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Effects of hypoxia on buoyancy control and the development of lordosis in physostomous and physoclistous fish species

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ABSTRACT

The swimbladder is a well-known adaptation with which many fish species create upward hydrodynamic forces to prevent them from sinking. Development and survival rates are adversely affected by deflated swimbladders. Based on morphology, an open swimbladder system (physostomous) and a closed swimbladder system (physoclistous) are distinguished. In this study two physostomous species, zebrafish (*Danio rerio*) and goldfish (*Carassius auratus)*, and two physoclistous species, tilapia (hybrid of *Oreochromis mossambicus* and *Oreochromis niloticus*) and the cichlid (*Haplochromis piceatus)* were exposed to severe chronic hypoxia. The zebrafish showed reduced buoyancy. X-ray pictures and MRI scans showed that all individuals exposed to severe hypoxia suffered from deflated swimbladders after three weeks. To maintain their position in the water column under hypoxic conditions, zebrafish redirect their swimming movements and swim at an angle of around 45 degrees, which ultimately leads to the development of lordosis. The other three tested species were able to keep their swimbladders inflated and maintained their buoyancy. As a result, none of them changed their swimming movements or developed lordosis. Our results demonstrate that coping with low oxygen levels is done in a species specific manner and that severe chronic hypoxia effects zebrafish on the long term.

KEYWORDS

Hypoxia;
Buoyancy control;
Swimbladder deflation;
Lordosis.

INTRODUCTION

Since living tissue in general has a higher density than water, fish must generate upward hydrodynamic forces to maintain their position in the water column. Continuous swimming or hovering with the pectoral fins are strategies that are used by several fish species to prevent them from sinking[1]. Another adaptation, which is energetically more favourable, is the incorporation of buoyancy devices, such as the storage of extra fats in the liver or the swimbladder.

The swimbladder is an air-filled sac, divided into two compartments, located at the dorsal side of many teleost fish. Its volume is compressible, changing the

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buoyancy of the fish according to the depth it is located at[2]. Gas is secreted from the blood into the swimbladder against the pressure gradient because of the combined action of the rete mirabile, a fine meshwork of blood vessels lying close to each other and running underneath the swimbladder's epithelium, and gas gland cells lining the swimbladder cavity[1]. Gas gland cells secrete CO_2 and lactic acid into the blood, thereby inducing a decrease in the oxygen-carrying capacity (Root effect) and lowering hemoglobin's affinity for O_2 (Bohr effect) which is then taken up by the swimbladder[1].

Based on the morphology of the swimbladder in the adult fish, there are two main types distinguished. Physostomous fish possess an open swimbladder, which still has its embryonic connection to the oesophagus, the pneumatic duct. The general assumption is that this type of swimbladder is inflated by gulping air from the water surface[3-8]. Later in development gland cells secrete gas into the swimbladder, but there are also several species known in which the air-gulping reflex persists throughout adulthood[9].

In physoclistous fish the connection between swimbladder and oesophagus is not present, resulting in a closed swimbladder system. Gas enters and leaves via the circulatory system, but the mechanisms of initial inflation of the swimbladder remain largely unknown[4,10]. Some physoclistous species have transient physostomous larvae, which possess a pneumatic duct in early developmental stages[11]. However, this does not mean that air gulping is used for initial swimbladder inflation in all transient physostomous species. It has already been shown that in the case of the transient physostomous larvae of the haddock, *Melanogrammus aeglefinus* (Linnaeus, 1758), initial swimbladder inflation is the result of secretion of CO_2 and lactic acid from the gas gland cells, leading to a gas influx into the swimbladder, and not from gulping air[12]. It thus appears that the systems for swimbladder inflation are species specific.

Swimbladder inflation is not always successful. Deflated or non-inflated swimbladders are a common problem in aquaculture, but also occur in natural populations[13]. Non-inflation of the swimbladder can have serious effects on development and survival. Delayed growth[14,15], increased metabolic rate[6], altered sensory capability and orientation[16], a strongly reduced resistance to environmental stress like hypoxia, and a reduced survival rate[15] have been reported.

