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Hypoxic tolerance of Chinese black sleeper *Bostrichthys sinensis* embryos at heartbeat stage

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Tolerance of hypoxia in Chinese black sleeper (*Bostrichthys sinensis*) embryos at heartbeat stage was examined at different oxygen concentrations. Embryonic response to hypoxic conditions was expressed in terms of the intensity of variation in heartbeat rate (V). Exposure of the embryos at 25°C to 0.5, 1.0 and 1.5 mg/l dissolved oxygen (DO), caused bradycardia, which was developed within the first 10 min of hypoxia, followed by a plateau, and lasted until termination of the hypoxia. The V values were significantly affected by DO concentrations ($P<0.01$). Exposure of the embryos to 0.2 mg/l DO at 25°C caused a periodic heartbeat (including a period of heartbeat and a period of silence). This phenomenon was first recorded in the present study. During the period of heartbeat, the heartbeat rates were faster at first (147 ±5 beats per min), and then decreased gradually until the period of silence. As the exposure time increased, the duration of heartbeat was prolonged significantly from 43.4 ±2.4 second to 126.2 ±8.2 second ($P<0.01$), and the duration of silence was also prolonged significantly from 68.0 ±5.5 second to 247.9 ±11.5 second ($P<0.01$). At the beginning of exposure, the primary heartbeat rates displayed tachycardia, and their V values were significantly lower than the V values of average heartbeat rates ($P<0.05$). However, the V values were not significantly different between primary heartbeat rate and average heartbeat rate after 90 min exposure ($P>0.05$).

INTRODUCTION

Numerous fish have adopted a fossorial mode of life, and the burrowing mode of life clearly offers a number of advantages, but burrowing fish must also be adapted to cope with the special problems that this life style presents. Fish which form burrows in intertidal sediments will experience more extreme conditions of hypoxia at low tide, when irrigation of the burrow is impossible (Atkinson & Taylor, 1991). There have been few studies on respiratory physiology of burrowing fish, but those available demonstrated that burrowing fish show quite a high tolerance of hypoxia, although tolerance of total anoxia may be low (Gordon et al., 1978). Very little, however, is known concerning tolerance to hypoxia of burrowing fish embryos.

Chinese black sleeper (*Bostrichthys sinensis* Lacepede) belongs to the Family Eleotridae, and is distributed in coastal waters and estuaries in south-east China. The fish species is a commercially important species in China (Hong & Zhang, 2003). It is an euryhaline and eurythermal species, living in the intertidal zone, and it is a seasonally breeding fish. During the non-spawning season, females and males live in individual burrows, but during the spawning season, a pair of fish mate and spawn inside the same burrow (Hong, 2005). After fertilization, eggs swell and form a big perivitelline space, and embryonic development proceeds inside the burrow. Eggs adhere to the substrate at the bottom of the burrow with attaching-filaments.

Compared with other fish embryos at the same stages of development, the rates of oxygen consumption of Chinese black sleeper were similar (e.g. Atlantic cod *Gadus morhua*, Finn et al., 1995; milkfish *Chanos chanos*, Swanson, 1996; seabass *Lates calcarifer*, Sivaloganathan et al., 1998; Senegal sole *Solea senegalensis*, Parra et al., 1999; Chen et al., 2006). However, our field investigation showed that DO concentrations inside the burrows of the Chinese black sleeper were as low as 0.20–1.57 mg/l at low tide (Hong, 2005). How do the Chinese black sleeper embryos cope with these extremely low oxygen concentrations? The aim of this study was to investigate the response of the embryos to hypoxia in terms of the intensity of variation in heartbeat rate.

MATERIALS AND METHODS

The experimental animals were caught in Quanzhou Bay, Fujian, China. After hormonal induction of maturation and ovulation, a semi-dry method was used for artificial fertilization. Fertilized eggs were washed three times to remove extra milt and then incubated in membrane-filtered (0.22 μm) seawater at 17‰ salinity, in the dark, at 25°C.

