Aerobic and anaerobic metabolism for the zebrafish, *Danio rerio*, reared under normoxic and hypoxic conditions and exposed to acute hypoxia during development

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Abstract

In order to verify the influence of chronic and acute ambient oxygen levels from egg to adult stage of the zebrafish, in vivo oxygen consumption (MO_2), critical tensions of oxygen (Pcrit), heart rate (f_H) and total body lactate concentration (Lc) were determined for *Danio rerio* (Hamilton, 1822) raised at 28 °C under normoxic (7.5 $mgO_2.L^{-1}$ or 80 $mm.Hg^{-1}$) and hypoxic conditions (4.3 $mgO_2.L^{-1}$) and exposed to acute hypoxia during different developmental stages. Our findings confirmed that very early stages do not respond effectively to ambient acute hypoxia. However, after the stage corresponding to the age of 30 days, *D. rerio* was able to respond to acute hypoxia through effective physiological mechanisms involving aerobic and anaerobic metabolism. Such responses were more efficient for the fishes reared under hypoxia which showed that *D. rerio* survival capability increased during acclimation to mild hypoxia. Measurements of body mass and length showed that moderate hypoxia did not affect growth significantly until the fish reached the stage of 60 days. Moreover, a growth delay was verified for the hypoxic-reared animals. Also, the *D. rerio* eggs-to-larvae survival varied from 87.7 to 62.4% in animals reared under normoxia and mild hypoxia, respectively. However, the surviving animals raised under moderated hypoxia showed a better aptitude to regulate aerobic and anaerobic capacities when exposed to acute hypoxia.

Keywords: hypoxia, anaerobic metabolism, *Danio rerio*, lactate, fish.

Metabolismo aeróbico e anaeróbico do paulistinha *Danio rerio*, mantidos sob normóxia e hipóxia moderada e expostos a hipóxia aguda durante diferentes estágios de desenvolvimento

Resumo

A influência de diferentes níveis de oxigênio no desenvolvimento (ovos a adulto) do peixe paulistinha *Danio rerio* (Hamilton, 1822) foi verificada por meio de medidas experimentais de consumo de oxigênio (MO_2), tensões críticas de oxigênio (Pcrit), taxa de batimentos cardíacos (fH) e concentração total de lactato nos tecidos (Lc), para os animais mantidos a 28 ºC sob níveis normóxicos de oxigênio (7.5 $mgO_2.L^{-1}$ ou 80 mmHg) e hipóxicos (4.3 $mgO_2.L^{-1}$) e submetidos a hipóxia ambiental aguda, em diferentes estágios de desenvolvimento. Os resultados obtidos indicam que os peixes em estágios iniciais do desenvolvimento não variam suas respostas fisiológicas em função das oscilações ambientais nos níveis de oxigênio, visto que tais respostas iniciaram-se somente no estágio de 30 dias de vida. A partir deste estágio *D. rerio* apresentou capacidade em responder à hipóxia aguda por meio de mecanismos fisiológicos efetivos envolvendo metabolismo aeróbico e anaeróbico. Tais respostas foram mais efetivas para os peixes mantidos sob hipóxia, o que mostrou que a capacidade de sobrevivência de *D. rerio* aumentou durante o período de aclimatação à hipóxia moderada. As medidas de massa e comprimento corpóreos mostraram que a permanência dos peixes em hipóxia durante o desenvolvimento não afetou esses parâmetro até os peixes atingirem o estágio de 60 dias. A partir deste estágio foi observado ligeiro atraso no crescimento dos espécimes mantidos sob hipóxia. A taxa de sobrevivência de *D.rerio* variou de 87,7 a 62,4% para os animais mantidos respectivamente sob níveis normóxicos e hipóxicos. No entanto, os animais mantidos sob hipóxia moderada, que sobreviveram, mostraram maior capacidade em regular seu metabolismo aeróbico e anaeróbico quando expostos à hipóxia ambiental aguda.

Palavras-chave: hipóxia, metabolismo anaeróbico, *Danio rerio*, lactato, peixe.

1. Introduction

Hypoxia is a constant environmental stressor for many fish species, particularly in freshwater habitats where oxygen concentrations suffer several daily oscillations (Bitar and Bianchini-Jr, 2002). Fish often react to hypoxia by invoking several protective responses (molecular, physiological and behavioural) aiming initially at oxygen uptake improvement through an enhancement of gill oxygen diffusing capacity (Cossins et al., 2006; Kong et al., 2004, Tsenga et al., 2008). Further fish responses usually include metabolic and heart rate reduction, increased circulation of red blood cells, enhancement of hemoglobin affinity, and anaerobic upregulation (Poon et al., 2001; Randall et al., 2004). Fish survival rate varies among different species depending on hypoxia degree and exposition period, and individual adaptation capacities (Anjos et al., 2008).