In this study we are interested in the effects of hypoxia on swimbladder inflation and buoyancy control in physostomous and physoclistous fish species. We expect that fish with an open swimbladder system will have more problems keeping their swimbladder inflated under hypoxic conditions. The loss of buoyancy might result in adapt swimming behaviour. Four fish species were studied: zebrafish (*Danio rerio* (Hamilton, 1822)), goldfish (*Carassius auratus* (Linnaeus, 1758)) (both physostomous) and two cichlidae, tilapia (hybrid of *Oreochromis mossambicus* (Peters, 1852) and Oreochromi niloticus (Linnaeus, 1758)) and *Haplochromis piceatus* (Greenwood & Gee, 1969) (both physoclistous). The size of the swimbladder of individuals from the different experimental groups was assessed by X-ray pictures and Magnetic Resonance Imaging (MRI) scans. According to our knowledge this is the first study which directly compares these two groups of morphologically fundamentally different fish on this aspect.

MATERIALS AND METHODS

Animal care and handling

Adult wild-type zebrafish (*Danio rerio* (Hamilton, 1822)) and goldfish (*Carassius auratus* (Linnaeus, 1758)) were obtained from a local pet store. Cichlids (tilapia, hybrid of *Oreochromis mossambicus* (Peters, 1852) and *Oreochromis niloticus* (Linnaeus, 1758), and *Haplochromis piceatus* (Greenwood & Gee, 1969)) have been collected in the Mwanza Gulf of Lake Victoria in 1984 and were bred in our laboratory for about 20 generations.

All fish were kept in identical aquaria of 100 L, at 28°C and with day/night light cycles of 12 h dark vs. 12 h light. All animals were handled in compliance with animal care regulations. Our animal protocols were approved by the review board of Leiden University in accordance with the requirements of the Dutch government.

Experimental design for hypoxia treatment

For hypoxia treatment, oxygen levels were gradually decreased in four days from 100% air saturated water to 40% (day 1), 30% (day 2), 20% (day 3) and

the final 10% air saturation (day 4). At 100% air saturation and 28°C the O_2 concentration is 8 mg/l and pO2 is 150 Torr. After day 4, the fish were kept for an additional 21 days at 10% air saturation (N = 50, in three independent experiments). For the assessment of the breathing rates one group of fish that were habituated to 10% hypoxia were put back under normoxic conditions on the day of the experiment (N = 25). The frequency of breathing movements of this group was compared to breathing rates of zebrafish that were still under hypoxia and a control group that had never been under hypoxic conditions. To test the kinetics of swimbladder deflation one group of zebrafish was directly exposed to 10% hypoxia (N = 25). Swimbladder deflation was assessed by X-ray photography and followed for one day. Another group was put back under normoxic conditions to test the re-inflation of the swimbladder (N = 25). Swimbladder re-inflation was assessed by X-ray photography and followed for six hours. Since the experimental set up did not allow the gulping of air the re-inflation of the swimbladder was due to gas exchange. One group of zebrafish was held at 10% hypoxia for six months to study long-term influence of low oxygen levels (N = 50, in three independent experiments). In parallel, a control group was kept at 100% air saturated water.

For comparison with other teleost species, goldfish (N = 10, in three independent experiments), tilapia (N = 10, in three independent experiments) and H. piceatus (N = 20, in three independent experiments) were subjected to severe chronic hypoxia (10%). The results of these individuals were compared with an equally large control group that were held under normoxic conditions.

The oxygen level at the hypoxia group was kept constant by a controller (Applikon Biotechnology, The Netherlands) connected to an O_2-electrode and solenoid valve in line with an air diffuser. The oxygen level in the tank was kept constant by adding oxygen via the diffuser and thereby compensating the oxygen consumption of the fish. In case of immediate hypoxia exposure, tanks were pre-equilibrated to the respective pO_2 concentration and fish were then directly set in the equilibrated aquaria.

X-ray photography and high-resolution magnetic resonance spectroscopy (MRI)

For the re-inflation experiment zebrafish were anesthetized with MS-222 tricaine methanesulfonate (Argent Chemical Laboratories, USA) and put back into the experimental containers after taking the photo. For other experiments the fish were euthanized with an overdose of anesthetic (MS-222 tricaine methanesulfonate). X-ray films were taken with a Philips Optimus M200 (maximum 150 frames/sec.), using a Kodak CFE film. The X-ray dose was continuously applied, 63 kV and 30 mA.