Water of desired oxygen concentration was prepared by bubbling nitrogen through the water column. Experimental water flow to the inlet/outlet manifold was supplied through glass pipes submerged in a thermoregulated water bath. Each test chamber was a Petri dish of 3.5 cm in diameter and 20 ml capacity. The shape of the test chambers prevented embryos from escaping with out-flowing water. Water flow was 8 ml/min and was supported by a peristaltic pump. Water temperature was measured at the outflow of the chambers. Water samples for oxygen concentration measurements were

taken from the outflow of the supply bottles and from the outflow of the test chambers. Dissolved oxygen concentrations were measured using YSI 55 oxygen electrodes, with an accuracy of 0.01 mg/l.

Before the test, embryos were checked under a low-power stereomicroscope and those without visible malformations at the developmental stages of heartbeat were chosen for the experiments. This is because the *Bostrichthys sinensis* embryo at heartbeat stage is more sensitive to hypoxic conditions than earlier stages (Chen et al., 2006), and the heartbeat rates can be used for measuring any discomfort to embryos caused by hypoxic conditions (Czerkies et al., 2002). For each test, six embryos were used. Successive observations of embryos were carried out at DO concentrations of 0.2, 0.5, 1.0 and 1.5 mg/l at 25°C. In the control treatment, embryos were exposed to normoxic water at a DO concentration of 6.0 mg/l.

The test chamber was placed under a low-power stereomicroscope and the heart rates of experimental embryos were measured after every 5 min of exposure. The time taken for 50 heartbeats was recorded and the frequency of heartbeats (F) was calculated (beats min^{-1}). The intensity of variation (V) was defined as the relative variation in heart rate:

$$V = [(F\mathrm{n} - F\mathrm{h})/F\mathrm{n}] \times 100$$

where V is intensity of variation (%); Fn is heart rate in normoxia; Fh is heart rate in hypoxia.

According to the equation, if the Fh is tachycardia, the value of V is negative; if the Fh is bradycardia, the value of V is positive.

The values are presented as mean ±standard deviation (SD). Analysis of variance (ANOVA) was used to analyse the differences in the V values between different experimental groups at the significance level of 0.05. All statistics were performed with the statistical software SPSS for windows (SPSS, Chicago, IL, USA).

RESULTS

All embryos were alive and showed no recognizable malfunctions during observation, and they hatched normally after the experiment.

Heart rates of the embryos exposed in test chambers containing normoxic water remained unchanged during the test. Exposure of the embryos to hypoxic conditions caused bradycardia. Bradycardia developed within the first 10 min of hypoxia (Figure 1). This was followed by a plateau and lasted until termination of the hypoxia. During the period of plateau, the V values were not positively correlated with exposure time ($P > 0.05$). However, the V values were affected significantly by DO concentrations ($P < 0.01$). The V values increased significantly as DO concentrations decreased. After exposure to hypoxia, the embryos were supplied again with normoxic water—this increased their heart rates, which tended to stabilize at the level observed in normoxia.

When embryos were exposed to a DO concentration of 0.2 mg/l, they displayed periodic heartbeat, namely heartbeat and silence alternately. To examine the dynamic patterns of periodic heartbeat, embryos were continuously observed for 15 min, and during periods of heartbeat, the

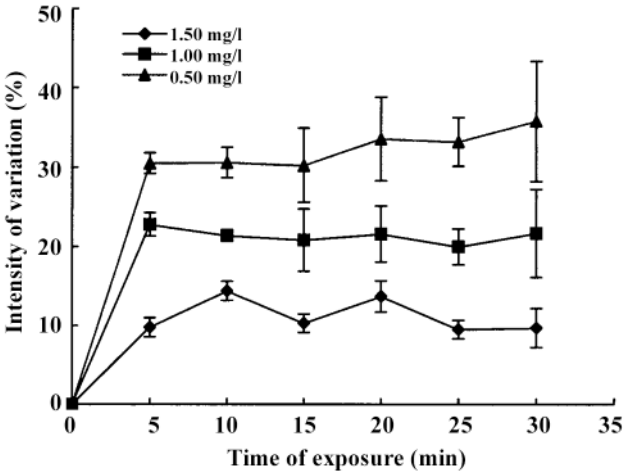

Figure 1. Development of bradycardia in Chinese black sleeper embryos at the developmental stage of heartbeat and erythroblast circulation, exposed at 25.0°C to DO of 1.5 mg/l, 1.0 mg/l and 0.5 mg/l. Data presented as mean ±SD of four to six replicate measurements.

