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Diel cyclic hypoxia alters plasma lipid dynamics and impairs reproduction in goldfish (*Carassius auratus*)

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Abstract Diel cyclic hypoxia occurs with varying frequency and duration in freshwater habitats, yet little is known about its effects on reproduction of freshwater fishes. The present study shows that long-term exposure of goldfish (*Carassius auratus*) to cyclic hypoxia (0.8 ± 0.2 mg/l dissolved oxygen) for 9 h or more, per day, altered plasma lipid and sex steroid profiles, which in turn directly or indirectly suppressed ovarian growth and viable spermatozoa production. Hypoxia decreased total cholesterol and high density lipoprotein (HDL $p < 0.05$) and elevated triglycerides (TG; $p < 0.05$) in both sexes. Plasma steroid concentrations particularly of 17α-hydroxyprogesterone (17-HP), estradiol (E2), testosterone (T) in females, and T and 11-ketotestosterone (11-KT) in males were attenuated under diel hypoxic conditions. Intriguingly, both diel and continuous hypoxia elevated plasma E2 and vitellogenin levels in males. However, neither lipid nor steroid profiles recorded any variation in a dose-dependent manner in response to diel hypoxia. The reduced GSI, decreased number of tertiary oocytes, and motile spermatozoa in hypoxic fish clearly indicate suppression of gametogenesis. Thereby, prolonged diel cyclic hypoxia may affect valuable fishery resources and fish population structure by impairing reproductive performances and inducing estrogenic effects in males.

Keywords Hypoxia · Diel cycle · Steroids · Lipids · Reproduction · Goldfish

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Introduction

Aquatic hypoxia has been considered a worldwide phenomenon affecting freshwater and coastal waters with severe consequences on aquatic life, including mortality and catastrophic changes, such as loss of biodiversity, forced migration, and habitat destruction. An assessment of literature shows that the number of coastal sites where hypoxia has been reported has increased with an exponential growth rate of 5.54% per year over time (Sunyer and Duarte 2008). The exponential growth rate of hypoxia is expected to increase further owing to combined effects of eutrophication and increase in

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temperature, caused by climate change. Oxygen depletion is increasing in frequency and severity in freshwater ecosystems, largely as a result of eutrophication (Smith 2003). Hypoxic conditions are also routine in aquaculture facilities where overstocking or overfeeding is common. In reservoirs, horizontal expansion of hypoxia tends to be greater than in lakes because of spatial variability in inflow, withdrawals, and loads of particulate organic matter (Thornton et al. 1990). The frequency of hypoxic events may be categorized as aperiodic, periodic, diel, seasonal, and persistent (Richards et al. 2009). Occurrence of diel cyclic hypoxia for variable periods is more frequent than long-term chronic or persistent hypoxia in freshwater systems. Deficiency in molecular oxygen (O_2) adversely affects a broad range of biochemical, physiological, developmental, and behavioral processes, including respiration, growth, metabolism, reproduction, and locomotion (Clayton 1993; Heath 1995). Studies have shown that, similar to endocrine disruptors (Kavlock 1999), hypoxia can also affect endocrine function in both mammals (De Angelis et al. 1996) and fish (Wu et al. 2003) by affecting the brain-pituitary-gonad axis (BPG) (Thomas et al. 2006, 2007; Landry et al. 2007; Fradette and Souich 2004; Shang et al. 2006).