For MR imaging, adult zebrafish were euthanized with an overdose of anesthetic (MS-222 tricaine methanesulfonate) and immediately embedded in Fomblin (perfluoropolyether). MR imaging was performed using a 400 MHz (9.4T) vertical bore system, using a 20 mm volume coil and a 1 Tm-1gradient insert (Bruker Analytic, Germany) as described previously[17]. For imaging a 2D gradient-echo sequence was used with following parameters: in plane resolution = 78 μm, field of view = 20 mm, slice thickness = 0.2 mm, TR = 175 ms, TE = 4.5 ms, averages = 8 with total scan time of 6 min.

RESULTS

Breathing and swimming behaviour in zebrafish under normoxic and hypoxic conditions

In previous studies we established that zebrafish and cichlids are able to survive in hypoxic conditions as severe as 10% of the normal oxygen content for several weeks to months[18,19]. For the zebrafish, a definite change in swimming behaviour was observed, characterized by a decrease in activities and a complete lack of rapid turns and movements[19]. Here, we describe in detail that whenever swimming movements were stopped, fish were seen to sink rapidly towards the bottom of the aquarium. The zebrafish had to compensate for this loss in buoyancy by adapting their swimming behaviour: in order to maintain their position in the water column, the fish had to direct their swimming movements upward, at an angle of around 45 degrees, and increase the frequency of their swimming movements.

Breathing rates were assessed in fish that were gradually habituated to severe hypoxic conditions, fish that were put back to normoxic conditions and a group that had never been under hypoxic conditions. The rates of breathing, displayed by the opening of the mouth,

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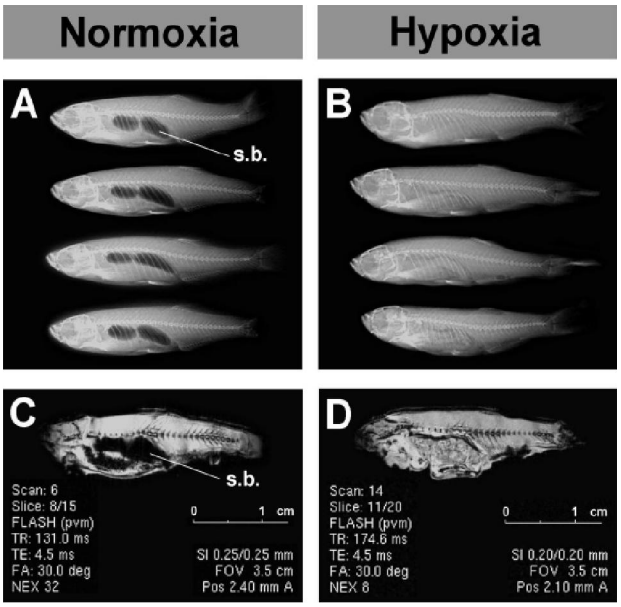

Figure 1 : Deflation of the swimbladder (s.b.) in zebrafish held under severe hypoxic conditions. Shown are zebrafish under normoxic conditions (A and C) and after three weeks under 10% hypoxia (B and D). A and B are showing examples of X-ray pictures and C and D show representative MRI images. Similar results were observed in three independent experiments (N = 50 fish for hypoxia and N = 50 for normoxia in each experiment)

were assessed at three time points, 30 minutes, 1.5 hours and 2.5 hours after the start of the experiment. It was observed that zebrafish under normal conditions had an average breathing frequency of three times per minute. The fish that lived under hypoxic conditions showed a dramatically increased breathing rate, on average 180 per minute, increasing even further during later timepoints. The lowest breathing rate was observed with the fish that had been adapted to hypoxia. After they were put back in normoxic conditions, they opened their mouths on average only once per minute.

Loss of buoyancy in zebrafish exposed to hypoxia is caused by swimbladder deflation

The observed loss of buoyancy in zebrafish exposed to severe hypoxia indicated a possible deflation of the swimbladder. Gradual reduction of oxygen levels to 10% of normal air saturated water in four days led to hypoxia without any induced lethality (see Material and Methods for the experimental procedure). Loss of buoyancy was apparent at day four of the treatment and X-ray photography revealed the deflation of the swimbladder (data not shown). The exposure to severe chronic hypoxia for three weeks leads to complete deflation of the swimbladder (Figure 1).

