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# Exposure to chronic moderate hypoxia impacts physiological and developmental traits of European sea bass (*Dicentrarchus labrax*) larvae

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**Abstract :**

Since European sea bass (*Dicentrarchus labrax*) larvae occurred in coastal and estuarine waters at early life stages, they are likely to be exposed to reduced dissolved oxygen waters at a sensitive developmental stage. However, the effects of hypoxia at larval stage, which depend in part on fish species, remain very poorly documented in European sea bass. In the present study, the impacts of an experimental exposure to a chronic moderate hypoxia (40 % air saturation) between 30 and 38 days post-hatching on the physiological and developmental traits of European sea bass larvae were assessed. This study was based on the investigation of survival and growth rates, parameters related to energy metabolism [Citrate Synthase (CS) and Cytochrome-c Oxidase (COX) activities], and biological indicators of the maturation of digestive function [pancreatic (trypsin, amylase) and intestinal (Alkaline Phosphatase "AP" and Aminopeptidase-N "N-LAP") enzymes activities]. While condition of hypoxia exposure did not induce any significant mortality event, lower growth rate as well as CS/COX activity ratio was observed in the Hypoxia Treatment group. In parallel, intestinal enzyme activities were also lower under hypoxia. Altogether, the present data suggest that sea bass larvae cope with moderate hypoxia by (1) reducing processes that are costly in energy and (2) regulating mitochondria functions in order to respond to energy-demand conditions. Both these effects are associated with a delay in the maturation of the digestive function.

**Keywords** : Hypoxia, Fish larvae, Energy metabolism, Maturation

**Abbreviations**

AP
Alkaline Phosphatase

C
Control group

COX
Cytochrome-c Oxidase

CS
Citrate Synthase

d.p.h.
Days post-hatching

FI
Feed intake

H
Head

HT
Hypoxia treatment

IS
Intestinal segment

N-LAP
Aminopeptidase-N

NS
No significant differences

PS
Pancreatic segment

RMR
Routine metabolic rate

s.e.m.
Standard error of mean

T
Tail

## Introduction

Since larvae of marine fish species hatch at an undeveloped stage, their development and ontogeny are liable to be greatly affected by biotic and abiotic conditions. This is especially true in coastal areas, which are nurseries for many marine fish species and where ecosystems are particularly impacted by human activities and global change. Among the environmental factors affecting the world's coastal waters, hypoxia is a major problem and is forecast to increase further under the combined effects of spreading coastal eutrophication and global warming (Diaz, 2001; Diaz and Rosenberg, 1995). Hypoxia episodes often coincide with periods of annual recruitment and the growth of benthic and pelagic fish species occurring in late spring and early summer (Breitburg, 1992; Pihl et al., 1991; Pihl et al., 1992). Understanding the impact of a low oxygen environment on the early life stages of fish is therefore of great importance, since fishery production relies on larval and juvenile recruitment.

Exposure of juveniles or adults from different fish species to hypoxia has been shown to notably affect fish routine metabolic rate (RMR), feed intake (FI), growth, condition factor and concentration of protein, *e.g.*, European sea bass (*Dicentrarchus labrax*) (Claireaux and Lagardere, 1999; Pichavant et al., 2001; Thetmeyer et al., 1999); turbot (*Scophthalmus maximus*) (Pichavant et al., 2001; Pichavant et al., 2000); Amazon oscar (*Astronotus crassipinis*) (Almeida-Val et al., 2011); and mummichog (*Fundulus heteroditus*) (Rees et al., 2012). It is supposed that some of these effects can be explained by the hypoxia-induced control of fundamental processes related to energy saving. Long-term exposure to hypoxia in fish has been also shown to affect egg development and hatching success, resulting in malformation, lower fitness and high mortality rates at larval and juvenile stages, *e.g.*, brown trout (*Salmo trutta*) (Massa et al., 1999; Roussel, 2007); black bream (*Acanthopagrus butcheri*) (Hassell et al., 2008); zebrafish (*Danio rerio*) (Padilla and Roth, 2001; Shang and Wu, 2004); and dogfish (*Scyliorhinus canicula*) (Diez and Davenport, 1990). These effects occur due to disturbances in a series of programmed, highly intricate and energy-consuming processes (Ozernyuk, 2011; Wu et al., 2006). Although it is generally accepted that early embryonic developmental stages are particularly sensitive to stresses in fish (Cameron and VonWesternhagen, 1997), post-hatching development also has windows of sensitivity to hypoxia. The effects of hypoxia exposure when applied at larval stages vary widely depending on fish species, hypoxia severity, exposure period and individual adaptive capacities (Anjos et al., 2008; Bickler and Buck, 2007). While Barrionuevo et al. (2010)

reported that zebrafish did not respond to moderate hypoxia before 30 days post-hatching (d.p.h.), Pelster (2002) showed that exposure of the same species to 7 days of moderate hypoxia affected larval cardiac activity, cardiac output, heart rate, organ perfusion and blood vessel formation. Since hatching of marine fish species occurs at earlier stages of development compared with freshwater species, their ontogenic processes during the larval phase are likely more sensitive to hypoxic episodes. Low tolerance to hypoxia has been observed over the period of metamorphosis in sea bream (*Pagrus major*) (Ishibashi et al., 2005); Japanese flounder (*Paralichthys olivaceu*) (Ishibashi et al., 2007); and bonefish (*Albula sp.*) (Pfeiler, 2001). It is supposed that this sensitivity is due to the increase of metabolic rates just before and just after the flexion stage, which is a period of dramatic molecular, physiological and behavioural changes for marine fish species such as European sea bass (Mazurais et al., 2011). The European sea bass is a highly valued fish that migrates from offshore areas to the coast just after flexion stage, which occurs between 22 and 25 d.p.h. (Dufour et al., 2009; Jennings and Pawson, 1992; Pickett and Pawson, 1994). This species can therefore be exposed to hypoxic events from this stage onward. However, few studies have been published on the effects of hypoxia on larval development of this marine fish species.

