as an  
23 indicator of MO<sub>2</sub>. Both MO<sub>2</sub> and LOC were found to increase exponentially with temperature  
24 (Q<sub>10</sub> =2.7 for MO<sub>2</sub> and 1.4 for LOC), while hypoxia acclimation resulted in a tendency for  
25 reduced MO<sub>2</sub>, but no lowering of LOC. The mean LOC at 6, 12, 16 and 18 °C were 2.9, 3.4,  
26 3.8 and 4.3 mg L<sup>-1</sup>, respectively. A strong correlation between MO<sub>2</sub> and LOS (LOC given in  
27 units of oxygen saturation) was found (R<sup>2</sup>=0.93), regardless of temperature, suggesting that  
28 measurements of MO<sub>2</sub> can be used to estimate the LOS of post-smolts. Vf was considered a  
29 reliable estimator of MO<sub>2</sub> in normoxic conditions, but not during reductions in oxygen, due to  
30 the increasing Vf, and relatively stable MO<sub>2</sub> as oxygen declined towards LOC.

31

32 **1. Introduction**

33 Temperature is the main controlling factor of fish metabolism (Fry, 1947, 1971), and  
34 is therefore essential when determining the dissolved oxygen (DO) requirement of cultured  
35 fish. The effect of temperature on the DO requirement of Atlantic salmon (*Salmo salar*) in the  
36 sea water phase is however largely unknown (reviewed by Thorarensen and Farrell, 2011),  
37 and makes it difficult for legislators and aquaculturists to assess whether observed DO levels  
38 in sea cages (e.g. Burt et al., 2012; Crampton et al., 2003; Johansson et al., 2006, 2007;  
39 Oppedal et al. 2011) are compromising fish performance and welfare.

40 Rates of biochemical processes and cost of oxygen transport to metabolising tissues  
41 increase with temperature (Mark et al., 2002), causing an exponential increase in the standard  
42 metabolic rate (SMR, the metabolic rate of fasted and resting fish) with temperature (Brett  
43 and Groves, 1979; Farrell et al., 2009). The maximum aerobic metabolic rate (MMR) also  
44 increases with temperature at lower and intermediate ranges, but levels off, and eventually  
45 decreases, at high temperatures (Farrell et al., 2009; Pörtner, 2010). The metabolic scope for  
46 activity, representing the difference between SMR and MMR, therefore increases with  
47 temperature up to the point where the increase in MMR no longer keeps up with that of the  
48 SMR. This turning point is referred to as the optimum temperature, allowing the largest  
49 capacity to feed, digest, assimilate nutrients, swim etc. (Fry, 1947, 1971; Neill and Bryan,  
50 1991). The thermal optimum for Atlantic salmon has been reported in the range of 16 to 20 °C  
51 (reviewed by Elliott and Elliott, 2010).

52 Oxygen is the main limiting factor of fish metabolism (Fry, 1971), and any DO that  
53 limit the metabolic scope can be defined as environmental hypoxia (Farrell and Richards,  
54 2009). As DO declines within the hypoxic zone, the oxygen uptake rate can be kept at the  
55 same level through increased gill ventilation and perfusion (Barnes et al., 2011; Ott et al.,  
56 1980; Perry et al., 2009), but the metabolic scope is gradually reduced as oxygen declines

57 (Fry 1971), causing reduced capacity for feeding and swimming (Kutty and Saunders, 1973;  
58 Remen et al., 2012). Eventually, the cost of maintaining  $MO_2$  exceeds the benefit, and  $MO_2$   
59 starts to decrease with further reductions in DO (see Perry et al., 2009, for review). Below this  
60 threshold, termed the limiting oxygen concentration (LOC) (Neill and Bryan, 1991), the rate  
61 of anaerobic metabolism increases sharply, anaerobic end-products accumulate and  
62 physiological as well as behavioural stress responses are elicited (Burton and Heath 1980;  
63 Perry and Reid, 1994; Remen et al., 2012; Van Raij et al., 1996; Vianen et al., 2001). Thus,  
64 for aquaculture purposes, the LOC for fish with routine  $MO_2$  can be considered the hypoxia  
65 tolerance threshold, and constitute a limit for reductions in DO that should be avoided in sea  
66 cages due to the hypoxic stress and time-limited survival at such DO levels (Nilsson and  
67 Nilsson, 2008).

68         According to the theoretical framework presented by Fry (1971) and reviewed by  
69 Wang et al. (2009), the LOC of fish can be expected to increase with any factor that increases  
70 the metabolic rate. The LOC of Atlantic salmon in a sea cage can therefore be expected to  
71 depend both on water temperature and the metabolic state of the fish (e.g. acclimation state,  
72 feeding status, swimming speed and stress level), and determination of LOC for aquaculture  
73 purposes therefore requires that the metabolic rate of fish is comparable to that of fish in sea  
74 cages. A recent study by Barnes et al. (2011) showed that individual  $MO_2$  was strongly  
75 correlated with LOC, regardless of experimental temperature, suggesting that LOC can be  
76 estimated from measurements of  $MO_2$ . This relationship is useful, as the LOC of Atlantic  
77 salmon over a range of temperatures and metabolic states can be estimated, based on  $MO_2$   
78 measurements presented in previous studies. However, as the measurements of Barnes et al.  
79 (2011) were performed on a relatively small selection of single, fasted fish in a respirometer  
80 at high temperatures (14-22 °C), the strong relationship between  $MO_2$  and LOC needs to be

81 validated for a wider temperature range, a larger group of fish and for experimental conditions  
82 more similar to the sea cage environment.

83         If LOC is determined by  $MO_2$ , it would be of high value to find an easily observable  
84 indicator of  $MO_2$  of fish in sea cages, in order to assess whether fish are provided with DO  
85 above their LOC, e.g. during short-term reductions in DO (Johansson et al., 2006). Millidine  
86 et al. (2008) suggest that gill ventilation frequency (Vf) may serve as an easily observable,  
87 and good predictor of  $MO_2$ , as a strong correlation between these two variables was found in  
88 Atlantic salmon juveniles. However, the effect of declining oxygen on Vf (Perry et al., 2009)  
89 was not taken into consideration in the study of Millidine et al. (2008), and the combined  
90 effect of temperature and oxygen on ventilation frequency needs to be evaluated in order to  
91 discuss the suitability of Vf as an indicator of  $MO_2$ .

92         Acclimation to hypoxia has been shown to both reduce the oxygen demand (Pichavant  
93 et al., 2000; 2001) and increase the capacity for oxygen uptake and -transport of fish (Lai et  
94 al., 2006; Soivio et al., 1980; Tetens and Lykkeboe, 1981). However, in spite of numerous  
95 physiological adjustments, LOC was not lowered in Atlantic cod (*Gadus morhua*) after 6-12  
96 weeks of acclimation to hypoxia (Peterson and Gamperl, 2010). Correspondingly, a recent  
97 study on Atlantic salmon post-smolts suggested that acclimation to periodic hypoxia did not  
98 increase hypoxia tolerance considerably, as the depression of feed intake and accumulation of  
99 lactate in hypoxia periods was relatively stable for 3 weeks (Remen et al., 2012). It is not  
100 known whether LOC is lowered as a result of hypoxia acclimation in Atlantic salmon.