In order to test the kinetics of swimbladder deflation we exposed adult zebrafish directly to severe hypoxia (10% of normal air saturated water). After 13 hours of exposure no significant decrease of swimbladder inflation was observed and the fish died overnight.

In experiments testing the re-inflation of the swimbladder, we were able to show that loss of swimbladder inflation is reversible and after six hours of exposure to air saturated (normoxic) water, zebrafish are able to fully inflate their swimbladder again (Figure 2).

Exposure to chronic constant hypoxia leads to curvature of the vertebral column

Long term experiments show that chronic constant hypoxia also has long term effects on zebrafish. Because the fish have to compensate for the loss of buoyancy, they direct their swimming movements in an angle of approximately 45 degrees towards the surface. This puts mechanical stress on the vertebral column and the dorsal muscles. After one month an inward curvature of the spine, also know as lordosis, becomes apparent in most of the fish. After six months, all fish in the experiment suffered from heavy lordosis (Figure 3). Zebrafish from the control group showed normal swimming behaviour, maintained their buoyancy, and none of the individuals developed lordosis.

Effects of severe hypoxia on swimbladder function in three different teleost species

Previous studies have shown that coping with hypoxia and swimbladder inflation is done in an highly species specific manner[3,8,20]. This highly species specific reaction might be explained by swimbladder morphology. To extend our data, experiments were performed with three other teleost species: goldfish (physostomous) and two cichlidae, tilapia and *Haplochromis piceatus* (both physoclistous).

Although goldfish are physostomous, like the zebrafish, they did not loose their buoyancy when they were adapted to 10% hypoxia. All individuals showed extensive breathing movements, opening their mouths wider and with a significantly higher frequency than the goldfish under normoxic conditions. Goldfish held under hypoxic conditions showed on average 105 breathing movements per minute, whereas goldfish held at

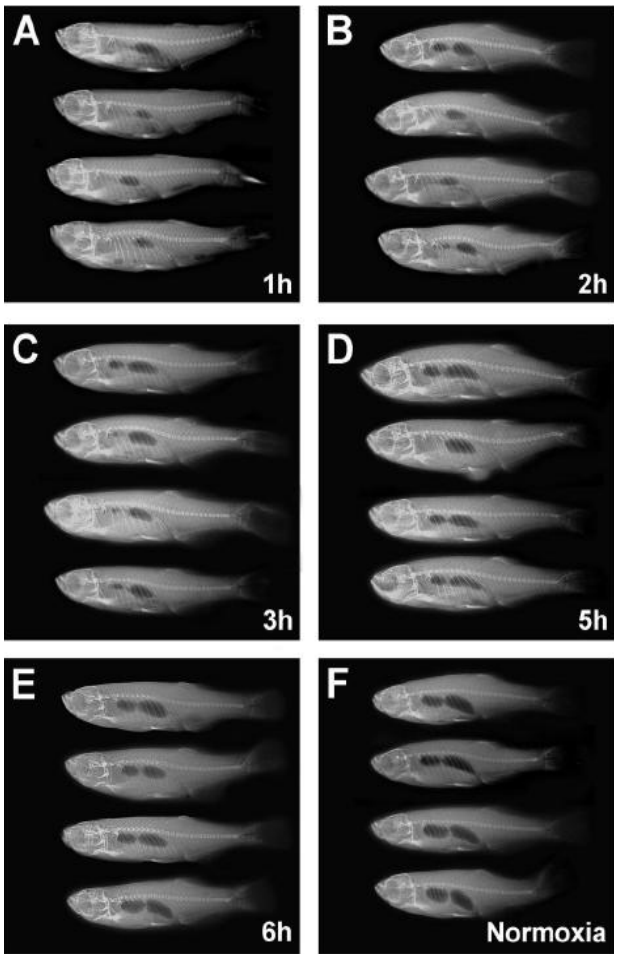

Figure 2 : Re-inflation of the swimbladder in zebrafish. Zebrafish under severe hypoxic conditions suffered from completely deflated swimbladders, but were able to gradually re-inflate the swimbladder within six hours. The state of re-inflation is shown after 1 hour (A), 2 hours (B), 3 hours (C), 5 hours (D), 6 hours (E) and also shown is a normoxic control group (F). Similar results were obtained in two independent experiments (N = 25 fish for hypoxia and N = 25 for normoxia in each experiment)

normal oxygen levels opened their mouths only 21 times per minute. The fish under hypoxic conditions also seemed to reduce their energy expenditure by reducing their swimming movements. Lordosis was observed in none of the goldfish.