The purpose of the present study was to evaluate the impact of a chronic (between 30 and 38 d.p.h., a natural hypoxia exposure window) moderate (40% air saturation) hypoxia exposure on growth, metabolism and maturation in European sea bass larvae. To gain insight into the energy metabolism of larvae, Citrate Synthase (CS) and Cytochrome c Oxidase (COX) activities were assessed. CS and COX are key mitochondrial enzymes localized in matrix or membrane which fluctuations provide information on the properties and the numbers of the mitochondria present (Guderley, 2007; Lucassen et al., 2003). CS/COX activity ratio was used in order to estimate changing in mitochondrial size and shape in response to energy-demand conditions as indicated in studies by Lucassen et al. (2003) and Ibarz et al. (2010). Finally, digestive enzyme activities [Trypsin, Amylase, Alkaline Phosphatase (AP) and Aminopeptidase-N (N-LAP)] were used as indicators of the developmental status of the fish larvae.

## Material and methods

### Larval rearing

Post-hatching, larvae of European sea bass were reared under normal oxygen conditions in 8 tanks at 19.1 ± 0.4 °C water temperature and 35 ± 0.2 ‰ salinity, and were fed with *Artemia* until 45 d.p.h according to Zambonino-Infante et al. (1996). The tanks were then divided into two groups of four tanks. The Control group (or C group) was maintained under normal oxygen condition (96.6 ± 1.3 % air saturation, 7.10 mg $O_2$. $L^{-1}$), while larvae from the challenged group, also named Hypoxia Treatment group (or HT group), were subjected to moderate hypoxia (40.1 ± 2.6 % air saturation, 2.95 mg $O_2$.$L^{-1}$) for 8 days (from 30 to 38 d.p.h., **Fig. 1**). These hypoxia conditions had previously been determined to not induce mortality. During hypoxia exposure larvae of each group were fed with *Artemia*, except 24 h prior to samplings where larvae were left undisturbed and unfed. After the hypoxia exposure, larvae continue to be fed with *Artemia* until the end of experimentations. It was not possible to evaluate feed ingestion throughout this experiment; however, we did not notice any visible change in feeding activity in HT group during or after hypoxia exposure.

### Survival rate and larval growth

Survival rate was estimated at 45 d.p.h. by counting the number of remaining larvae in each group and taking into account the larvae collected for sampling during the experiment.
Growth was evaluated on four pools (1 pool from each tank) of approximately 40 larvae per sample from the beginning of hypoxia exposure (30 d.p.h.) until 45 d.p.h. (*i.e.* at 30, 32, 35 and 38 d.p.h – during hypoxia exposure – and at 42 and 45 d.p.h. – after the return to normoxic conditions).

### Larvae sampling

During hypoxia exposure, five samplings (at 30, 32, 35 and 38 d.p.h. and 42 d.p.h.) were performed in order to analyse CS and COX activities. For this purpose, in each treatment group, 4 samplings (1 per tank) of 35 or 40 pooled larvae were for protein concentration and enzymes activities assays. Larvae were first placed on a glass maintained at 0°C and dissected under microscope into four parts: head “H” (just behind the eye), pancreatic segment “PS” (just behind the gills) and tail “T” (point between the end of the digestive tract and the anal tail) were discarded, and the intestinal segment “IS” conserved (**Fig. 2**). For each group,

pooled IS were then weighed, placed in ice-cold lysis buffer and stored at –80°C until proteins extraction.

For digestive enzymes assays, four samplings (one per tank) of 35 larvae per group were performed at 38 d.p.h. and stored at –20°C. Larvae were placed on an ice-chilled glass and dissected under a microscope into 4 parts: head (H), pancreatic segment (PS) intestinal segment (IS) and tail (T) (**Fig. 2**). PS and IS were then stored at –20°C for later assay of their respective enzymes, as previously described by Cahu and Zambonino-Infante (1994).

**Extractions of proteins from larvae for analysis of CS and COX activities**

Proteins were solubilized by adding 3-6 mL of ice-cold lysis buffer containing 150 mM NaCl, 10 mM Tris, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% Igepal, 1 tablet of complete EDTA-free protease inhibitor cocktail (Roche Pharma; Basel, Switzerland) in 25 mL buffer, 1% phosphatase inhibitor cocktail III (Sigma-Aldrich®; St. Louis, MO, USA) and 5mM NaPPi, pH 7.4. Total proteins and enzymes were then extracted as described by Le Foll et al. (2007). Briefly, total proteins and enzymes lysates were obtained after homogenization with a Pro Polytron® (BioBlock Scientific; Illkirch , Bas-Rhin , France) tissue disruptor. The homogenates obtained were then centrifuged at 3,000 *g* for 1 h at 4°C. At the end of the centrifugation, the interphase containing proteins and enzymes was collected by carefully pipetting and then centrifuged at 10,000 *g* for 45 min at 4°C in order to ensure total lipid removal. The new interphase was carefully collected, aliquoted and stored at −80°C.