101         The main purpose of this study was to investigate the effect of temperature and  
102 hypoxia acclimation on LOC for Atlantic salmon post-smolts kept in experimental conditions  
103 resembling production conditions. Further, we aimed to evaluate whether  $MO_2$  determines  
104 LOC, allowing LOC estimation based on assessment of  $MO_2$ , and whether  $MO_2$  can be  
105 estimated from ventilation frequency.

106

## 107 **2. Material and methods**

108

### 109 *2.1. Fish material and experimental conditions*

110 This study is based on two separate experiments. The effects of temperature on the  
111 oxygen consumption rate ( $MO_2$ ) and the limiting oxygen concentration (LOC), and the  
112 combined effect of temperature and dissolved oxygen concentration (DO) on ventilation  
113 frequency ( $V_f$ ) was studied in Experiment I (referred to as Exp I). The effects of acclimation  
114 to hypoxia of varying severity on  $MO_2$  and LOC were studied in Experiment II (referred to as  
115 Exp II). Both experiments were carried out at the Institute of Marine Research, Matre,  
116 Norway using Atlantic salmon post-smolts (*Salmo salar* L., AquaGen strain) hatched in  
117 January 2008. Out-of-season smolts were produced according to standard procedures. This  
118 involves constant illumination (LL) from first-feeding until smoltification was initiated by a  
119 winter signal (6 weeks of L:D, 12:12). The parr-smolt process was completed by another 6  
120 weeks of LL before sea transfer on 22 September 2008 (e.g. Oppedal et al., 2007).

121 In both Exp I and Exp II, the water flow rate, temperatures and feeding (Arvotec  
122 feeding units, Arvo-Tec T drum 2000, [www.arvotec.fi](http://www.arvotec.fi)) in experimental tanks were controlled  
123 from custom made computer software (SD Matre, Normatic AS, Nordfjordeid, Norway),  
124 which also recorded temperature (TST 487-1A2B temperature probes), flow through rates  
125 (Promag W flow meters Endress + Hausser), oxygen level (Oxyguard 420 probe, Oxyguard  
126 International, Denmark, <http://www.oxyguard.dk>) and salinity (Liquisys MCLM223/ 253  
127 probes) continuously (1 minute averages). Oxygen probes were calibrated in air once a week.  
128 Illumination was constant and provided by one fluorescent light tube per tank.

129

### 130 *2.2. Experimental design*

131 *2.2.1. Experiment I*

132 137 post-smolt Atlantic salmon were transferred from outdoor tanks to indoor, squared  
133 tanks (~460 L) fitted with lids on 21 January 2009. Weights ( $291 \pm 4$  g) and lengths  
134 ( $28.2 \pm 0.1$  cm) were measured on 11 February (Table 1). Upon transfer, fish were kept in the  
135 same water quality as in the outdoor tank (salinity  $34 \text{ g L}^{-1}$ , temperature  $8\text{-}9$  °C), and  
136 temperature was gradually increased ( $1$  °C per day) to  $12$  °C by 24 January. A water flow  
137 through rate of  $20 \text{ L min}^{-1}$  kept oxygen levels above  $7 \text{ mg L}^{-1}$  (measured in tank outlet) until  
138 30 March, the day before experiment start-up. From this day on and throughout the  
139 experiment, oxygen levels were maintained at ~100% of air saturation by an automatically  
140 controlled addition of super-saturated sea water (~400% of air saturation), except during LOC  
141 measurements.

142 The fish in all four experimental tanks were subjected to three subsequent changes in  
143 temperature, from  $12$  to  $18$  °C (day 0),  $18$  to  $12$  °C (day 20) and  $12$  to  $6$  °C (day 29), and were  
144 allowed to acclimate to the new temperature for 8-15 days before measurements of  $\text{MO}_2$  and  
145  $V_f$  were performed (days 15, 28 and 42). During the entire experimental period, fish were fed  
146 to satiation twice daily (09:30-10:30 and 14:00-15:00), aiming at ~40% surplus of feed. On  
147 LOC measurement days, fish were fed to satiation 1-2 h before the initial reduction in oxygen  
148 below 100% of air saturation, and the feed intake was estimated according to the method  
149 described by Helland et al. (1996). The weights and lengths of fish were recorded on day -48  
150 and four days after the last LOC measurement (day 46), following the procedure described in  
151 Remen et al. (2012) (see Table 1). Mean weights ( $\pm$ SEM) on LOC measurement days were  
152 estimated to be  $425 \pm 7$  g ( $18$  °C),  $460 \pm 8$  g ( $12$  °C) and  $501 \pm 10$  g ( $6$  °C), based on overall  
153 specific growth rates.

154

155 *2.2.2. Experiment II*



156            Approximately 1300 post-smolts ( $209\pm 1$  g) were transferred from outdoor tanks and  
157 distributed among 12 indoor circular tanks ( $\text{Ø}=3$  m,  $\sim 5600\text{L}$ ) supplied with  $9^\circ\text{C}$  sea water ( $34$   
158  $\text{g L}^{-1}$ ) on 9-10 February 2009. Temperature was gradually increased until  $16^\circ\text{C}$  was reached  
159 on 16 March, and maintained throughout. Flow rates were kept at  $80\text{ L min}^{-1}$  and increased to  
160  $105\text{ L min}^{-1}$  on 26 March, maintaining a minimum of  $6.4\text{ mg L}^{-1}\text{ O}_2$  (80% of air saturation) in  
161 tank outlets prior to the acclimation period.