Oxygen transference from the aquatic ambient to the fish cells decrease gradually in the direction of the cellular ambient, generating a respiratory cascade (Wood and Lenfant, 1979; Hughes, 1984; Soares et al., 2006) that presents as main points of resistance the gill membranes, the blood circulation itself and the cellular membranes of target tissues (Hughes, 1973). Therefore, the lower the aquatic oxygen concentration, even lower will be the oxygen concentration delivered to the metabolising tissues. Considering such characteristics, the cutaneous gas exchange in fish embryos and larvae seems to be quite efficient. Due to the shorter diffusion distance, the larvae skin is as effective as any other organ of gas exchange. Moreover, the partial pressure of oxygen gradient that exists between the blood (less oxygenated) and the ambient water increases the gas diffusion capacity (Rombough, 1988).

Long term exposure to hypoxia affects the development of eggs and larvae which results in malformation, birth defects and high mortality rates (Burggren, 1991; Molles, 1999; Kendra and Jon, 2004). These outcomes occur due to disturbances in normal histogenesis and organogenesis, which are dependent on a series of programmed, highly intricate processes (Wu et al., 2004).

The zebrafish, *Danio rerio* (Hamilton-Buchanan, 1992), is a small sized aquarium fish naturally found in South-Asiatic rivers (Laale, 1977). This cyprinid species has been subjected to several studies since the thirties due to its availability, low cost and ease of care in laboratory settings. Laboratory methods for its husbandry are well established (Westerfield 1994, Wixon 2000). Under appropriate conditions, *D. rerio* reproduces easily and generates large amounts of immersible, non-adherent and transparent eggs. Another advantage is the short development span from fertilisation to hatching (approximately 96 hours, at 28 °C). Just after hatching, the embryo becomes a free-swimming larva, which displays a wide range of behaviours: it swims, swallows, shows escape responses, is touch sensitive, and, by day 5, it appears able to respond to acoustic stimuli (Briggs, 2002). As a result of all these mentioned characteristics, the zebrafish has been the focus of several developmental biology and genetics studies and has emerged as a major model organism for biomedical research (Raymond et al., 2007). Physiological studies include the analysis of sensory neurons, cardiac rhythm disturbances, gastrointestinal function (Farber et al., 2001; Denvir et al., 2008), and studies of the developing organs (Majumdar and Drummond, 2000.). Despite the fact that studies about *D. rerio* physiology have considerably increased during the last decade (Barrionuevo and Burggren, 1999; Bagatto et al., 2001), we are still far from elucidating all its regulatory mechanisms when exposed to ambient oscillations during development. The purpose of this study was to verify the aerobic and anaerobic metabolism of *D. rerio* maintained chronically under normoxic and hypoxic conditions and exposed to acute variations of oxygen concentrations during different development stages.

2. Material and Methods

Danio rerio laid eggs (n ~ 2000, < 24 hours) were obtained from commercial suppliers and reared in 2 litre flasks (n = 100 eggs) at normoxic (~140 $mm.Hg^{-1}$) and moderated hypoxic levels (80 $mm.Hg^{-1}$). A chronic hypoxic condition was maintained by bubbling the aquaria water with the appropriate mixture of oxygen and nitrogen through a precise oxygen control system connected to flow metres. Fishes were kept at 28 °C in 14:10-hours light/dark conditions (Ton et al., 2003). A thermo pump was used to circulate the water and to keep the temperature constant at 28 °C. After 30 days post-hatching the animals were gently distributed into ten 50-litre tanks (100 animals/tank), with the same water temperature and oxygen tensions at which they were previously maintained. Feeding started 4 days post-hatching, according to diet recommendations for each specific age (Westerfield, 1994). Larvae in jars were fed four times daily with *Paramecium* or newly hatched *Artemia* (Bagatto et al. 2001) while the older animals were fed twice daily with TetraMin flakes, except for the 24 hours period preceding measurements. Water from flasks and tanks were filtered daily using a filter with sand and zeolites to eliminate ammonia nitrogen. Water changes (20%) were slowly carried out every 3 days.

2.1. Experimental procedure

In order to verify the influence of chronic and acute ambient oxygen levels on each developmental stage of the zebrafish (embryos and at ages of 10, 20, 30, 40, 50, 60, 70 and 100 days), in vivo oxygen consumption (MO_2), critical tensions of oxygen (Pcrit), heart rate (f_H) and total body lactate concentration (Lc) were determined for *Danio rerio* raised at 28 °C under normoxic (7.5 $mgO_2.L^{-1}$ or 80 $mm.Hg^{-1}$) and hypoxic conditions (4.3 $mgO_2.L^{-1}$) and exposed to acute hypoxia during different developmental stages. During experimental procedures, 10 animals were randomly caught from the

rearing tanks and transferred to the different setups used for each analysed parameter. After measurements, the animals were not returned to the tanks. Animal handling and experiments were conducted according to a protocol approved by the University of Nevada Las Vegas Ethics Committee.