**Enzymatic activities assays (aerobic metabolism and larval maturation)**

CS activity was assessed in Nunc™ 96-well microplates (Thermo Scientific Inc.; Waltham, MA, USA). Data were obtained with a microplate reader (Bio-Tek® Synergy™ HT; Colmar, Haut-Rhin, France) and then treated with KC4 v3 software. The assay was adapted from Guderley et al. (2011). Briefly, CS activity was assayed for 10 min at wavelength $\lambda$ = 412 nm and temperature T = 25°C. The microplate was prepared by mixing 20 µL of sample with 160 µL of a mix consisting of 0.25 mM Acetyl-coA, 0.125 mM DTNB and 86.25 mM Tris-HCl, pH = 8. The reaction was initiated by adding 20 µL 5 mM oxaloacetate to each well. The control used for the assay was a commercial enzyme of CS (Sigma-Aldrich® Inc.; St. Louis, MO, USA).

In parallel, COX activity was assessed using Cytochrome c Oxidase Assay Kit, CYTOCOX1 (Sigma-Aldrich® Inc.; St. Louis, MO, USA) following manufacturer's instructions. Briefly,

COX activity was assayed for 1 min at wavelength λ = 550 nm and temperature T = 25°C. The reaction was prepared by mixing 350 µL of assay buffer (10 mM Tris-HCl, 120 mM KCl, pH = 7.0), 80 µL of enzyme buffer (10 mM Tris-HCl, 250 mM sucrose, pH = 7.0) and 20 µL of sample. The reaction was then initiated by adding 50 µL of 0.22 mM Ferrocytochrome c substrate solution.

Larval development was evaluated as described by Cahu and Zambonino-Infante (1995). Protocols for the homogenisation of the pancreatic segments and intestine, brush border purification, protein dosages and enzymatic assay procedures for Amylase, Trypsin, AP and N-LAP are described in Zambonino-Infante et al. (1997). Enzyme activities were expressed as specific activities, *i.e.*, units per mg of proteins.

**Statistical analyses**

Data are presented as means ± s.e.m. (Standard Error of Mean). Statistical analyses were performed using R software (R Core Team, 2013). For all analyses, all response variables were checked for normality with the Shapiro test and for equality of variances using the Levene test. A regression was used in order to determine the influence of oxygen treatment on survival. A two-way ANOVAs were then used (except for survival and maturation analyses, for which a regression and a one-way ANOVA were used, respectively) to determine the influence of oxygen treatment, age of larvae (as main effects) and their interaction on each response variable. When the interaction between oxygen treatment and the age of larvae was significant, Student-test were used to determine significant differences among groups within each sampling time. An ANCOVA was used for CS/COX activity ratio analyses in order to determine the influence of oxygen treatment (as main effect) with age of larvae as co-factor. In addition, a Student-test was used to determine significant differences among groups within each sampling time for CS/COX activity ratio and at 30, 38 and 45 d.p.h. for growth rate. Finally, a one-way ANOVA was used in order to determine the influence of oxygen treatment on larval development. Differences were considered significant at the 95% confidence level (p-value < 0.05).

## Results

No variable differed significantly between the Control (C) and the Hypoxia Treatment (HT) groups prior to the hypoxia exposure, *i.e.* at 30 d.p.h. just before the beginning of exposure.

### Survival and growth of larvae

*Survival*

No significant mortality events were observed in the tanks during and after the period of exposure to hypoxia. This was confirmed by the survival rate estimated at 45 d.p.h., which was not significantly different between the two groups (C = 44.9 ± 7.2%, HT = 44.7 ± 5.3% ; p = 1).

*Larval growth*

Growth of larvae was monitored from the beginning of hypoxia exposure (30 d.p.h.) until 45 d.p.h. (after the return to normoxic conditions). Data were transformed using cubic root following recommendations of Bureau et al. (2000) (**Fig. 3**). Globally, in both groups, the larval growth rate increased with the age of larvae. Statistical analyses revealed that larval growth was affected by oxygen treatment (two-way ANOVA: $p < 0.001$) and by the age of larvae (two-way ANOVA: $p < 0.001$). The interaction between oxygen treatment and the age of larvae was significant (two-way ANOVA: $p < 0.01$). While the mean larval masses were similar between the two groups at the beginning of hypoxia exposure (30 d.p.h.) (C = 4.66 ± 0.17 mg ; HT = 4.67 ± 0.16 mg ; Student-test: $p > 0.5$), they were found significantly different at the end of hypoxia exposure (38 d.p.h.) (C = 13.26 ± 2.29 mg ; HT = 9.23 ± 1.73 mg ; Student-test: $p < 0.01$). This data can be related to the significant impact of hypoxia exposure on the growth rate (two-way ANOVA interaction: $p < 0.01$). Furthermore, growth tend to be higher in HT group after hypoxia exposure resulting in similar mass between the two groups at 45 d.p.h. (C = 31.40 ± 2.97 mg ; HT = 27.79 ± 4.18 mg ; Student-test: $p > 0.09$).