162            Prior to measurements of  $\text{MO}_2$  and LOC, the post-smolts were acclimated to periodic  
163 hypoxia of different severities for 33 days at  $16^\circ\text{C}$ . Hypoxic periods were chosen over  
164 constant hypoxia, as this is more likely to occur in on-growing production in sea cages (e.g.  
165 Burt et al., 2012; Johansson et al. 2006, 2007), and the frequency of hypoxia was set to mimic  
166 hypoxic periods occurring during the turn of tidal currents (Johansson et al., 2006). Starting  
167 on 24 April 2009, four triplicate groups (tanks) of post-smolts (overall initial weight  $383\pm 2$  g)  
168 were either kept at constant  $6.4\text{ mg O}_2\text{ L}^{-1}$  (80% of air saturation, referred to as “control” and  
169 “normoxia”), or subjected to 1 h and 45 minutes periods of reduced DO every 6 h, to either  
170  $5.6$  (70% of air saturation),  $4.8$  (60% of air saturation) or  $4.0\text{ mg O}_2\text{ L}^{-1}$  (50% of air  
171 saturation) (Fig. 1). Groups were termed 80:80, 80:70, 80:60 and 80:50, based on the oxygen  
172 saturation in normoxia: hypoxia. The desired oxygen levels were maintained by controlling  
173 tank water flow rates, while the water current in the tank was upheld using a submerged pump  
174 (capacity of  $120\text{ L min}^{-1}$ ) varying in supply depending on the amount of inflowing water.  
175 Throughout the acclimation period, fish were fed to satiation ( $\sim 25\%$  surplus of feed) twice  
176 daily in normoxic periods. Before the LOC measurement on day 33, the latest hypoxic period  
177 and the morning feeding period were finished approximately 6 and 4 hours prior to the initial  
178 reduction in DO below 100% of air saturation, respectively. The weights and lengths of fish  
179 were registered on the day following LOC measurements (Table 1), according to the  
180 procedures described in Remen et al. (2012).

181

182 2.3. Open respirometry

183 Both in Exp I and Exp II, the oxygen consumption rates ( $MO_2$ ) of post-smolts were  
184 measured during a progressive decline in DO, by using the experimental tanks as open  
185 respirometers, in order to find the limiting oxygen concentration (LOC). In brief, the water  
186 flow through rates in the holding tanks were reduced to a minimum (some flow was necessary  
187 for proper function of oxygen probes), and without disturbing the fish, oxygen gradually  
188 declined as a result of fish consumption. This was allowed to continue until the rate of oxygen  
189 decline was clearly lowered, indicating that  $MO_2$  was reduced and that LOC had been passed.  
190 No fish lost equilibrium during the LOC trials.

191 In Exp I, DO in tanks was elevated to 115-125% of air saturation by increasing the  
192 supply of oxygen-supersaturated water, before the supply was turned off, and the water  
193 exchange rate ( $Flow$ ) was reduced to  $2 \text{ L min}^{-1}$  (12 and 18 °C), or  $1 \text{ L min}^{-1}$  (6 °C). The  
194 oxygen consumption rate per tank ( $MO_2$ ,  $\text{mg O}_2 \text{ min}^{-1}$ ) was found from the equation:

195

196 
$$MO_{2_t} = Vol \cdot Sol \cdot \frac{Sat_t - Sat_{t-\delta t}}{\delta t \cdot 100} + Flow \cdot Sol \cdot \frac{100 - Sat_t}{100},$$

197

198 where  $Vol$  is the tank volume (~460 L) and  $Sol$  is the solubility of oxygen at prevailing  
199 temperature and conductivity conditions.  $Sat_t$  is the oxygen saturation at time  $t$ . The average  
200 saturation in 5 minute intervals was used ( $\delta t=5$  minutes). The oxygen flux over the water  
201 surface during the progressive decline in oxygen was investigated by measuring the DO  
202 change in tanks without fish, after the water had been oxygen-stripped using  $N_2$  gas. Flow  
203 rates corresponded to that used in experiments. The contribution of oxygen flux to the DO  
204 development was modeled as

205 
$$O_{2\text{flux}} = k \cdot (100 - Sat_t) \cdot Sol \cdot \frac{Vol}{100}$$

206 For the lidded experimental tanks in Exp I, the influx was found to be so small that it  
207 could not be identified and therefore is considered negligible.

208 In Exp II, DO in tanks was elevated to 110-120% of air saturation by addition of  
209 supersaturated water, before this supply was turned off and flow reduced to 3 L min<sup>-1</sup>. For the  
210 large experimental tanks used in Exp II, the influx of oxygen at DO below air saturation was  
211 significant, and added to the calculation of tank MO<sub>2</sub>. The diffusion constant, *k*, was estimated  
212 to be 0.00135, by finding the value of *k* that maximized the correlation between the observed  
213 and modeled increase in oxygen saturation after oxygen-stripping (R<sup>2</sup>=0.9997).

214

#### 215 *2.4. Gill ventilation frequency*

216 The ventilation frequency (Vf, gill movements per minute) was monitored for all LOC  
217 measurements in Exp I. Vf was registered in each tank for approximately every 10% decrease  
218 in oxygen saturation, by measuring the time needed to perform 14 gill movements in 10 fish  
219 and calculating the average.

220

#### 221 *2.5. Calculations and statistics*

222 The specific growth rates (SGR) that was used to estimated weights of fish on LOC  
223 measurement days in Exp I, was calculated according to  $SGR = (e^g - 1)100$ , where  $g = (\ln M_2 -$   
224  $\ln M_1) (T_2 - T_1)^{-1}$ , and where  $M_1$  is the mass at the start of the growth period ( $T_1$ ) and  $M_2$  is the  
225 mass at end ( $T_2$ ) (Houde and Schekter, 1981). Condition factor (CF) was calculated by the  
226 formula  $CF = 100ML^{-3}$ , where  $M$  is the mass (g) and  $L$  is the fork length (cm) of the fish.

227 The temperature effect of metabolism,  $Q_{10}$ , was calculated as

228  $Q_{10} = \left( \frac{MO_{2T_2}}{MO_{2T_1}} \right)^{\frac{10}{T_2 - T_1}}$ , where  $MO_{2T_1}$  and  $MO_{2T_2}$  are oxygen consumption rates ( $\text{mg kg}^{-1} \text{min}^{-1}$ )

229 at temperatures  $T_1$  and  $T_2$ , respectively (Schmidt-Nielsen, 1997).

230 The break-point in the relationship between ambient DO ( $\text{mg L}^{-1}$ ) and  $MO_2$ ,  
231 representing the limiting oxygen concentration (LOC), was found using the “segmented”-  
232 package in the free software programme R 2.14.0 (The R Foundation for Statistical  
233 Computing © 2011, [www.r-project.org](http://www.r-project.org)). This method simultaneously estimates slope  
234 parameters and turning point(s) within a standard linear model framework (Muggeo, 2003;  
235 2008) (see Fig. 2A). Maximum number of iterations was set to 30. Only  $MO_2$  values for  $DO \leq$   
236 the concentration equivalent to 90% of air saturation was used at all temperatures, and the  
237 normoxic  $MO_2$  was determined by averaging all 5 minute values for  $MO_2$  above the LOC. A  
238 Davies test was used to test for difference in slopes, and results were not included in the  
239 manuscript for  $p > 0.05$  (Muggeo, 2008).