Research on lipid dynamics in relation to endocrine disruption has been gaining attention of scientists in recent decades, because cholesterol is the precursor to all steroid hormones, such as estrogens, androgens, and corticosteroids (Scott 1987). Most steroidogenic tissues (such as ovary and adrenal) obtain cholesterol through receptor-mediated lipoprotein endocytosis, while some tissues (such as testis) differentially utilize de novo synthesized cholesterol as a substrate for steroid biosynthesis (Pederson 1988; Gwynne and Strauss 1982). In addition, lipid dynamics are closely associated with seasons and reproductive cycle in teleosts (Babin and Vernier 1989). In goldfish, estrogen-mimicking compounds like β-sitosterol have been reported to affect endocrine end points and cholesterol concentrations differently in male and female goldfishes at different reproductive state and exposure durations (Sharpe and MacLatchy 2007). Recent research efforts have been focused particularly on physiological, developmental, and reproductive impairment resulting from continuous hypoxia (Nikinmaa and Rees 2005; Wu et al. 2003; Thomas et al. 2007; Shang et al. 2006; Wang et al. 2008). There is little information on effects of hypoxia on plasma lipid concentrations in relation to gonadal development or on the responses of sexually active fish to naturally occurring diel cyclic hypoxia. This has prompted us to investigate the effects of diel cyclic hypoxia on plasma lipid dynamics, sex steroid levels, and reproduction in the hypoxia-tolerant goldfish, *Carassius auratus*. Goldfish, a hypoxia-tolerant species (Thillart 1982), is a classic model for neuroendocrine studies. It has well-characterized spawning cycles regulated by both photoperiod and temperature. The periodic effect of day length shows an opposite effect at two different temperature regimes. Long photoperiod is stimulatory for oogenesis, when associated with low temperature; in contrast, higher temperature induced gonadal regression in the ovary occupied with advanced stages of oocytes, under long photoperiod (Gillet et al. 1978). Moreover, circadian light-sensitive phase has been demonstrated in fish, suggesting that photoperiodic regulation of gametogenesis is mediated through endogenous daily rhythm. Reproduction is controlled by a well-defined BPG axis, wherein titer of circulating gonadotropin (GtH) increases with initiation of gonadal recrudescence. GtH binds to germ cell receptors and stimulates gonadal steroidogenesis, which in turn regulates maturation, ovulation/spermiation, and spawning (Kobayashi et al. 1987). Male androgens, such as testosterone (T) and 11-ketotestosterone (11-KT) (but not estrogens), rise prior to spawning, whereas both androgens and estrogens are present in females during the spawning season (Schreck and Hopwood 1974). The present study aims to understand the effect of cyclic hypoxia on reproduction of a hypoxia-tolerant fish in general and will be helpful for predicting the reproductive damage caused by this abiotic stressor in other species. Specifically, plasma lipid and steroid profiles were evaluated in relation to reproductive parameters in goldfish following exposure to either diel cyclic hypoxia of variable duration or continuous hypoxia for 60 days.

Material and methods

Fish stock and hypoxic exposure

Adult goldfishes (body weight 25.0 ± 0.5 g), obtained from a commercial fish farm in Mumbai, India, were acclimated in aerated water for 2 weeks (22.5 ± 1.5 °C temperature, 6.4 ± 0.2 mg O_2/l, and 14L/10D cycle) prior to experiments. One hundred eighty fishes, comprising females and males in a 1:1 ratio, were distributed

equally in 15 glass aquaria of 30 l capacity and categorized into four experimental groups and one control group in triplicate. The four treatment groups were T1 [6 h hypoxia (H)/18 h normoxia (N)], T2 (9 h H/15 h N), T3 (12 h H/12 h N), and T4 (24 h H). The control groups (C) were maintained at 24 h normoxic condition (6.4 ± 0.2 mg O_2/l). Hypoxic level (0.8 ± 0.2 mg O_2/l) was controlled by using a dissolved oxygen controller coupled with two solenoid valves that regulated the flow of N_2 and air, respectively. Two 24-h timers, equipped with 15-min-interval switching valves, were installed to activate those solenoid valves for introducing oxygen and nitrogen into the test aquaria and holding the tank, respectively, at predetermined intervals. The hypoxic water was incorporated in each treatment aquaria at 5.30 a.m. daily, and within half an hour, each treatment aquarium was transformed from normoxic to hypoxic condition, thereafter maintained for different intervals of time as mentioned for different treatment groups. All the test aquaria were provided with timer-regulated light systems to maintain a light/dark cycle of 14L/10D (lights on 6.00 a.m.) throughout the experimental period. Average water quality parameters in the treatments were maintained as follows: pH 6.5–7.5, NO_2-N 0.001–0.004 mg/l, NO_3-N 0.02–0.06 mg/l. Fish were fed ad libitum twice daily with a commercial feed (protein 35%). At the end of an exposure period of 60 days, 36 fishes (18 males and 18 females) were sampled from each treatment, anesthetized, and weighed individually and tissues were collected thereafter for further analysis. Blood samples collected into heparinized microcentrifuge tubes were centrifuged at 3000 rpm at 4 °C for 5 min (to obtain plasma) and stored at −20 °C till further analysis.