### Regulation of Citrate Synthase and Cytochrome c Oxidase activities

CS and COX activities in the IS of larvae were assessed from the beginning of hypoxia exposure (30 d.p.h.) until 42 d.p.h. (after the return to normoxic conditions) (**Fig. 4**). Overall, CS and COX specific activities increased during larval development in both groups (**Fig. 4 A**

**and Fig. 4 B**) (ANCOVA: $p < 0.001$ and $p = 0.001$, respectively). During hypoxia exposure, *i.e.* from 30 to 38 d.p.h., CS specific activity was not impacted by hypoxia exposure while COX specific activity was higher in the HT group (ANCOVA: $p < 0.001$). Globally, the CS/COX activity ratio exhibit significant fluctuations throughout the experiment (**Fig. 4 C**) (ANCOVA: $p < 0.001$). During hypoxia exposure, *i.e.* from 30 to 38 d.p.h., CS/COX activity ratio was lower in the HT group (ANCOVA: $p < 0.001$). The effect of the oxygen treatment on CS/COX activity ratio is particularly marked at 32, 35 and 38 d.p.h. (Student-test: $p < 0.001$, $p < 0.001$ and $p < 0.05$, respectively). There was no more difference in CS/COX activity ratio between C and HT groups 96h (42 d.p.h.) after the return to normoxic conditions (Student-test: $p > 0.05$).

**Maturation of larvae digestive functions**

Activities of digestive enzymes were determined in both the C and HT groups at 38 d.p.h. (**Table 1**). For pancreatic enzymes, larvae from the HT group exhibited 25% lower trypsin specific activity and showed a significantly 150% higher amylase specific activity than the C group (one-way ANOVA: $p_{Trypsin} < 0.01$, $p_{Amylase} < 0.05$). For the intestinal enzymes, larvae exposed to hypoxia exhibited AP and N-LAP specific activities significantly lower than those from the C group by 78% and 53%, respectively (one-way ANOVA: $p_{AP} = 0.01$, $p_{N\text{-}LAP} < 0.01$).

## Discussion

Since they arrive on the coastline around one month after hatching (Dufour et al., 2009; Jennings and Pawson, 1992), European sea bass (*Dicentrarchus labrax*) are likely to experience hypoxia episodes at the end of the larval period. However, the impact of hypoxic events on their physiology at this developmental stage is still largely unknown. The present study was intended to assess the effects of moderate hypoxia (40% air saturation) applied for a duration of 8 days on 30 day-old sea bass larvae. The hypoxia treatment did not induce any significant mortality. Moreover no significant impact on skeletal deformity rate was observed in juveniles originated from larvae of HT group (data not shown). The absence of effect on survival and malformation rates in the preset study differs from previous data showing that moderate hypoxia induces lower survival rates and higher frequency of vertebral column deformities in first feeding yellowfin tuna (*Thunnus albacares*) (Wexler et al., 2011) and Atlantic salmon alevins (*Salmo salar*) (Sanchez et al., 2011) respectively. This apparent discrepancy of response is consistent with the well admitted species-specific as well as stage-specific response of fish to hypoxia constraint (Richards et al., 2009).

We observed a lower mass in larvae from the HT group at the end of hypoxia exposure resulting from a reduction of growth during the exposition compared to the C group. Evaluation of food intake in larvae is not really easy and necessitates the use of $^{14}$C-labelled feeds (Kolkovski et al., 1997), which was not possible to perform during this experiment. However, it has been well described that hypoxia usually induces reduction in food ingestion (Lakani et al., 2013; Pichavant et al., 2001; Pichavant et al., 2000; Thetmeyer et al., 1999). Seven days after the return under normoxic conditions (*i.e.* at 45 d.p.h.), larvae from HT group recovered the same weight as those from the C group (**Fig. 3**) suggesting a potential compensatory growth. Yet, such a compensatory growth after hypoxia exposure was previously shown in Chinese shrimp (*Fenneropenaeus chinensis*) (Wei et al., 2008); it is likely that a higher number of sampling points after the return under normoxic conditions would have permitted to point out compensatory growth in our study.

In the present study, the lower trypsin specific activity in larvae originated from the HT group suggested that one week moderate hypoxia exposure impaired ingestion of fish. As mentioned by Zambonino-Infante and Cahu (2001), pancreatic maturation in sea bass larvae has been completed by 25 d.p.h. and trypsin activity can be directly related to the dietary protein intake from 35 d.p.h. Therefore, a decrease in trypsin specific activity observed after 35 d.p.h. is generally related to undernutrition (Zambonino-Infante and Cahu, 2001). Again, in the present

study, even if we observed that larvae exposed to 40% air saturation hypoxia continued to have a certain feeding activity, we cannot exclude that they reduced their food consumption, compared to control larvae. Furthermore, in our study, the larvae which have experienced hypoxia exposure exhibited higher amylase specific activity at the same stage of development than larvae from C group. It is well documented that amylase specific activity decrease throughout the larval stage (Péres et al., 1996; Ribeiro et al., 1999; Zambonino-Infante and Cahu, 2001). This difference indicates that the normal decrease of amylase specific activity in larvae was delayed in the HT group. In parallel, the lower AP and N-LAP specific activities in larvae from the HT group revealed that the maturation of the enterocytes was impaired by the hypoxia episode. In marine fish species, particularly European sea bass, it is well admitted that the settlement of an efficient intestinal membrane digestion (characterised by the concomitant increase in AP and N-LAP specific activities) occurred around 30 d.p.h. at 19°C (Cahu and Zambonino-Infante, 1994; Zambonino-Infante and Cahu, 2001). In the present study, these developmental delays could also have contributed to a growth depression as long as the sea bass larvae were exposed to hypoxia, as already observed in fish larvae (Perez-Dominguez and Holt, 2006; Wexler et al., 2011).