240 In order to find the ventilation frequency in normoxia ( $Vf_{\text{norm}}$ , at DO equivalent to  
241 90% of air saturation at all temperatures), the maximal  $Vf$  ( $Vf_{\text{max}}$ ) and the limiting oxygen  
242 concentration for increased  $Vf$  ( $LOC_{Vf}$ ), a third order polynomial relationship was fitted to  
243 plots of  $Vf$  against DO for each tank using Microsoft® Office Excel® 2007 (© 2006  
244 Microsoft Corporation). By replacing  $x$  in the resulting polynomial function with the DO ( $\text{mg}$   
245  $\text{L}^{-1}$ ) equivalent to 90% of air saturation,  $Vf_{\text{norm}}$  was found. By derivation of the third-order  
246 polynomial function and solving the equation for  $Vf=0$ ,  $LOC_{Vf}$  was found. Then,  $Vf_{\text{max}}$  was  
247 calculated by replacing  $x$  in the third-order polynomial function with  $LOC_{Vf}$  (see Fig. 2B).

248 All further statistical tests were performed using Statistica© (StatSoft, Inc., USA).  
249 Fixed non-linear regression was used to test the non-linear relationships between temperature  
250 and the parameters feed intake,  $MO_2$  and LOC, and the effect of periodic hypoxia severity on  
251 feed intake,  $MO_2$  and LOC was tested using regression analysis. Differences between LOC

252 and  $LOC_{VF}$  at 6, 12 and 18 °C were tested using One-Way ANOVA. The correlation between  
253  $MO_2$  and LOC was tested using correlation analysis.

254 For comparison of linear relationships between  $MO_2$  and LOC obtained in the present  
255 experiment and the study of Barnes et al. (2011), LOC from both studies was expressed as  
256 LOS (limiting oxygen saturation, % of air saturation), due to the use of different temperatures.  
257 For a given oxygen concentration, the oxygen saturation increases with temperature, and as  
258 the saturation (or the corresponding oxygen tension) determines the gradient for oxygen  
259 diffusion over the gills (Davis, 1975), this denomination was considered more appropriate  
260 than the oxygen concentration for the relatively wide range of temperatures used. It should be  
261 noted that one observation from the study of Barnes et al. (2011) was left out of the  
262 comparison due to the lower weight (49 g) and long time used to perform the LOC  
263 measurement (29 h). Whether the relationship between  $MO_2$  and LOS in the present study  
264 differed from that of Barnes et al. (2011), was analyzed using Analysis of Covariance, with  
265 study origin as a categorical, random predictor variable,  $MO_2$  as the continuous predictor  
266 variable and LOS as the dependent variable.

267

### 268 **3. Results**

269

#### 270 *3.1. The effect of temperature on feed intake, $MO_2$ and LOC*

271 The feed intake (FI, % of biomass) of post-smolts during the meal preceding LOC  
272 measurements in Exp I increased with temperature, and a logarithmic relationship between  
273 temperature and FI was found ( $R^2= 0.93$ ,  $p<0.001$ ) (Fig. 3A). The normoxic oxygen  
274 consumption rate ( $MO_2$ ,  $mg\ kg^{-1}\ min^{-1}$ ), was found to increase exponentially with temperature  
275 ( $R^2=0.95$ ,  $p<0.001$ ), and was equivalent to  $1.2\pm 0.0$ ,  $2.3\pm 0.1$  and  $3.8\pm 0.2\ mg\ kg^{-1}\ min^{-1}$  at 6, 12

276 and 18 °C, respectively (Fig. 3B).  $Q_{10}$  for temperatures ranging from 6 to 18 °C was found to  
277 be 2.7. The exponential relationship between temperature and  $MO_2$  was modeled as

278

$$279 \quad MO_2 = 0.6564e^{0.0977T}$$

280

281 The limiting oxygen concentration (LOC) was found to increase exponentially with  
282 temperature ( $R^2=0.80$ ,  $p<0.001$ ) with LOC equivalent to  $2.9\pm 0.1$ ,  $3.4\pm 0.1$  and  $4.3\pm 0.2$  at 6, 12  
283 and 18 °C, respectively (Fig. 3C). The  $Q_{10}$ -value for the increase in LOC with temperature  
284 was 1.37, and LOC was modeled as

285

$$286 \quad LOC = 2.3812e^{0.0314T}$$

287

288 During the time period needed to reduce DO from normoxic levels to LOC (1.5-4.5 h),  
289  $MO_2$  was generally found to decline. The time used to reduce DO to LOC, and the reduction  
290 in  $MO_2$  within this period is presented in Table 2. Two out of 12 LOC measurements were  
291 excluded due to non-significant Davies tests, and in one case, no breakpoint could be  
292 estimated.

293

### 294 3.2. The relationship between $MO_2$ and LOS

295 A strong correlation between  $MO_2$  and LOC was found ( $R^2=0.86$ ,  $p<0.001$ , Fig. 4A).  
296 The recalculation of LOC into LOS (limiting oxygen saturation, expressed as % of air  
297 saturation) (Fig. 4B) reduced residual error, increasing  $R^2$  to 0.93 ( $p<0.001$ , Fig. 4B). In order  
298 to determine whether LOS can be determined by  $MO_2$ , across experimental temperature and –  
299 conditions, data from the present study was compared with data from Barnes et al. (2011),  
300 using ANCOVA. Results from Barnes et al. (2011) were found to yield slightly lower LOS

301 for a given  $MO_2$  ( $p=0.004$ ), equivalent to a 3-6% reduction (in oxygen saturation units) within  
302 the range of  $MO_2$  overlap ( $2.4-4.2 \text{ mg kg}^{-1} \text{ min}^{-1}$ ). The relationship between  $MO_2$  and LOS in  
303 the present study was determined as

304

$$305 \quad LOS = 9.785MO_2 + 17.873$$

306

### 307 *3.3. The effect of hypoxia acclimation on feed intake, $MO_2$ and LOC*

308 In Exp II, the feed intake of post-smolts during the meal preceding LOC  
309 measurements was reduced according to the DO in hypoxic periods ( $R^2=0.46$ ,  $p<0.05$ ), and  
310 was equivalent to a 20% reduction in FI of fish from the 80:50 group compared to the control  
311 (Fig. 5A). A tendency for increased  $MO_2$  with DO in hypoxic periods was observed, but not  
312 statistically significant ( $R^2=0.31$ ,  $p=0.068$ ) (Fig. 5B). The linear trend-line suggest that  $MO_2$   
313 was reduced by 10% when DO was reduced from 80% to 50%  $O_2$  in hypoxic periods. The  
314  $MO_2$  calculated when DO had been reduced to LOC, showed that the difference in  $MO_2$   
315 between groups had been reduced to none at this point of time ( $R^2=0.01$ ,  $p=0.749$ ). No effect  
316 of hypoxia acclimation status on the limiting oxygen concentration was found ( $R^2=0.01$ ,  
317  $p>0.90$ ) (Fig. 5C). The overall LOC was  $3.76 \pm 0.06$ .