2.2. Body mass and length

In order to verify the influence of hypoxic conditions on *D. rerio* growth, measurements of body mass (wet) and length (snout to tail) were performed immediately after each series of metabolic experiments, according to the procedure used by Barrionuevo and Burggren (1999). For the early stages the animals were weighed with a microbalance. For body mass measurements embryos were first removed from the egg capsule, leaving the yolk sac intact. The collective weight of 10 individual embryos was then determined using a microbalance, and the average weight of that group was calculated. This procedure was repeated 10 times, and consequently data were acquired from an average of 100 eggs. Body length measurement was not conducted in the embryos, which are normally in a highly curled natural posture within the egg. The larvae body length measurements were made to the nearest 0.1 mm using a dissecting microscope fitted with a calibrated scale in one eyepiece. Older fish length measurements were performed with a ruler.

2.3. Oxygen consumption

Oxygen consumption (MO_2) was measured in unhatched *Danio rerio* embryos (<72 hours) and at the ages of 10, 20, 30, 40, 50, 60, 70 and 100 days for the animals kept at 28 °C under normoxic and moderate hypoxic conditions. The animals were introduced into the closed respirometers which consisted of glass syringes with 5 to 50 mL of sterile tap water, according to the animal size (1 g animal/1 mL water). The syringes were then placed in a temperature-controlled water bath. All chambers were covered with opaque material to minimise visual disturbances to the animals. Subsequently all animals rested for 2 hours in the respirometer before experimental procedures, and measurements were made according to Barrionuevo and Burggren (1999). Considering their small size, embryo and 10-day larvae MO_2 was not calculated individually, but for several animals of the same developmental stage. The total mass of several individuals was used to calculate the overall MO_2 within the syringe. This result was then divided by the number of individuals to yield a single point for mass-specific MO_2 for an individual fish at the developmental stage. For the subsequent stages of days 20-100, individual fishes were placed into each respirometer. Furthermore, 50% of the syringe's water was slowly exchanged by air-saturated water (normoxia). Prior to initiating MO_2 measurements, initial water volume was recorded and initial water PO_2 (in $mm.Hg^{-1}$) of each respirometer was measured in a 100 µL water sample injected directly from the respirometer to a water-jacketed O_2 electrode (Microelectrodes Inc.) connected to a Radiometer pHM71 gas meter. Water PO_2, which began to decline due to the MO_2 of the fish, was measured over eight successive 20 minutes intervals in each respirometer. Considering the mass of the fish in each respirometer, along with O_2 capacitance with water at 28 °C, the MO_2 for individual fishes was calculated based on the rate of decrease in water PO_2, according to fish oxygen consumption. Each MO_2 value generated according to each fish metabolism was assigned to one of eight 15 $mm.Hg^{-1}$ wide bins, described by a mean PO_2 of 130, 115, 100, 85, 70, 55, 40, or 25 $mm.Hg^{-1}$ (Barrionuevo and Burggren, 1999). The Pcrit for each studied group was obtained graphically from the intersection of the least-square linear regression line plotted with the decreasing values of MO_2 and the horizontal line passing through the mean MO_2 above those tensions (Hastings and Burggren, 1995; Barrionuevo and Burggren, 1999).

2.4. Heart rate

Heart rate (f_H) was measured in different developmental stages of *D. rerio* reared in either normoxic and hypoxic conditions as previously described. f_H measurements in embryos and day 10 larvae were carried out on individuals placed in a water-filled chamber assembled with Petri dishes. Water flow was maintained through the chamber, with controlled water temperature (28 °C) and PO_2 (130 $mm.Hg^{-1}$). The experimental chamber was placed under an optical microscope connected to a Javelin JE3010 colour camera. All animals were allowed to rest for 2 hours before measurements. In addition, the in vivo f_H was visually recorded through the translucent bodies of the embryos and early larvae. Water PO_2 was then lowered over a 5 minutes period to 110 $mm.Hg^{-1}$ and maintained for 30 minutes before re-measuring f_H. This process was repeated in sequence for PO2 values of 90, 70, 50, 30, and 10 $mm.Hg^{-1}$. Water PO_2 flowing into the holding chamber was controlled by equilibration with gas produced by a Cameron GF-3 gas mixing flowmeter.