We also found a down-regulation of CS/COX activity ratio in the HT group during hypoxia exposure. This down-regulation disappeared after the return in normoxic conditions (**Fig. 4 C**). This down-regulation of CS/COX activity ratio was mainly due to a significant increase in COX specific activity (**Fig. 4 B**) more than a decrease in CS specific activity (**Fig. 4 A**). The CS is a key enzyme localized in the mitochondrial matrix and is involved in the Krebs' cycle whereas COX is the terminal electron acceptor of the electron transport chain located in the mitochondrial inner membrane. These two enzymes are of great importance in aerobic metabolism and provide information on respiration metabolism capacity. The fluctuations of these two key mitochondrial enzymes provide information on the properties and the numbers of the mitochondria present (Guderley, 2007; Lucassen et al., 2003). CS/COX activity ratio could be used in order to estimate changes in mitochondrial size and shape in response to energy-demand conditions (Ibarz et al., 2010). In consequence, the decrease in CS/COX activity ratio observed in our study during the hypoxia period could reflect a lower citrate synthesis over respiratory chain capacities of mitochondria in the larvae from the HT group (Lucassen et al., 2003) as well as a modification of the size and the shape of mitochondria (Ibarz et al., 2010). The similar CS/COX activity ratio observed at 42 d.p.h. indicated that such mitochondrial changes were transient.

311 It is interesting to note that the decrease in CS/COX activity ratio in HT group coincided with
312 a growth depression, but we were not able to establish a significant relationship between
313 growth rate and CS/COX activity ratio. Such relationship has yet been reported by Mathers et
314 al. (1992) in the saithe (*Pollachius virens*).

315 **Conclusion**

316 Altogether, the results obtained suggest that sea bass larvae are able to implement
317 physiological regulations in order to cope with a moderate decrease in ambient oxygen.
318 However, these regulations seemed to have a metabolic cost that impacted growth and
319 development, but not survival. Such consequences could have a strong impact on fish larvae
320 activities, especially on their capacities to escape predation, which could adversely affect the
321 recruitment of sea bass.
322

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**Author contributions**

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**Competing interests**

No competing interests declared.

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**Figures captions:**

**Fig. 1 Schema of hypoxic conditioning at 40% air saturation.** Sea bass larvae were divided into two groups : "C" represents the Control group and "HT" represents the Hypoxia Treatment group in which the level of oxygen was reduced to 40% air saturation for 8 days. Samplings were performed during hypoxia exposure (*i.e.* at 30, 32, 35 and 38 d.p.h.) and after the end of exposure phase (*i.e.* at 42 and 45 d.p.h.)

**Fig. 2 Dissection performed on sea bass larvae at each sampling.** Larvae were dissected into 4 parts: Head (***H***), Pancreatic Segment (***PS***), Intestinal Segment (***IS***) and Tail (***T***). H and T were systematically removed. PS were only sampled on 38-day-old sea bass larvae for pancreatic enzymes activity measurements. IS were used for all enzyme assays

**Fig. 3 Sea bass larvae mass for Control "C" (–) and Hypoxia Treatment "HT" (– –) groups.** Results are expressed as mean mass larvae ± s.e.m. (n = 4 pools of 40 larvae). Statistical analyses revealed that larval growth rate was affected by the oxygen treatment (Two-way ANOVA: $p < 0.001$) and the age of larvae (Two-way ANOVA: $p < 0.001$). Significant interaction between the oxygen treatment and the age of larvae (Two-way ANOVA: $p < 0.01$) indicated that the effects of time on growth (*i.e.* the growth rate) depend upon the oxygen treatment. Additional statistical analyses (represent by asterisks) indicated significant differences in mass between the Control and Hypoxia Treatment groups (Student-test: significant codes : * $p < 0.05$, *** $p<0.001$, NS = No Significant differences). Refer to "Material and methods" section for more details on the statistical analyses