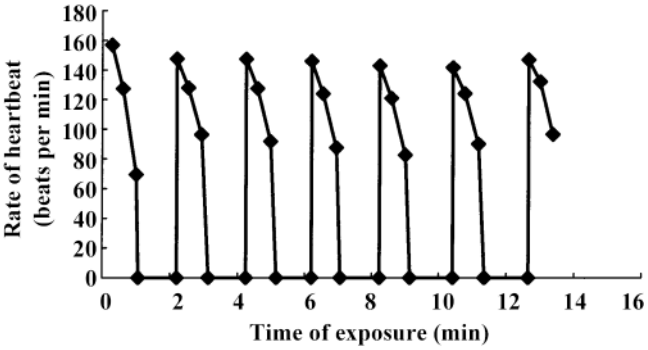

Figure 2. Heartbeat rates in Chinese black sleeper embryos at the developmental stage of heartbeat and erythroblast circulation, exposed to 0.20 mg/l DO at a temperature of 25°C. Data presented as one sample.

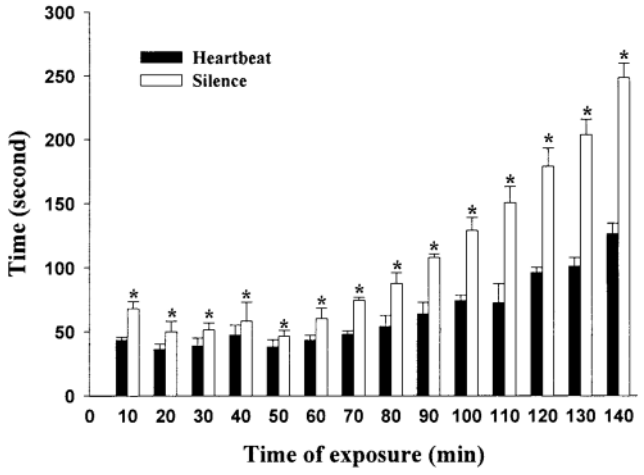

Figure 3. Variation of the time of heartbeat and silence in Chinese black sleeper embryos at the developmental stage of heartbeat and erythroblast circulation, exposed to 0.20 mg/l DO at a temperature of 25°C. Data presented as mean ±SD of four to six samples. *Indicates data with significant difference between duration of silence and heartbeat ($P < 0.05$).

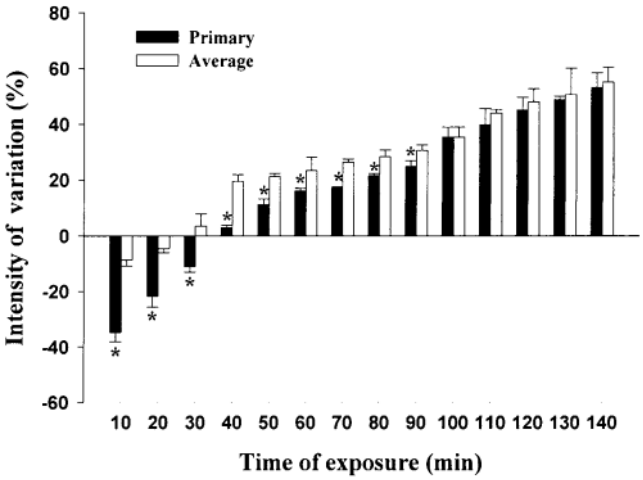

Figure 4. Development of *V* value of first 50 heartbeats and all heartbeats in Chinese black sleeper embryos at the developmental stage of heartbeat and erythroblast circulation, exposed to 0.20 mg/l DO at a temperature of 25°C. Data presented as mean ±SD of four to six embryos. *Indicates data with significant difference between the *V* value of primary heartbeat rate and average heartbeat rate ($P<0.05$).

time of every 50 heartbeats was recorded and then the heartbeat rates were calculated. The results showed that the heartbeat rates were fastest at the beginning of each period of heartbeat (147 ±5 beats per min), and then decreased gradually until silence (Figure 2).