Older fish (20 days to adult) f_H measurements were registered in individuals placed in 15 mm diameter glass tubes irrigated with water at a rate of 30 mL/min. The water PO_2 and temperature were controlled as previously described. Animals were allowed to rest overnight before measurements. However, 20 days to adult fish bodies were no longer translucent and appropriate illumination clearly revealed cardiac-induced pulsations of the ventral body wall, which were recorded with a Canon video camera mounted vertically under the glass tube. Water PO_2 reduction was carried out following the same protocol described above. For all experiments, the f_H recorded was counted and analysed 20 times for each animal at each respective development stage and water PO_2.

2.5. Lactate concentration

Whole body lactate content was measured at different *D. rerio* developmental stages (unhatched embryos, at the ages of 10, 20, 30, 40, 50, 60, 70 and 100 days). The

animals reared under normoxic (control) and hypoxic chronic conditions during development were exposed to acute ambient oxygen depletion. Before the experimental procedure, animals at each different stage (n = 20) were transferred to distinct aquaria containing oxygen saturated water (140 mm.Hg^{-1}). Subsequently, the water PO_2 was lowered 15 mm.Hg^{-1} at each 20 minute interval through a nitrogen-oxygen injection controlled system until water PO_2 reached 25 mm.Hg^{-1}. In order to verify *D. rerio*'s capacity to use anaerobic metabolism during development, whole body lactate content was calculated for each developmental group (n = 10) after they were maintained for 1 hour at normoxic (140 mm.Hg^{-1}) or hypoxic (25 mm.Hg^{-1}) level into the experimental tank.

As regards to the measurement of whole body lactate content the animals were withdrawn from tanks and immediately grounded, weighed, and homogenised in perchloric acid 5% (5 mL percloric acid per mg of animal). Then, the content was centrifuged for 10 minutes and the supernatant was removed and mixed to the Sigma 826-UV reagents. The lactate concentration was determined through a spectrophotometer and calculated according to the Sigma 826-UV catalogue.

All studied parameters were confirmed by ANOVA analysis ($P < 0.05$), the Newman-Keuls multiple rank test (NKS), and t-test with 95% significance level.

3. Results

Table 1 shows changes in *Danio rerio* wet body mass and body length as a function of chronological age (0–100 days) and ambient PO_2. The animals were reared at different normoxic (PO_2 = 140 mm.Hg^{-1}) and hypoxic (PO_2 = 80 mm.Hg^{-1}) oxygen levels. For body mass, significant difference was not observed for early stages (embryos to 50 days) reared under both normoxic and hypoxic conditions. Afterwards, at any given chronological age body mass was lowest for the group reared under hypoxic conditions. For body length there was no significant difference at 10-days-stage. Furthermore there was a significant difference in all stages, being higher for the group reared at hypoxic conditions at 20-days-stage and higher for the group reared at normoxic conditions in all further stages. Concomitantly the *D. rerio* eggs-to-larvae (embryo-to-20-days-stage) survival changed from 87.7% to 62.4% in animals reared under normoxia and mild hypoxia, respectively. After the 20 day-stage, no zebrafish death in the stock tanks was observed. In this paper we use the terms "embryo" to describe an individual within an unhatched egg and "larva" for an individual after hatching up to day 20, according to Barrionuevo and Burggren, 1999).

Figures 1 to 3 summarise the oxygen consumption (MO_2), critical tensions of oxygen (Pcrit), heart rate (f_H), and whole body lactate content (L_C) determined for *Danio rerio* (embryo to adult) raised under normoxia, mild hypoxia, and exposed to acute hypoxia. Embryo MO_2 increased approximately 6 times with hatching and started to progressively decline from the stage of day 10 to day 100 for both experimental groups (Figure 1). The effect of development on MO_2 was highly significant ($P < 0.05$) for all different stages. Conversely, the effect of mild hypoxia rearing did not affect significantly ($P > 0.05$) the MO_2 during all development stages. A progressive decreasing Pcrit as a function of increasing body mass was also observed, indicating a more effective response to hypoxia for the animals with greater body mass. Moreover, there was no significant difference ($P > 0.05$) in Pcrit for both groups from embryo to 20-days stages. Afterwards, Pcrit values were significantly lowest ($P < 0.05$) for the animals reared under mild hypoxia showing a better metabolic response for this group.

Figure 2 illustrates the influence of chronic and acute hypoxic exposition on *D. rerio*'s f_H as a function of development. At normoxic levels both groups presented an increase in f_H of ~40% during hatching, followed by a peak in day 10 and a further progressive decrease until the animals reached the adult stage. Though at normoxic levels the group raised at mild hypoxic presented a slightly but significantly ($P < 0.05$) higher f_H than the control

Table 1. Body mass and length verified for *Danio rerio* reared under normoxia (140 mm.Hg^{-1}) and mild hypoxia (80 mm.Hg^{-1}).