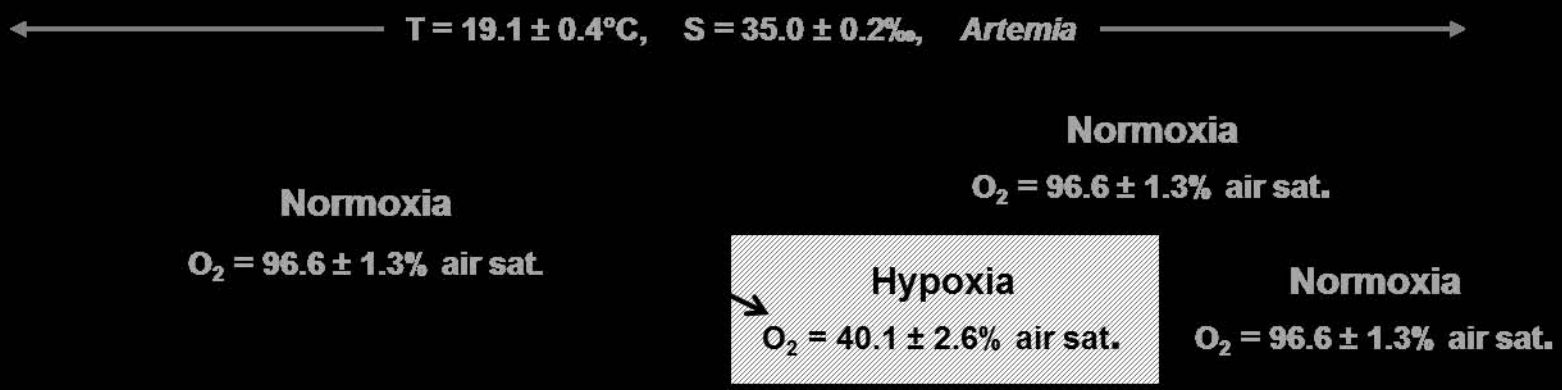
**Fig. 4 Changes in Citrate Synthase (CS) and Cytochrome c Oxidase (COX) activities** for Control "C" (white bars) and Hypoxia Treatment "HT" (grey bars) groups. **A**) Specific enzymatic activity of CS, **B**) Specific enzymatic activity of COX and **C**) Ratio between enzymatic activity of CS and enzymatic activity of COX. Results are given ± s.e.m. (n = 4 pools of 40 larvae). Statistical analyses revealed that COX specific activity and CS/COX activity ratio was affected by the oxygen treatment (ANCOVA: $p < 0.001$ and $p < 0.001$, respectively) and by the age of larvae (ANCOVA: $p = 0.001$ and $p < 0.001$, respectively). In parallel, CS specific activity was not affected by the oxygen treatment (ANCOVA: $p > 0.7$) but was affected by the age of larvae (ANCOVA: $p < 0.001$). Additional statistical analyses

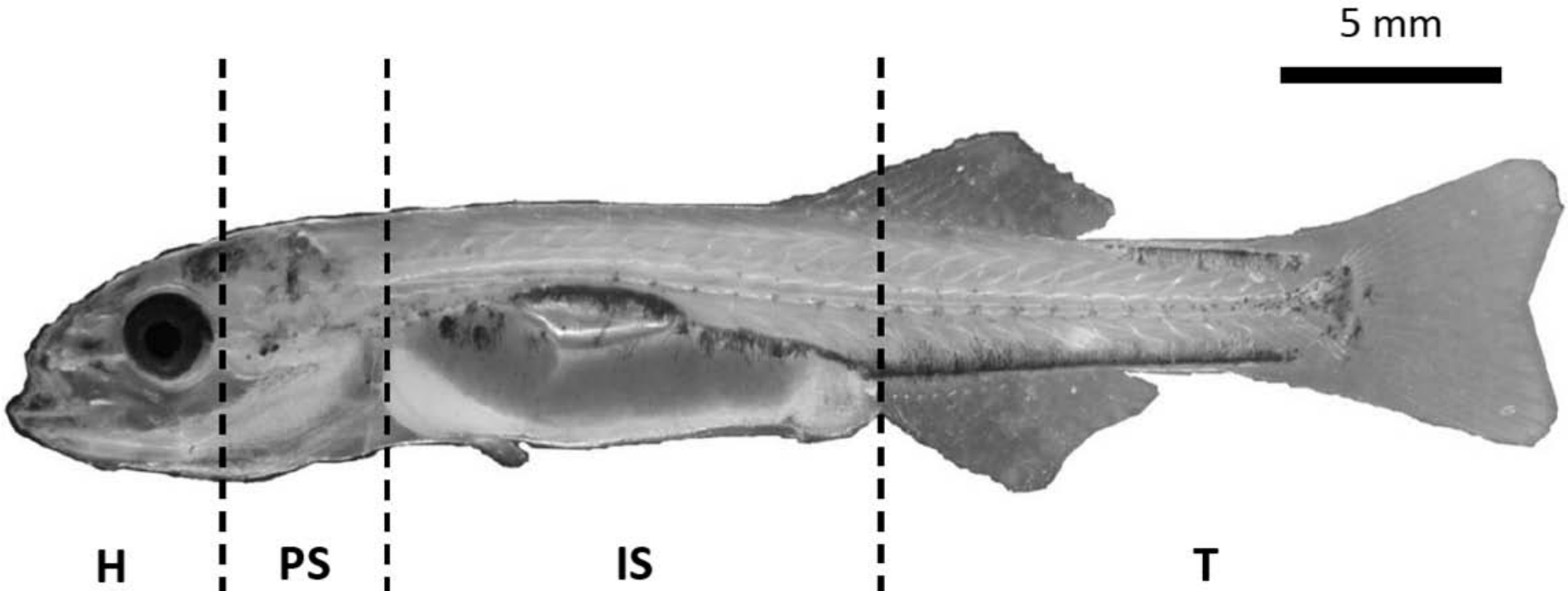
(represent by asterisks) indicated significant differences between the Control and Hypoxia Treatment groups (Student-test: significant codes : * $p<0.05$, ** $p<0.01$, *** $p<0.001$, NS = No Significant differences)