318

### 319 *3.4. The combined effect of temperature and oxygen on $V_f$*

320 The ventilation frequency ( $V_f$ ) increased both with temperature and declining oxygen  
321 levels, until  $LOC_{V_f}$  was reached. Below  $LOC_{V_f}$ ,  $V_f$  declined (Fig. 6A). No significant  
322 differences between LOC and  $LOC_{V_f}$  were found at any of the temperatures (One-way  
323 ANOVA,  $p>0.05$ ) (Table 2). Both the  $V_f$  in normoxia ( $V_{f_{norm}}$ ;  $V_f$  at 90% of air saturation)  
324 and at  $LOC_{V_f}$  ( $V_{f_{max}}$ ) were closely related to  $MO_2$ , and data were fitted with second-order  
325 polynomial relationships ( $R^2=0.98$  and  $0.99$ ) (Fig. 6B). For data obtained at 18 °C, the

326 polynomial curve suggest that  $V_{f_{max}}$  reaches a plateau at  $MO_2$  equal to  $4.2 \text{ mg kg}^{-1} \text{ min}^{-1}$  (Fig.  
327 6B).

328

## 329 **4. Discussion**

### 330 *4.1. The relationship between temperature, $MO_2$ and LOC*

331 The results from the present experiment show a clear exponential relationship between  
332 temperature and oxygen consumption rate ( $MO_2$ ) of Atlantic salmon post-smolts, similar to  
333 what have been found in previous studies on salmonids (Barnes et al., 2011; Brett, 1971; Brett  
334 and Glass, 1973). The  $Q_{10}$ -value for the entire temperature range (2.7) was somewhat higher  
335 than values reported in studies of other, similar-sized salmonids (1.2-2.4) (Brett and Glass,  
336 1973; Evans, 1990; Ott et al., 1980), as well as a recent study on Atlantic salmon post-smolts  
337 (2.2) (Barnes et al., 2011). The higher  $Q_{10}$ -value can be explained by the inclusion of low  
338 temperature ( $6 \text{ }^\circ\text{C}$ ), as the effect of temperature on salmonid metabolism appears to be higher  
339 at low temperatures (Evans, 1990; Fivelstad and Smith, 1991; Henry and Houston, 1984).  
340 Further, the fish in our study were fed, and as feed intake increases with temperature, the  
341 effect of feeding on  $MO_2$  (Forsberg, 1997) can explain the more steep increase in  $MO_2$  with  
342 temperature observed in the present study.

343 The measured oxygen consumption rates ( $MO_2$ ) of post-smolts at  $6$  and  $12 \text{ }^\circ\text{C}$  were  
344 close to what can be obtained for fed post-smolts (500 g) swimming at low speed ( $0.3$  body  
345 lengths,  $BL, \text{ s}^{-1}$ ), using the model presented by Forsberg (1994). At higher temperatures,  
346 observed  $MO_2$  was higher than that predicted by Forsberg's model, which does not predict  
347 exponential increase with temperature. The measured  $MO_2$  was also comparable to the lower  
348 end of the  $MO_2$  range observed in fed Atlantic salmon post-smolts in a study by Bergheim et  
349 al. (1991), as well as to the  $MO_2$  of fasted ( $>12 \text{ h}$ ), resting post-smolts found by Wilson et al.  
350 (2007). Together, these results suggest that the  $MO_2$  of post-smolts observed in the present



351 study was at the lower end of the range for what can be expected for post-smolts kept under  
352 production conditions in sea cages. Both the swimming speed and feed intake of fish were  
353 probably at relatively low levels, due to the low water current speed in the tanks and repeated  
354 experiences of hypoxic stress (Bernier and Craig, 2005; Remen et al., 2012).

355         Compared to the only other study that has investigated LOC in Atlantic salmon post-  
356 smolts (Barnes et al. 2011), LOC was 25% higher in the present experiment, at the  
357 temperature common of both studies (18 °C). This is likely to be a result of using fed fish,  
358 with higher metabolic rates, compared to the use of fasted fish in the experiment by Barnes et  
359 al. (2011), as both studies show a strong, positive correlation between  $MO_2$  and LOC. The  
360 plots of limiting oxygen saturation (LOS) values against  $MO_2$ , showed that only slight  
361 differences in LOS was obtained for a given  $MO_2$  when these two studies were compared,  
362 suggesting that relatively good estimates of LOS can be made from  $MO_2$  measurements,  
363 across experimental conditions and -temperatures. The stronger linear relationship between  
364  $MO_2$  and LOS ( $R^2=0.93$ ) than between  $MO_2$  and LOC ( $R_2=0.86$ ), suggests that branchial  
365 oxygen transfer is more dependent on water oxygen tension than oxygen concentration, at  
366 least when DO is reduced to levels around LOC, where gill ventilation is at its maximum.  
367 Therefore, assessment of whether Atlantic salmon are provided with DO above their hypoxia  
368 tolerance threshold may be more accurate using oxygen saturation than concentration.

369

#### 370 *4.2. Hypoxia tolerance thresholds for post-smolts in production conditions*

371         Using the relationship between  $MO_2$  and LOS presented here, the hypoxia tolerance  
372 threshold (LOS) of post-smolts, with metabolic rates as similar to fish under production  
373 conditions in sea cages as possible, can be estimated based on previously published  $MO_2$   
374 measurements. Several studies have investigated the  $MO_2$  of post-smolts (100-800 g) in large  
375 production tanks, or experimental tanks, where fish are fed to satiation, and where swimming

376 activity ( $\sim 0.4\text{--}1.7$  body lengths  $\text{s}^{-1}$ ) varies according to the water current speed in the tanks  
377 (Atkins and Benfey, 2008; Bergheim et al., 1991; Castro et al., 2011; Fivelstad and Smith,  
378 1991; Forsberg, 1994). The resulting LOS values at different temperatures are summarized in  
379 Fig. 7. Generally, the estimated LOS values are higher than the LOS obtained in the present  
380 study (up to 73% higher), and suggest that the hypoxia tolerance threshold should be  
381 increased by at least 40% compared to the LOS obtained in the present study, in order to serve  
382 as practical guidelines for Atlantic salmon aquaculture (see Fig. 7). For practical use, it should  
383 be emphasized that the hypoxia tolerance threshold represents a threshold for hypoxic stress  
384 (Vianen et al., 2001) and compromised survival (Nilsson and Nilsson, 2008), and that  
385 negative effects on parameters of production performance (e. g. feeding, digestion and  
386 growth) are expected at higher levels of DO (e.g. Remen et al., 2012). Knowledge of both  
387 hypoxia tolerance thresholds and thresholds for maintained feeding and growth at various  
388 temperatures would enable an approximation of the graded negative effect of hypoxia  
389 between these two thresholds. Further, it should be emphasized that in some situations, the  
390 hypoxia tolerance threshold can be expected to increase beyond the guideline presented here,  
391 for example if fish are subjected to acute stress, (Folkedal et al., 2012), or if forced to swim at  
392 high speed (Wilson et al., 2007). The relationships between temperature,  $\text{MO}_2$  and LOS  
393 should be validated for larger Atlantic salmon ( $> 800$  g) in the sea water phase and  
394 investigated further for higher temperatures.