Age, days post fertilisation	Mass, mg			Length, mm		
	Normoxia	Hypoxia	*t*-test	Normoxia	Hypoxia	*t*-test
Embryo	0.4 ± 0.1	0.5 ± 0.2	P = 0.142	-	-	-
10	0.4 ± 0.2	0.5 ± 0.1	P = 0.302	3.1 ± 0.8	3.6 ± 0.4	P = 0.093
20	2.0 ± 0.8	1.5 ± 0.8	P = 0.099	3.1 ± 0.3	3.5 ± 0.5	P = 0.033
30	4.1 ± 0.8	3.1 ± 0.8	P = 0.060	6.3 ± 1.2	5.2 ± 0.7	P = 0.001
40	8.3 ± 2.2	6.4 ± 2.2	P = 0.064	8.9 ± 7.0	6.9 ± 1.1	P < 0.001
50	10.4 ± 2.5	8.1 ± 2.5	P = 0.076	10.9 ± 1.7	8.6 ± 2.1	P = 0.013
60	89.7 ± 3.3	59.4 ± 3.3	P < 0.001	14.2 ± 2.1	11.2 ± 2.5	P = 0.009
70	236.4 ± 7.3	87.1 ± 7.3	P < 0.001	18.4 ± 3.3	13.0 ± 2.9	P < 0.001
80	352.2 ± 4.4	124.7 ± 4.4	P < 0.001	23.9 ± 3.9	15.8 ± 3.1	P < 0.001
90	473.3 ± 5.8	211.5 ± 5.8	P < 0.001	28.1 ± 2.9	20.5 ± 3.3	P < 0.001
100	501.3 ± 5.4	321.5 ± 5.4	P < 0.001	30.6 ± 3.2	26.2 ± 3.2	P = 0.006

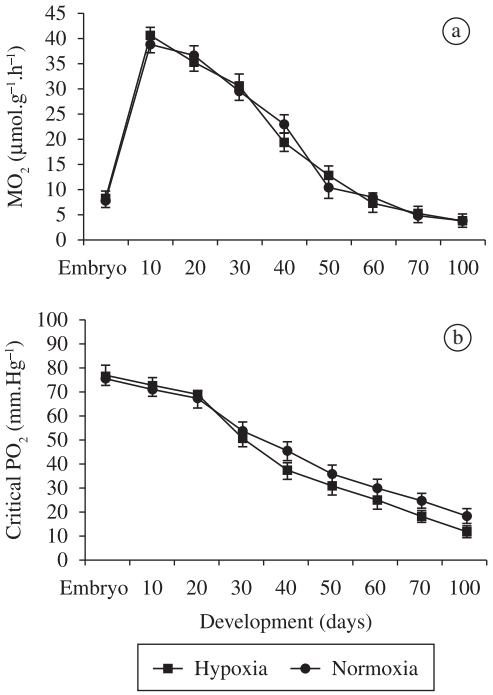

Figure 1. Oxygen consumption (MO_2) and critical oxygen tension (PO_2), determined for *Danio rerio* raised under normoxia and mild hypoxia, and exposed to acute hypoxia during different developmental stages (embryo to adult).

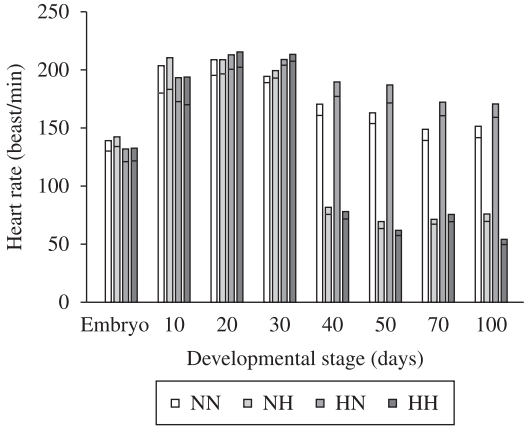

Figure 2. Heart rate measurements verified for *Danio rerio* during different developmental stages. NN: animals raised and kept at normoxic water levels; NH: animals raised in normoxia and exposed to acute hypoxia; HN: animals raised in mild hypoxia and exposed to normoxic water levels; HH: animals raised in mild hypoxia and exposed to acute hypoxia.

group after the animals reached the stage of day 20. After exposure to acute hypoxia bradycardia was not verified in both groups until they reached the stage of day 30. For all subsequent stages, bradycardia was also present in response to ambient progressive hypoxia. Such response under acute hypoxia approximately paralleled changes