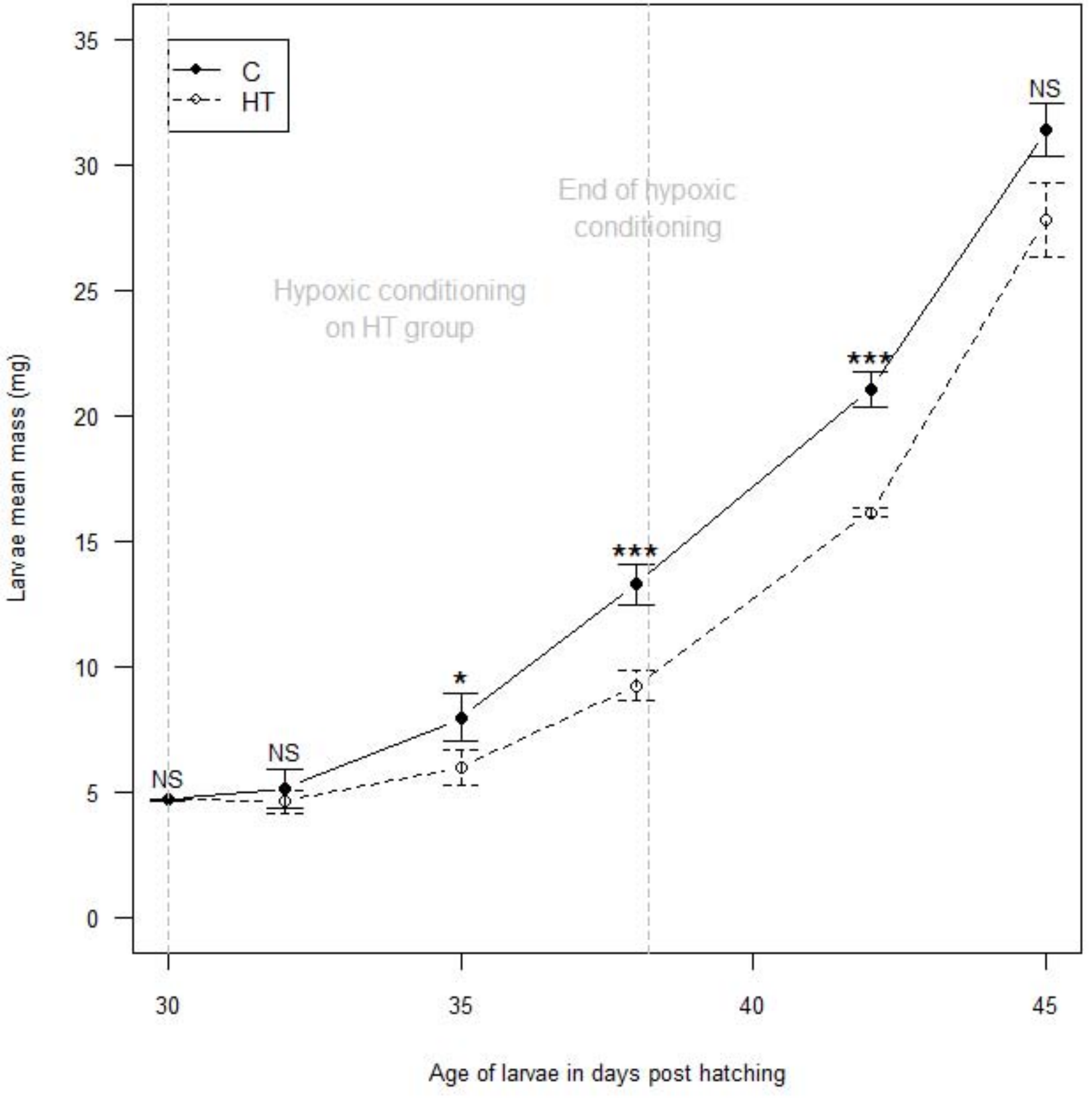
## Tables

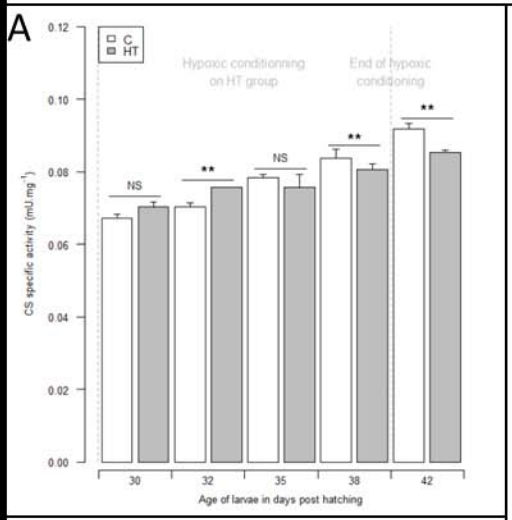
**Table 1: Specific activities of digestive enzymes.** Results are given in milli-units per mg of protein (mU.mg protein$^{-1}$) ± s.e.m. (n = 4 * 35 larvae). Within any one column, means with the same superscript letter are not significantly different (p-value > 0.05). C = Control group; HT = Hypoxia treatment group; AP = Alkaline Phosphatase; N-LAP = Aminopeptidase-N.