Plasma lipid profiles

Total plasma cholesterol (CHL), low-density lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL), and triglyceride (TG) concentrations were measured by Ecoline assay kit (Merck India Ltd.) specific for individual lipids, using an auto blood analyzer (Selectra Junior, Merck India Ltd.), following the principle of enzymatic calorimetric test. A 120-μl aliquot of the serum sample was taken to analyze CHL, LDL, HDL, and TG following the manufacturer's instructions. Aliquots of serum were diluted eight times in 0.9% NaCl to adjust the concentration within the sensitivity range of the instrument. The absorbance of CHL and TG was measured at 546 nm, whereas the absorbance of HDL and LDL was taken at 600 nm. Values were expressed in millimoles per milliliter (Sharpe and MacLatchy 2007).

Sex steroids and vitellogenin measurement

Estradiol (E2), testosterone (T), and 11-ketotestosterone (11-KT) levels in plasma were assayed using enzyme-linked immunosorbent assay (ELISA, Cayman Chemical Company, MI, USA). The detection limit of E2, T, and 11-KT were 20.0, 6.0, and 1.3 pg/ml, respectively. 17α-Hydroxyprogesterone (17-HP) was analyzed by enzyme immunoassay (EIA, Diagnostic Systems Laboratories, Texas, USA). The detection limit was 9.5 pg/ml. Circulating vitellogenin (VTG) concentrations were analyzed by competitive ELISA using carp vitellogenin ELISA Kit (Biosense Laboratories, USA) as described earlier (Spanò et al. 2004).

Spermatozoa motility and count

Spermatozoa motility assessment was carried out by following the method described in carp by Perchec et al. (1995) and Verma et al. (2009). Milt was diluted with sterile water (1:100) at room temperature (29 °C) on a glass slide, and the movement of goldfish spermatozoa was recorded at 25 frames/s using a camera fixed onto a dark-field microscope (Olympus BH-L obj. ×20). The relative motility (score of 5) in this experiment was evaluated according to the criteria established earlier (Kruger et al. 1984) for the closely related common carp, *Cyprinus carpio*. Counting of sperm was done by diluting milt 1000 times with a suitable extender (Bozkurt 2006) and observing 20 μl of the mixture in a hemocytometer slide under an inverted microscope. Sperm count was expressed as number of spermatozoa per milliliter.

GSI and gonad histology

Gonadosomatic index (GSI) was calculated as the percentage of gonad weight to body weight. Gonads were fixed in formalin, embedded in paraffin, sectioned at 7 μm, and stained with hematoxylin–eosin stains. Oocytes were classified into peri-nucleolus (PN), cortical alveoli (CA), primary yolk stage (PYS), secondary yolk stage (SYS), and tertiary yolk stage (TYS), following Thomas et al. (2007).

Statistical analysis

Statistical significance of different parameters was analyzed using one-way analysis of variance (ANOVA) via SPSS 19.0 for Windows. Duncan's multiple range test was used for post hoc comparison of mean ($p < 0.05$) among hypoxic and control groups. All data presented in the text, figures, and tables are means ± standard error of means (SEM), and statistical significance for all statistical tests was set at $p < 0.05$.