395

#### 396 *4.3. Ventilation frequency as an indicator of $\text{MO}_2$*

397 The increasing ventilation frequency ( $V_{f_{\text{norm}}}$ ) with temperature in Atlantic salmon  
398 agreed with Millidine et al. (2008), and the increasing, and then decreasing  $V_f$  as oxygen  
399 declined towards, and then below LOC, is in agreement with previous observations in a range  
400 of teleosts (see Perry et al., 2009, for review). Our results support Millidine et al. (2008) in

401 that  $V_f$  is a good indicator of  $MO_2$  in normoxic conditions, but not during reductions in  
402 oxygen, due to the increasing  $V_f$ , and relatively stable  $MO_2$  as oxygen declines towards LOC.

403

#### 404 *4.4. The effect of acclimation to periodic hypoxia on $MO_2$ and LOC*

405 In fish acclimated to cyclic hypoxia, the tendency for reduced  $MO_2$  with reduced DO  
406 in hypoxic periods (~10% reduction) corresponded well with the reduction in  $MO_2$  that can be  
407 expected from the 20% reduction in feed intake, using a model presented by Forsberg et al.,  
408 1997) for adult Atlantic salmon. Their results suggest that  $MO_2$  is reduced by 10% when  
409 ration is reduced by 20%. Our results are in line with previous reports of reduced  $MO_2$  as a  
410 consequence of reduced feed intake in turbot, European sea bass and Atlantic cod subjected to  
411 hypoxia (Claireaux et al., 2000; Pichavant et al., 2000; 2001), although marginal in the  
412 present experiment. Feeding fish in normoxic periods are likely to have reduced the negative  
413 effect of periodic hypoxia on feed intake and  $MO_2$ , compared to experiments where fish are  
414 fed in hypoxia (e.g. Remen et al., 2012). The tendency for reduced  $MO_2$  did not result in  
415 lowered LOC. This is in line with previous findings in Atlantic cod (Peterson and Gamperl,  
416 2010, 2011) and Atlantic salmon post-smolts (Remen et al., 2012), and suggest that frequent  
417 exposure to severe hypoxia does not reduce the hypoxia sensitivity of Atlantic salmon.

418

#### 419 *4.5. Conclusions and perspectives*

420 In conclusion, the limiting oxygen concentration (LOC) of Atlantic salmon post-  
421 smolts was found to increase exponentially for temperatures ranging from 6 to 18 °C, and a  
422 close correlation between the oxygen consumption rate ( $MO_2$ ) and the limiting oxygen  
423 saturation (LOS) was found within and across temperatures. LOS values for different  
424 temperatures found in the present experiment are likely to be at the lower end of the LOS  
425 continuum that can be expected at a given temperature in a sea cage, and may be implemented

426 by the salmon farming industry as hypoxia tolerance thresholds for fish with relatively low  
427 metabolic rates (e.g. when feeding ration is restricted). Hypoxia tolerance thresholds for fish  
428 with higher metabolic rates were estimated. Acclimation to hypoxia did not lower LOC,  
429 suggesting that negative effects of severe hypoxia are not reduced as a result of physiological  
430 adjustments.

431

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436

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589  
590

591 **Tables**

592

593 **Table 1.** Fish weights (g), lengths (cm, fork length) and condition factors (CF) of Atlantic salmon post-smolts,  
 594 measured on days -48 and 46 after first change of temperature (day 0) in Experiment I, and on the day following  
 595 LOC measurements in Experiment II. The number of replicate fish tanks and total number of fish measured per  
 596 treatment are provided. For Experiment II, group names indicate the oxygen saturation in normoxic and hypoxic  
 597 periods, respectively.

	<b>Day</b>	<b>Group</b>	<b>Weighth (g)</b>	<b>Length (cm)</b>	<b>CF</b>	<b>Replicate tanks</b>	<b>Total number of fish</b>
<b>Exp I</b>	-48		291±4	28.2±0.09	1.29±0.01	4	137
	46		513±10	34.6±0.21	1.24±0.01		
<b>Exp II</b>	34	80:50	569±15	36.0±0.3	1.20±0.01	3	293
	34	80:60	576±3	36.3±0.1	1.18±0.02	3	294
	34	80:70	578±9	36.2±0.2	1.20±0.01	3	294
	34	80:80	608±18	36.8±0.3	1.20±0.01	3	270

598

599 **Table 2.** The time used to reduce the dissolved oxygen concentration (DO) from normoxic levels to LOC  
600 (duration; minutes), the decline in oxygen consumption rates ( $MO_2$ ) during this time period ( $MO_2$  decline; % of  
601 normoxic levels) and the limiting oxygen concentration for increased ventilation frequency ( $LOC_{Vf}$ ,  $mg L^{-1}$ ) at  
602 the different temperatures in Experiment I, and in fish acclimated to periodic hypoxia of varying severity (group  
603 names indicate oxygen saturation in normoxic and hypoxic periods) at 16 °C for 33 days in Experiment II. The  
604 number of verified observations is indicated by number of replicate tanks (N). NA= not applicable.

	<b>T</b> (°C)	<b>Group</b>	<b>Duration</b> (min)	<b><math>MO_2</math> decline</b> (%)	<b><math>LOC_{Vf}</math></b> ( $mg L^{-1}$ )	<b>N</b> (replicate tanks)
	6	NA	252±9	1±6	2.4±0.4	3
<b>Exp I</b>	12	NA	167±0	6±3	3.0±0.1	3
	18	NA	99±2	10±4	5.1±0.2	4
	16	80:80	236±12	5±2	NA	3
<b>Exp II</b>	16	80:70	266±23	6±2	NA	3
	16	80:60	271±12	11±4	NA	3
	16	80:50	228±3	19±8	NA	3

605

606

607 **Figure captions**

608 **Fig. 1.** Schematic overview over the daily fluctuations in oxygen concentration ( $\text{mg L}^{-1}$ ) in the four  
609 experimental groups during the acclimation period (33 days) preceding LOC measurements in Exp II. Shaded  
610 areas represent feeding periods.