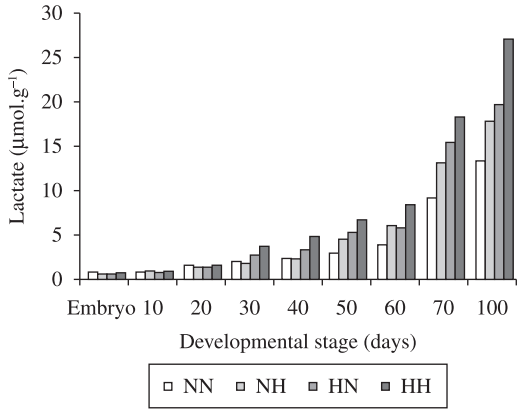

Figure 3. Lactate concentration determined for *Danio rerio* (embryo to adult) raised under normoxia and mild hypoxia, and exposed to acute hypoxia during development. NN: animals raised and kept at normoxic water levels; NH: animals raised in normoxia and exposed to acute hypoxia; HN: animals raised in mild hypoxia and exposed to normoxic water levels; HH: animals raised in mild hypoxia and exposed to acute hypoxia.

in L_C (Figure 3) which presented no significant differences ($P > 0.05$) in their values for both experimental groups at embryo-to-20 days stages exposed to acute hypoxia. Afterwards (stages 30 to 100 days), acute hypoxic conditions caused a significant increase in L_C in both groups. However, such increase was significantly higher ($P < 0.05$) for the group reared under mild hypoxia.

4. Discussion

Hypoxia occurs over large areas in aquatic systems worldwide, and may affect aquatic animals, leading to population decline and changes in community by elimination process of sensitive species (Shang and Wu, 2004). It is generally accepted that embryonic and larval development are much more sensitive to stress than adult stages (Rand et al. 1995; Rolland et al. 1995; Connell et al. 1999) since normal histogenesis and organogenesis depend on a series of programmed, highly intricate processes. According to Wu et al. (2004), disturbance of any of these processes is likely to result in malformation and birth defects, which may ultimately affect fish growth and survival.

Changes in body mass and body length are simple and inexpensive direct means of indicating actual influence of hypoxia on fish growth (Cheung et al., 2007). In our study we found that moderate hypoxia did not affect *D. rerio* growth significantly until they reach 60 days. A growth delay was then verified in the animals reared under hypoxia when compared with the control group which confirms the significant interaction between environmental oxygen concentrations and *D. rerio* growth. Similar results were obtained by Jacob et al. (2002) who studied zebrafish embryo-to-larvae and did not find any growth and morphology differences for the animals

raised under either normoxic or hypoxic conditions during two weeks.

Several other researchers have investigated the effect of hypoxic conditions on fish development and survival. Burton et al. (1980) observed that a small variation in degrees of hypoxia greatly affected the growth rates of juvenile spot, since exposure to 0.8 $mgO_2.L^{-1}$ resulted in 5% mortality, and exposure to 0.6 $mgO_2.L^{-1}$ resulted in 95% mortality. Randall et al. (2004) investigated the effect of an extreme hypoxic condition (0.5-0.8 $mgO_2.L^{-1}$) on zebrafish survival and found that egg production was reduced to only 9/fish after the first day, compared to 52/fish in the control group. Roesner et al. (2006) observed a decrease in the survival rate of more than 80% in early zebrafish reared at ~0.9 $mgO_2.L^{-1}$. In addition, Shang and Wu (2004) also verified that sublethal levels of hypoxia (0.5 $mgO_2.L^{-1}$) could produce teratogenic effects as well as delayed embryonic development in zebrafishes. Similar results were found by Wu et al. (2004) for the carp *Cyprinus carpio* embryos reared under hypoxia (1.0 ± 0.2 mgO_2L^{-1}). Carp eggs to larvae survival decreased from 92.3% in the normoxic group to only 4.4% in the hypoxic group. In our study we verified lesser effects of the hypoxic condition on fish growth and survival since fish were only reared under moderate hypoxic levels (4.3 $mgO_2.L^{-1}$ or 80 $mm.Hg^{-1}$). In such conditions, *D. rerio* eggs-to-larvae survival changed from 87.7 to 62.4% in animals reared under normoxia and hypoxia, respectively.