Result

GSI and gonadal histology

Gonadosomatic indices in both male and female goldfish under normoxic conditions (C) had reached 3.2 and 9.2%, respectively, after 8 weeks, whereas the fishes exposed to various hypoxic treatments (T2–T4; Figs. 1a and 2a) attained 0.62–1.24% and 2.50–3.98% GSI in males and females, respectively. The GSI values in both the sexes did not change significantly ($p > 0.05$) at 6 h of cyclic hypoxia (T1). However, there was dramatic reduction in the development and growth of vitellogenic oocytes in the fish exposed to cyclic hypoxia (T2–T4), compared to control (Fig. 1c–e). TYS and SYS were abundant in the ovaries of normoxic groups at the end of 8 weeks, while the percentage (%) of both these stages was reduced significantly ($p < 0.05$) in groups exposed to 9 h (T2) or 12 h (T3) of daily cyclic or chronic hypoxia (T4) (Fig. 1b). Similarly, gonadal recrudescence was significantly ($p < 0.05$) impaired in hypoxic male groups. There was a marked decrease in spermatogenesis and production of spermatozoa in males exposed to hypoxia for nine or more hours daily as compared with the control (Fig. 2d–f). Among goldfish exposed to hypoxia for nine or more hours daily, there were no significant duration-dependent differences in GSI values (Fig. 2a) or histological appearances of gonads between groups (Fig. 2d–f).

Plasma lipid profiles

Plasma cholesterol and HDL decreased significantly ($p < 0.05$) over the period of recrudescence in both males and females (Fig. 3). The concentrations of serum CHL, HDL, LDL, and TG in normoxic females were 12.28, 3.87, 2.1, and 6.00 mmol/ml, respectively. Similar levels were observed in normoxic male groups, except the concentration of plasma TG, which was significantly higher than that of female (Fig. 3a–d). A significant decrease ($p < 0.05$) in plasma cholesterol and HDL, with a concomitant increase in TG, was clearly evident in both the sexes exposed to hypoxia for nine or more hours daily for 8 weeks and plasma LDL remained unchanged over the period in both the sexes. However, 6 h cyclic hypoxia did not cause any significant difference in the lipid parameters examined in both the sexes (Fig. 3). The plasma lipid profiles exhibited significant differences ($p > 0.05$) in between cyclic (T2 and T3) and chronic hypoxic groups of either sex (Fig. 3).

Sex steroids and vitellogenin

Female goldfishes exposed to hypoxia for nine or more hours daily for a period of 8 weeks showed a significant reduction ($p < 0.05$) in plasma 17-HP and E2 (Fig. 4a, d). Reduced plasma E2 attenuated circulating Vtg levels in females in spite of unaltered plasma T levels in all the hypoxic groups compared with the normoxic group over the period (Fig. 4b, c). In males (in spite of no changes in plasma 17α-hydroxyprogesterone levels), hypoxia for nine or more hours daily inhibited ($p < 0.05$) plasma T and 11-KT in males with a marked increase ($p < 0.05$) in E2 except in T1 (Fig. 5). Plasma vitellogenin levels showed a significant increase ($p < 0.05$) in males exposed to either diel cyclic (T2 and T3) or chronic hypoxia (Fig. 2b). Plasma T and E2 levels were found to be highly correlated in males ($r = 0.99$) and females ($r = 0.81$), and the E2/T ratio showed an increasing trend in males compared to the opposite trend in females following hypoxia exposure (Fig. 2c). There were no marked differences either in plasma steroid concentrations or in vitellogenin levels in both the sexes exposed to 6 h hypoxia (T1) in comparison to normoxic groups (C) (Figs. 2b, 4, and 5). Continuous hypoxia showed greater impact compared to diel hypoxic groups (T2 and T3) with regard to all these parameters.