Tilapia, a physoclistous fish species, showed behaviour similar to the goldfish. Individuals under hypoxia were frequently opening their mouths, while in tilapia from the control group breathing movements were not that strong. Tilapia at 10% hypoxia opened their mouths on average 99 times per minute, while tilapia held under normoxic conditions had a breathing rate of 13 times per minute. There was no sign of adapted swimming behaviour. The second cichlid species, H. piceatus, seemed to be the least influenced by low oxygen levels. The fish under 10% hypoxia showed the same swimming and breathing behaviour as the individuals under normoxic conditions. Again, buoyancy seemed to be unaffected by these low oxygen levels.

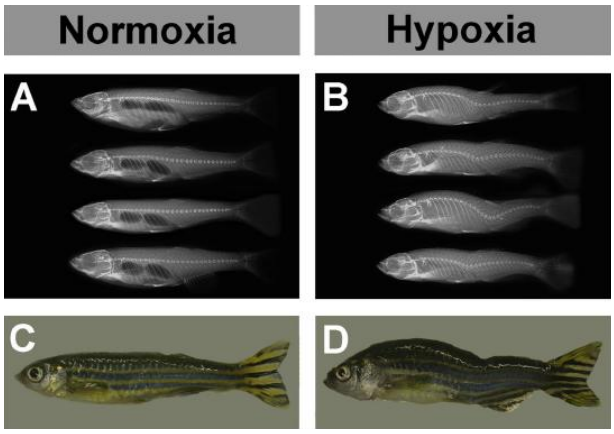

Figure 3 : Long-term effects of severe hypoxia in zebrafish. Zebrafish held under normoxic conditions (A and C) and zebrafish held under 10% hypoxia for six months (B and D). The latter show a skeletal malformation, known as lordosis. Three independent experiments showed comparable results (N = 50 fish for hypoxia and N = 50 for normoxia in each experiment)

X-ray pictures confirmed that only zebrafish and none of the other tested species suffered from a deflated swimbladder after 3 weeks under 10% hypoxia (Figure 4). We further measured the area of the swimbladder in X-ray photographs of three species using IMAGEJ Software. Ten individuals for normoxic and ten individuals for hypoxic conditions were investigated per species. For zebrafish the average area of the swimbladder under normoxic conditions was 20.9 mm2 (s.d. = 2.7) versus 0 mm2 (s.d. = 0) under hypoxic conditions. For goldfish the area under normoxic conditions was 738.2 mm2 (s.d. = 149) versus 792 mm2 (s.d. = 139) under hypoxic conditions and for tilapia 1010.3 mm2 (s.d. = 82) versus 912 mm2 (s.d. = 167) under hypoxic conditions.

DISCUSSION

Our study has shown that adult zebrafish (physostomous) are unable to keep their swimbladder inflated under severe hypoxic conditions. X-ray pictures and MRI scans show full deflation of the swimbladder after three weeks under severe hypoxia.