4.1. Metabolic rate

Fish metabolic rates tend to vary continually during development primarily due to differentiation of biological structures and to the increasing animal size, which affects the metabolic rate per se and the physiological process of gas convection and diffusion (Adolph, 1983). According to Wieser et al. (1985), the oxygen consumption (MO_2) in fish eggs is usually low and increases steeply until hatching due to the beginning of embryo muscles and cardiac activities (Gerald and Cech, 1970). Furthermore, the usually higher metabolism maintained in early larvae is probably due to the energy spent with body mass increment. As animals develop, energy expenditure is reduced primarily for its usage in regular body activities. In general, the pattern of metabolic increase or decrease after hatching varies. Wieser and Forstner (1986) verified a decrease just after hatching in three species of cyprinids *(Rutilis rutilis, Scardinius erythrophthalmus,* and *Leuciscus cephalus*) and De Silva et al. (1986) noticed a similar pattern of decrease in *Oreochromis niloticus.* A more common pattern consists of a continuous MO_2 increase until the endogenous food (yolk sac) is almost finished (Lasker and Theilacker, 1962, Wieser and Forstner,1986; Rombough, 1986). In such studies larvae were not fed, so a faster decrease in MO_2 occurred as soon as endogenous food was used. According to Rombough (1988), depending on the studied species, during larvae feeding the MO_2 tends to increase during metamorphosis, and it gradually decreases until the animal reaches the adult age. Such behaviour of reaching an early peak of mass-specific metabolism early in overall development followed by a decline of metabolic change may be a general vertebrate pattern since it has also been demonstrated for anuran amphibians (Burggren and Just, 1992), reptiles (Burggren and Pinder,1991), and chickens (Howe et al., 1995). In our study the MO_2 in normoxia increased 6 times just before hatching. This occurred probably due to organogenesis and to the increase in the animal's movements, which continued relatively high until ~10 days. Further, the MO_2 values decreased gradually until the animal reached the adult stage. The fall in MO_2 in zebrafish from day 10 to day 100, also observed by Barrionuevo and Bruggren, (1999), may be attributed in part to allometry, since body mass rose nearly 400 fold during this period.

4.2. Critical oxygen tensions (Pcrit)

Under increasing ambient hypoxia fish try to maintain their standard metabolic rate until they reach the critical partial pressure (Pcrit) of oxygen. Beyond the critical point, the fish start to lose capacity to regulate their standard metabolic rate and use anaerobic metabolism. Under such conditions, fish survival is usually limited to few minutes or hours (Steffensen, 2006). Therefore, the lower the Pcrit, the greater tolerance to hypoxic conditions the animal presents. Rombough (1988) reported that in larval steelhead, *Salmo gairdneri*, Pcrit declines from a peak of 140 $mm.Hg^{-1}$ (just under air saturation) at hatching to a value of 75 $mm.Hg^{-1}$ after 30 days post fertilisation. A fall in Pcrit with development has also been documented in larvae of the anuran amphibian *Xenopus laevis*, where Pcrit at 20 °C falls from near air saturation in water breathing larvae to ~75 $mm.Hg^{-1}$ once air breathing begins, terminating at a value of just 30 $mm.Hg^{-1}$ in adult frogs (Hastings and Burggren, 1995.). In the present study specimens of *D. rerio* reared under hypoxia presented a lower Pcrit when compared to the control group, after they reach the 30 days stage, which demonstrate that their ability to maintain aerobic metabolism has increased during acclimation to hypoxia. Another important find was that adult zebrafish either reared under normoxia or hypoxia presented a low Pcrit when compared to other teleosts. Such behaviour displayed a high physiological capacity of this species to respond to ambient oxygen oscillations.

4.3. Heart rate

The embryonic circulation of lower vertebrates has been a fascinating issue for a number of researches. Because the heart is easily observed through the embryonic body wall most developmental studies are carried out considering heart rate (f_H) changes throughout all development stages. According to Burggren and Pinder (1991), the pattern of f_H varies between species and does not correlate with vertebrate class or even with families. In the present work, *D. rerio* embryos presented ~40%

increase in f_H at hatching, followed by a peak in days 10 and 20, and further presented a progressive decrease until the animals reached adult stage. A similar pattern of change in resting f_H during larval development occurs in brown trout (Grodzinsky, 1950.) and rainbow trout, in which the f_H increases at hatching but then declines again a few days after hatching (Holeton, 1971). However, the typical maximum f_H peaks appear much earlier around hatching and then decreases with further development for other teleosts such as walleye and Arctic chair (Burggren and Pinder, 1991). Although different vertebrate species show considerable variation in f_H patterns during development, there is a general tendency for the f_H of lower vertebrates to rise sharply during early development until it reaches an apex, and then it presents a decline (Burggren and Warburton, 1994). The cause of these general changes in resting f_H during development remains unknown, but it may involve the animal's movement, increase in body mass, organogenesis, and changes in the membrane permeability of the cardiac pacemaker as well as the onset of sympathetic and parasympathetic cardiac tone (Barrionuevo and Burggren, 1999).

In order to identify the point in the development when cardiac activity becomes responsive to hypoxia, we raised zebrafish under normoxic and hypoxic conditions. At ambient normoxia the groups raised either at normoxic and hypoxic levels presented an increased f_H during hatching and a progressive decrease after the stage of day 20. However, the f_H was slightly but significantly higher at any developmental stage from 20 to 100 days in the group raised under mild hypoxia. Different results were verified by Moore et al. (2006) who found that hypoxia-reared zebrafish had significant lower heart rates. Jacob et al. (2002) studied early zebrafish stages and found that cardiac activity in the small zebrafish larvae already becomes responsive to hypoxia at the time of hatching. They also verified an increase in both stroke volume and in cardiac output at days 4 to 6 after hatching in animals raised under hypoxic conditions. Nevertheless convective oxygen transport became crucial for aerobic metabolism only ~2 weeks after fertilisation.