611 **Fig. 2 A-B.** Examples of (A) measured oxygen consumption rates ( $\text{MO}_2$ ,  $\text{mg kg}^{-1} \text{min}^{-1}$ ) and (B) gill ventilation  
612 frequencies ( $V_f$ , gill movements  $\text{s}^{-1}$ ) of Atlantic salmon post-smolts plotted against the dissolved oxygen  
613 concentration ( $\text{DO}$ ,  $\text{mg L}^{-1}$ ) in one replicate tank during a progressive decline in  $\text{DO}$ . A segmented regression  
614 model was fitted to the plot of  $\text{MO}_2$  against  $\text{DO}$  and used to estimate the limiting oxygen concentration (LOC). A  
615 third order polynomial function was fitted to the plot of  $V_f$  against  $\text{DO}$  and used to estimate  $V_f$  in normoxia ( $8.7$   
616  $\text{mg L}^{-1}$ , termed  $V_{f_{\text{norm}}}$ ), the LOC for compensatory gill ventilation ( $\text{LOC}_{V_f}$ ) and the maximum ventilation  
617 frequency ( $V_{f_{\text{max}}}$ ). The data presented are from fish acclimated to  $12^\circ\text{C}$  and fed to satiation  $\sim 2$  h prior to the  
618 progressive reduction in  $\text{DO}$  (Exp I).

619 **Fig. 3 A-C.** The effect of temperature ( $^\circ\text{C}$ ) on A) the feed intake ( $\text{FI}$ , % of biomass) of Atlantic salmon post-  
620 smolts during the morning meal preceding LOC measurements, B) the normoxic oxygen consumption rate  
621 ( $\text{MO}_2$ ,  $\text{mg kg}^{-1} \text{min}^{-1}$ ) and C) the limiting oxygen concentration (LOC) of undisturbed Atlantic salmon post-  
622 smolts fed to satiation. A logarithmic curve was fitted to the data in A, while exponential curves were fitted to  
623 the data in B and C.

624

625 **Fig. 4 A-B.** A) The limiting oxygen concentration ( $\text{LOC}$ ,  $\text{mg L}^{-1}$ ) and B) the corresponding limiting oxygen  
626 saturation ( $\text{LOS}$ , % of air saturation) of Atlantic salmon post-smolts in response to oxygen consumption rates  
627 ( $\text{MO}_2$ ,  $\text{mg kg}^{-1} \text{min}^{-1}$ ) at different temperatures (see legend), obtained in the present study (filled symbols) and a  
628 study of Barnes et al. (2011) (open symbols). Linear regression lines were fitted to both data sets, and multiple  
629  $R^2$  are shown in the figures.

630

631 **Fig. 5.** The effect of hypoxia severity on A) the feed intake ( $\text{FI}$ , % of biomass) ingested during the meal  
632 preceding LOC measurement), B) the oxygen consumption rate ( $\text{MO}_2$ ,  $\text{mg kg}^{-1} \text{min}^{-1}$ ) and C) the limiting oxygen  
633 concentration ( $\text{LOC}$ ,  $\text{mg L}^{-1}$ ) of Atlantic salmon post-smolts subjected to cyclic hypoxia ( $\sim 2$  h every 6 h,  $\text{DO} =$

634 5.6, 4.8 or 4.0 mg L<sup>-1</sup>, control kept at constant 6.4 mg L<sup>-1</sup>) for 33 days. Post-smolts were held at 16 °C and  
635 continuous lighting, and feed was provided in normoxic periods.

636

637 **Fig. 6.** The data presented in A) show the effect of oxygen (DO, mg L<sup>-1</sup>) on the gill ventilation frequency (Vf,  
638 movements s<sup>-1</sup>) of Atlantic salmon post-smolts at 6, 12 and 18 °C. Temperature is indicated by the use of  
639 different symbol shapes (see legend). Data points represent the average Vf (±SEM) for DO at intervals  
640 corresponding to 10% of air saturation, calculated from the polynomial relationships between DO and Vf  
641 obtained for each replicate tank (n=4). The data presented in B) show the relationships between oxygen  
642 consumption rate (MO<sub>2</sub>, mg kg<sup>-1</sup> min<sup>-1</sup>) and the normoxic Vf (Vf<sub>norm</sub>; filled symbols, DO equivalent to 90% of air  
643 saturation) and the maximal Vf (Vf<sub>max</sub>; open symbols, DO equivalent to the limiting oxygen concentration for  
644 compensatory gill ventilation; LOC<sub>Vf</sub>). Temperature is indicated by the use of different symbol shapes (see  
645 legend).

646

647 **Fig. 7.** The limiting oxygen saturation (LOS, % of air saturation) of fed Atlantic salmon post-smolts at  
648 temperatures ranging from 6-18 °C obtained in the present study (black line), compared to LOS estimated from  
649 MO<sub>2</sub> measured in other studies of Atlantic salmon smolts/post-smolts fed to satiation and swimming at various  
650 speed (~0.4-1.7 body lengths, BL, s<sup>-1</sup>) (broken lines). LOS was estimated using LOC=9.785\*MO<sub>2</sub>+17.873.  
651 Based on this comparison, it is suggested that LOS should be increased by at least 40% (grey line) in order to  
652 serve as a practical guideline for the salmon farming industry. The number next to broken lines indicate the  
653 literature reference; 1) Fivelstad and Smith, 1991 (200-800 g post-smolts, swimming speed ~0.4-0.7 body  
654 lengths, BL, s<sup>-1</sup>), 2) Bergheim, 1991 (520-800 g post-smolts, ~1.2-1.7 BL s<sup>-1</sup>), 3) Castro et al., 2011 (100-200 g  
655 post-smolts, 0.7 BL s<sup>-1</sup>), 4) Forsberg, 1994 (500 g post-smolts, 1 BL s<sup>-1</sup>) and 5) Atkins and Benfey, 2008 (~150  
656 g parr/smolts, swimming speed unknown).

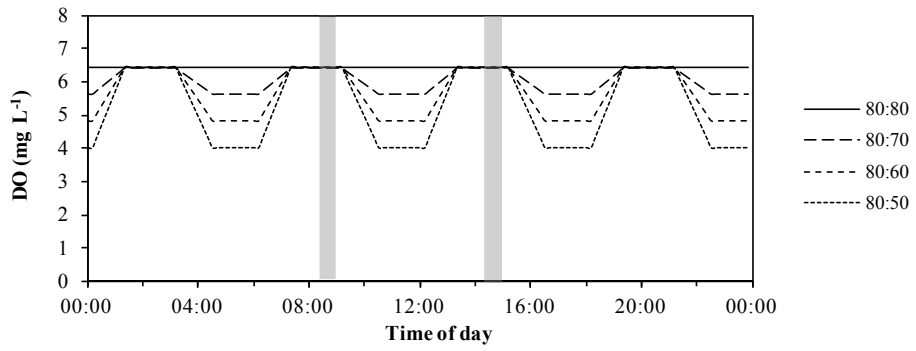
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659 **Figures**