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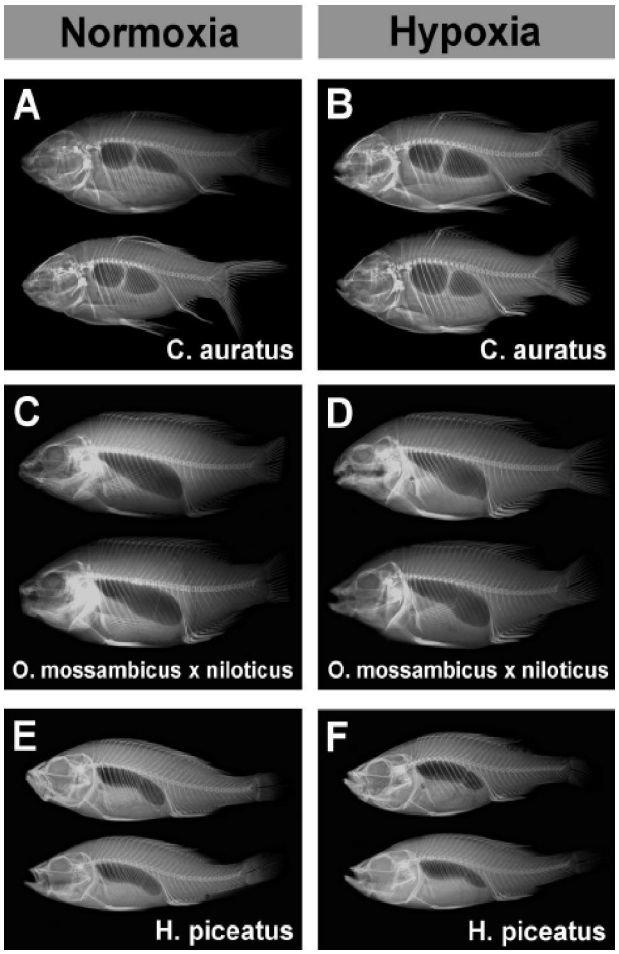

Figure 4 : Three other teleost species that were held under severe hypoxic and normoxic conditions. Shown are examples for goldfish (C. auratus) (A and B), tilapia (hybrid of O. mossambicus and O. niloticus) (C and D) and H. piceatus (E and F). For all three species no significant loss of swimbladder inflation was observed. Three independent experiments showed comparable results (for goldfish and tilapia N = 10 and for H. piceatus N = 20 in each experiment)

This deflation is reversible and zebrafish was capable to fully re-inflate their swimbladders within six hours after they were put under normoxic conditions.

Zebrafish under hypoxia showed abnormal swimming behaviour and appeared to compensate for the loss in buoyancy by increased swimming movements. In order to maintain their position in the water column, the fish had to direct their swimming movements upward, at an angle of around 45 degrees, and increased the frequency of their swimming movements. Our hypothesis is that this unusual angle leads to reorientation of muscle tissue and ultimately to the observed skeletal malformations associated with lordosis. It already has been reported that skeletal malformations such as lordosis can occur in sea bass (Dicentrarchus labrax (Linnaeus, 1758)) and sea bream (*Sparus auratus* (Linnaeus, 1758)) that were forced to swim against a water current[21]. The occurrence of lordosis has also been reported in Japanese sea bream (Chrysophrys major)[22-24], sea bream (*S.auratus*)[25] and sea bass (*D. labrax*)[26] cultures. Further, it is likely that this change in behaviour and the increased movements in zebrafish lead to higher energy expenditure.

Reasons for the absence of a functional swimbladder can be very diverse, ranging from deflation to congestion of the air space because of extreme proliferation and hypertrophy of the cuboidal epithelial cells of the gas gland and proliferation of the rete mirabile[25]. In all cases buoyancy is influenced and the fish have to adopt an aberrant swimming behaviour, like the behaviour that was witnessed in our study. Lordosis is ultimately caused by mechanical stress. To the best of our knowledge, we show here for the first time that exposure to chronic constant hypoxia induces lordosis in an adult fish species, the zebrafish.

The other three fish species examined showed less severe effects under hypoxic conditions. Goldfish and tilapia exposed to hypoxic conditions both showed more frequent breathing motions and, in the case of goldfish, also reduced swimming movements. The other cichlid species, *H. piceatus*, seemed to be less affected by severe hypoxia. All three species maintained their buoyancy and X-ray pictures showed no significant change in the size of the swimbladder.

CONCLUSION

In this study we compared the effects of chronic constant hypoxia on two physostomous and two physoclistous species. Zebrafish, a physostomous species, were unable to keep their swimbladder inflated under chronic severe hypoxia. After four days under 10% hypoxia they showed abnormal swimming behaviour caused by the decrease in buoyancy, which ultimately lead to the skeletal malformations associated with lordosis.

None of the other tested fish species showed equally severe effects under hypoix conditions. All other species were able to keep their swimbladder inflated and maintained their buoyancy. We conclude that fish display a highly species specific response to hypoxic conditions.

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