During progressive exposure to hypoxia many lower vertebrate respond with a reflex bradycardia of varying magnitude (Burggren and Pinder, 1991). According to Farrell (2007), bradycardia can afford a number of direct benefits to the fish heart. Considering that the oxygen supply to the spongy myocardium by venous blood is precarious the bradycardia increases the diastolic residence time of blood in the lumen of the heart, increasing time for diffusion and improving cardiac contractility. Moreover, an increase in stroke volume stretches the cardiac chambers, which potentially reduces the diffusion distance for oxygen. In spite of such benefits, bradycardia appears to be absent in early embryo fishes (Holeton, 1971; Barrionuevo and Burggren, 1999; Jacob et al. 2002). However, the decrease in f_H during hypoxic events that some embryo vertebrate shows is usually considered a direct cardiac depression caused by tissue hypoxia rather than a reflex slowing of heart beat (Burggren and Doyle, 1986; Burggren and Pinder, 1991). Nevertheless, even in the absence of bradycardia, some larval fish can develop a mild tachycardia (McDonald and McMahon, 1977; Burggren and Pinder, 1991; Jacob et al., 2002) which indicates that larval heart is not completely irresponsive to ambient hypoxia. In addition, Pelster (1997) demonstrated the early presence and functioning of oxygen sensors due to the stimulation of environmental hypoxia in very early fish larvae. In the present study, both experimental and control groups presented bradycardia from the stage of day 30 to adult stage when exposed to progressive hypoxia. Such response coincided with the other studied parameters (MO_2, Pcrit and lactate contents) and indicated that physiological regulation was mostly maintained after *D. rerio* reached stage of day 30.

4.4. Anaerobic metabolism

Under severe hypoxia, physiological adjustments do not occur efficiently enough to allow the organism to maintain its energetic needs solely via aerobic metabolism. As a result, several fish larvae have developed the ability to supply metabolic demand through anaerobic metabolism producing lactic acid as the end product (Gnaiger, 1979; Gnaiger et al., 1981). According to Rombough (1988), fish embryos have typical high anaerobic resistance due to their ability to obtain energy through anaerobic metabolism. However, Nilsson and Östlund-Nilsson (2008) defended that large individuals exposed to extreme hypoxia present a clear advantage over smaller ones due to the fact that small fishes reach lethal levels of anaerobic end-products much faster because of their higher mass-specific metabolic rate. The results of the present investigation demonstrated that a significant increase in whole body lactate content in *D. rerio* occurred from unhatched embryo to adult stages. Nonetheless, the animals started to respond to acute hypoxia only after they reach the stage of day 30. Afterwards both groups raised under normoxic (control) and hypoxic levels responded to acute hypoxia by increasing their whole body lactate content. However, the group raised under lower oxygen concentration presented much higher anaerobic capability. Such findings confirm other previous studies made by Jacob et al. (2002) and Pelster et al. (2003) with early zebrafish (unhatched egg to 21 day stages), where the authors verified that early *D. rerio* did not respond to acute hypoxia. During the stage of day 21, an increase in vascularisation and mitochondria concentration occurred, which indicated tissue oxygenation enhancement. Subsequent studies showed that chronic exposition of *D. rerio* to hypoxia during development triggered a progressive expression of genes involved in anaerobic metabolism (Roesner et al., 2006) and an increase in red blood cell concentration two weeks after hatching (Schwerte et al., 2003). Barrionuevo and Burggren (1999) also verified that the *D. rerio* cardio-respiratory responses became effective only after they reached the stage of day 30.

Our findings confirm that very early stages do not respond to hypoxia. However, after the stage of day 30, *D. rerio* is able to respond to acute hypoxia through effective physiological mechanisms involving anaerobic metabolism. Such responses were more effective in the group raised under chronic hypoxic exposition. Therefore we conclude that acclimation to moderate hypoxic conditions improves *D. rerio* ability to tolerate acute ambient hypoxia. Such results are in accordance with genetic studies that found that genes encoding enzymes for glycolysis and fermentation were more strongly expressed after long term hypoxia in adult goby fish, *Gillichthys mirabilis* (Gracey et al., 2001), and in adult zebrafish (van der Meer et al., 2005).

In general the animals raised at moderated hypoxia presented a slight decrease in survival and growth rate, but the surviving animals displayed a better aptitude to regulate aerobic and anaerobic capacities when exposed to acute hypoxia.

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