660

661 **Fig. 1**

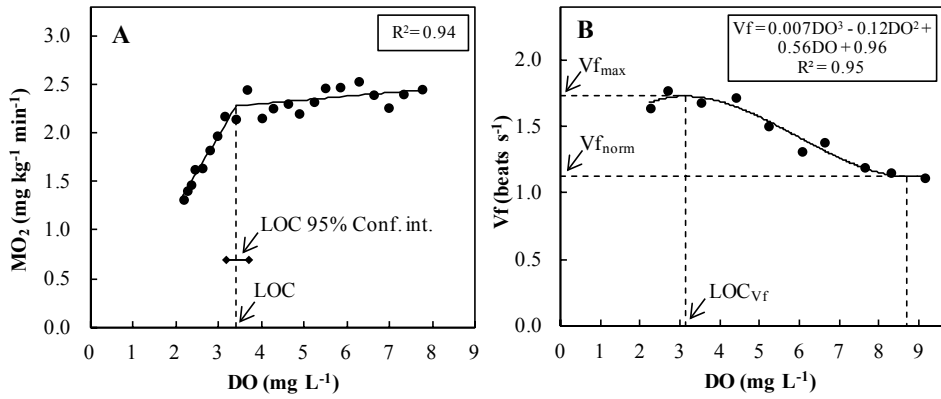


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665 Fig. 2 A-B



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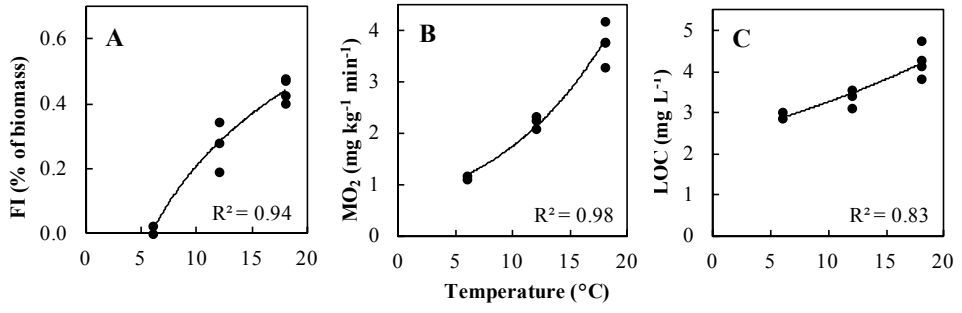
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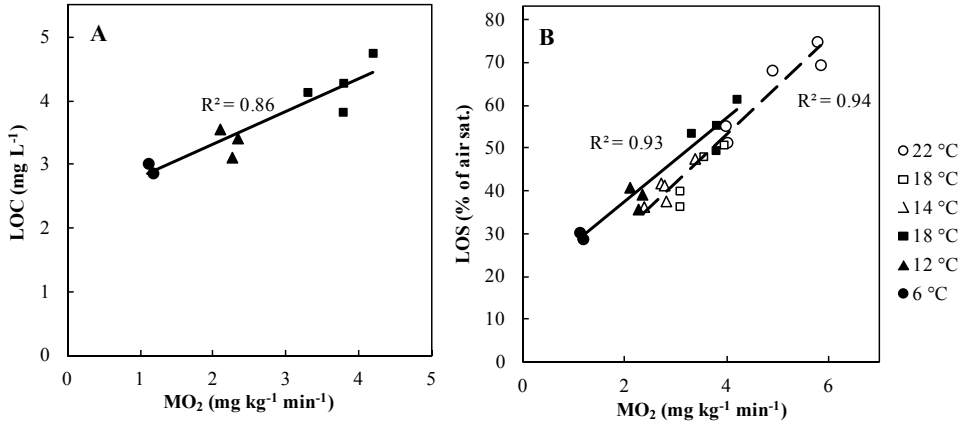
670 Fig. 3 A-C



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672

673 Fig. 4 A-B

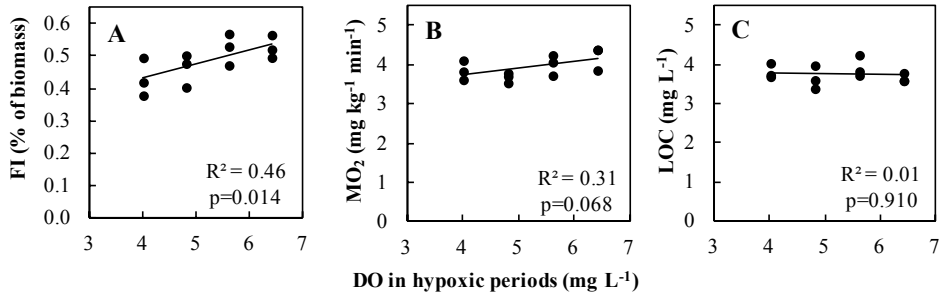


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676 Fig. 5 A-C

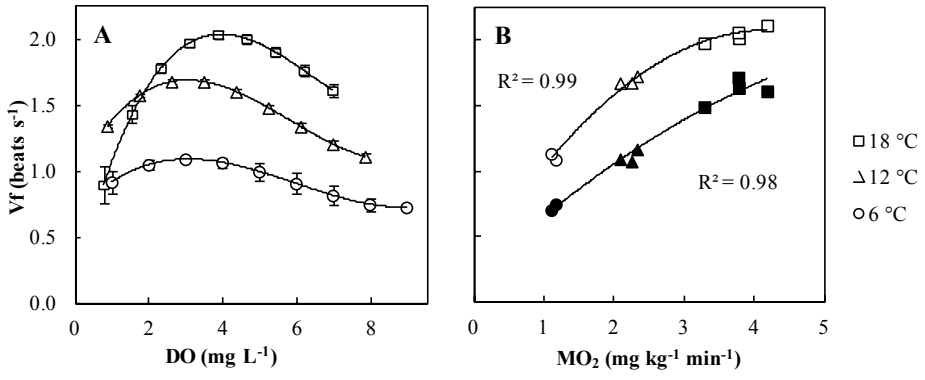
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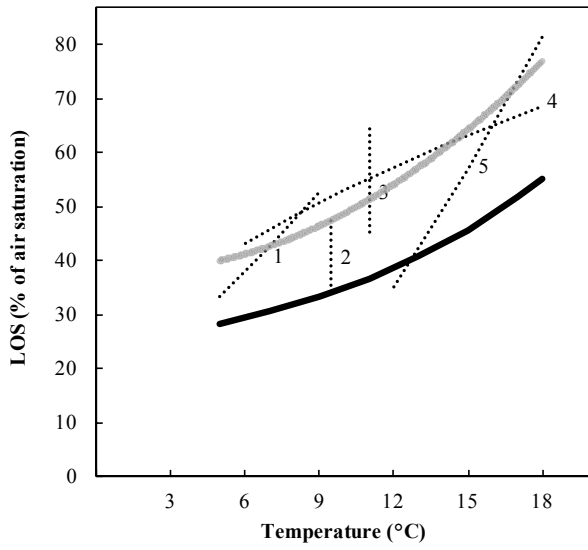
680 Fig. 6 A-B



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683 Fig. 7



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