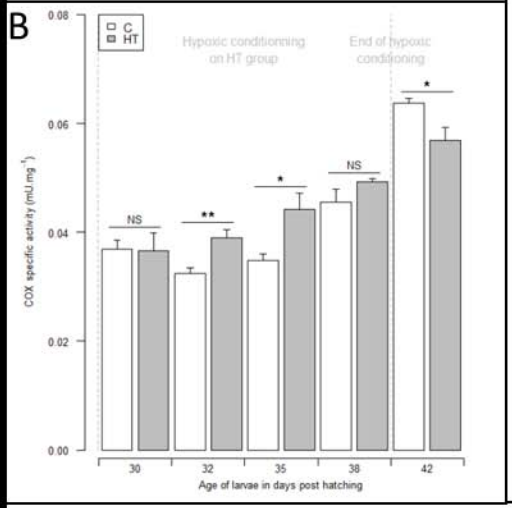
| | Pancreatic enzymes | | Intestinal enzymes | |
|---|---|---|---|---|
| | Trypsin | Amylase | AP | N-LAP |
| C | 42.03 ± 4.00$^{a}$ | 243.89 ± 22.56$^{a}$ | 1242.59 ± 253.57$^{a}$ | 204.12 ± 43.10$^{a}$ |
| HT | 31.41 ± 2.21$^{b}$ | 608.16 ± 112.74$^{b}$ | 276.96 ± 89.67$^{b}$ | 96.84 ± 6.81$^{b}$ |

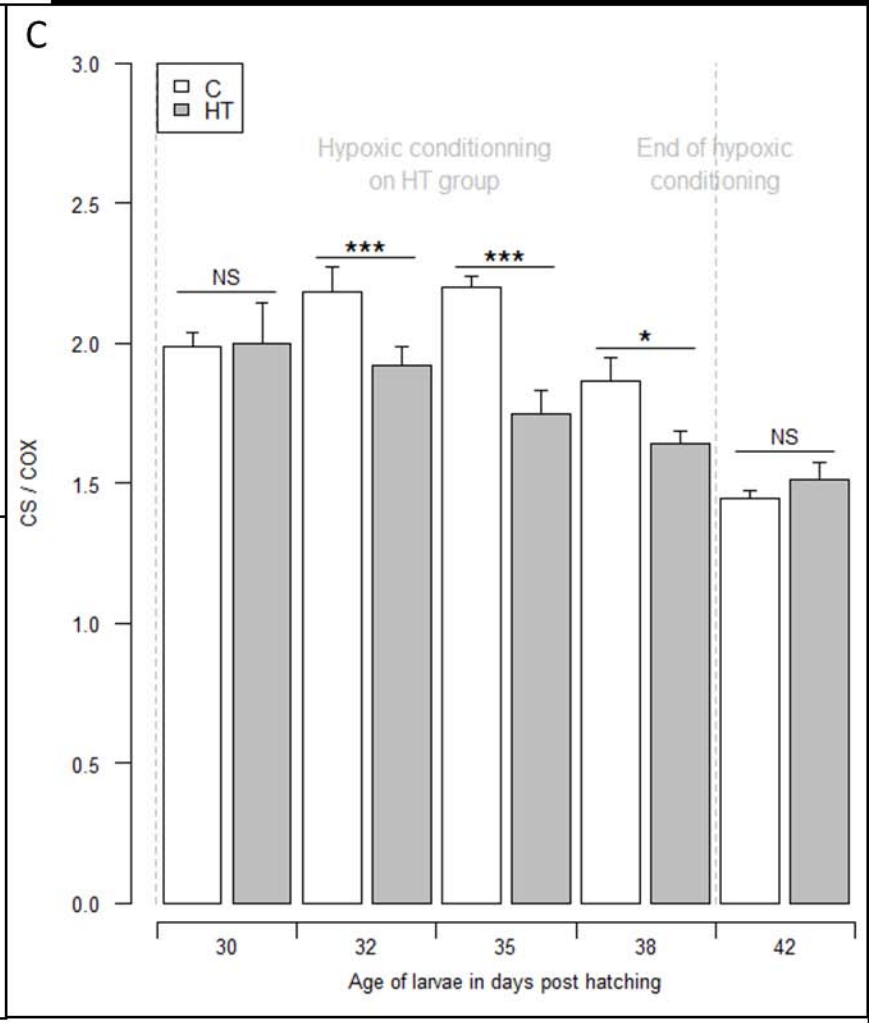

T = 19.1 ± 0.4°C, S = 35.0 ± 0.2‰, Artemia
Normoxia
O2 = 96.6 ± 1.3% air sat.
Normoxia
O2 = 96.6 ± 1.3% air sat.
Hypoxia
O2 = 40.1 ± 2.6% air sat.
Normoxia
O2 = 96.6 ± 1.3% air sat.


5 mm
H
PS
IS
T


C
HT
End of hypoxic conditioning
Hypoxic conditioning on HT group
NS
NS
*
***
***
NS
Larvae mean mass (mg)
Age of larvae in days post hatching
0
5
10
15
20
25
30
35
30
35
40
45


A
C
HT
Hypoxic conditionning on HT group
End of hypoxic conditioning
CS specific activity (mU.mg-1)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
NS
**
NS
**
**
30
32
35
38
42
Age of larvae in days post hatching


B
C
HT
Hypoxic conditionning on HT group
End of hypoxic conditioning
COX specific activity (mU.mg-1)
0.08
0.06
0.04
0.02
0.00
NS
**
*
NS
*
30
32
35
38
42
Age of larvae in days post hatching


C
C
HT
Hypoxic conditionning on HT group
End of hypoxic conditioning
CS / COX
3.0
2.5
2.0
1.5
1.0
0.5
0.0
NS
***
***
*
NS
30
32
35
38
42
Age of larvae in days post hatching