To examine whether the exposure time affected the duration of both heartbeat and silence, a cycle of periodic heartbeat was observed every 10 min, and the duration of both heartbeat and silence were recorded. During a cycle of periodic heartbeat, the duration of heartbeat was significantly lower than that of silence ($P<0.05$; Figure 3). As the exposure time increased, the duration of heartbeat was prolonged significantly from 43.4 ±2.4 second to 126.2 ±8.2 second ($P<0.05$), and the duration of silence was also prolonged significantly from 68.0 ±5.5 second to 247.9 ±11.5 second ($P<0.05$) (Figure 3).

Every 10 min the time of the first 50 heartbeats of a period of heartbeat was recorded. Then the heartbeat rates were calculated and expressed as primary heartbeat rates. The average heartbeat rates were calculated during the period of heartbeat. Both the primary heartbeat and average heartbeat rates were expressed as *V* values. The results showed that at the beginning of exposure, the primary heartbeat rates displayed tachycardia, and their *V* values were significantly lower than the *V* values of average heartbeat rates ($P<0.05$; Figure 4). As exposure time increased, the *V* values were not significantly different between primary heartbeat rates and average heartbeat rates after 90 min exposure ($P>0.05$; Figure 4); the *V* values of primary heartbeat rates increased significantly from −34.7 ±3.4 to 53.1 ±5.3 ($P<0.01$), and the *V* values of average heartbeat rates also increased significantly from −8.8 ±2.2 to 55.0 ±5.4 ($P<0.01$; Figure 4).

DISCUSSION

In this study, under hypoxic conditions (1.5 mg/l, 1.0 mg/l and 0.5 mg/l), the intensity of variations in heartbeat rate increased rapidly within 5–10 minutes, then a plateau was attained and this lasted until termination of the hypoxia. This result is consistent with previous work involving embryos of vendace (*Coregonus albula*) and whitefish (*C. lavaretus*) (Czerkies et al., 2002). But when the value of DO was as low as 0.2 mg/l, the embryo displayed periodic heartbeat, and the heartbeat rate was faster at first but then decreased until silence in each period of heartbeat. To our knowledge this phenomenon has not been reported before. It is generally assumed that hypoxia induced reduction in cardiac activity most likely represents a direct effect of oxygen shortage on cardiac muscle cells (Pelster, 1999), and low oxygen levels would result in reduction of ATP (Padilla & Roth, 2001). Based on these viewpoints, one hypothesis to account for periodic heartbeat is that it could be elicited by low oxygen levels, which reduce oxidative phosphorylation in cardiac muscle and result in ATP depletion during the heartbeat period, and then ATP is replenished during the silent period. This hypothesis can also explain why bradycardia was kept on a plateau under hypoxic conditions (1.5 mg/l, 1.0 mg/l or 0.5 mg/l), because the heartbeat rates must match with the rates of production of ATP by cardiac muscle. If this hypothesis is correct, it would be of interest to determine at what level the ATP can stop and/or elicit the heartbeat.

In the present study, at the beginning of exposure to 0.20 mg/l DO, the first 50 heartbeat rates displayed tachycardia, but decreased gradually to bradycardia 40 min after exposure. This phenomenon could be due to hormonal effects. Several studies have shown that cholinergic and adrenergic receptors are present and functional in the vascular system of vertebrate larvae at the time of hatching or even earlier, and hormonal control of peripheral resistance and of cardiac activity is established prior to hatching. Using this control system, larvae are capable of modifying cardiac activity and peripheral resistance in response to environmental changes, such as hypoxia (Pelster, 2003).

It takes 5–6 days for the Chinese black sleeper embryos to hatch at water temperatures of 23–25°C (Hong, 2005). Hence, a long embryonic development inside the burrows in the intertidal mudflats means that the embryos of this species will experience more extreme conditions of hypoxia at low tide. In order to adapt to the hypoxic environment of the burrows at low tide, the embryos of this species have genetically developed a physiological mechanism that is capable of modifying cardiac activity to cope with hypoxia.

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