Sperm count and motility

Hypoxia for nine or more hours daily caused marked inhibition ($p < 0.05$) in spermatogenesis as evident by spermatozoa count and sperm motility (Table 1) compared to the control. The average level of reduction was in the range of 69% in hypoxic males compared to the normoxic group. Most spermatozoa were motile with

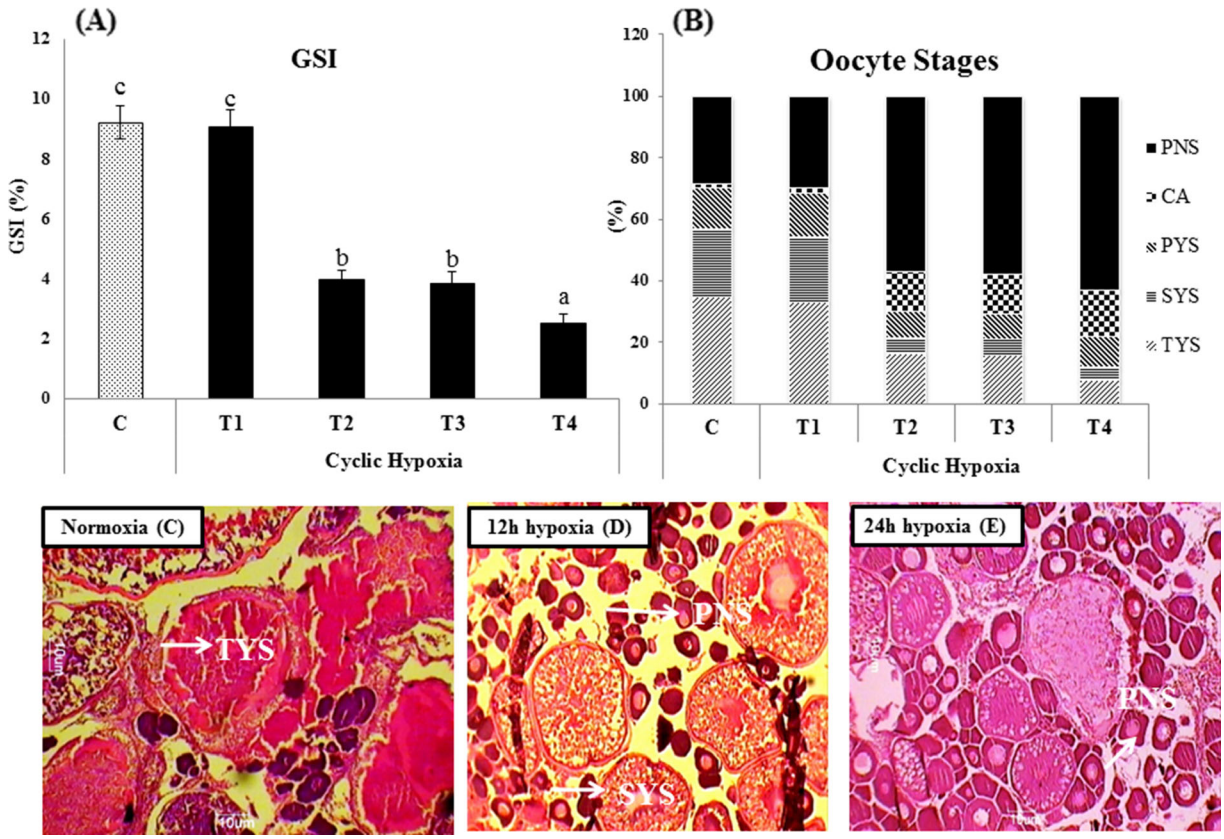

Fig. 1 Ovarian development in goldfish under hypoxia. **a** Oocyte stages. **b** GSI (ovarian growth). **c-e** Histological appearances of respective ovaries. *PNS* peri-nucleolus stage, *CA* cortical alveoli, *PYS* primary yolk stage, *SYS* secondary yolk stage, *TYS* tertiary yolk stage. *Different superscripts* indicate significant difference ($p < 0.05$) among different treatments and control. Values are expressed as mean ± SEM ($n = 18$)

vigorous flagellar movements or exhibited strong vibration in locomotion during normoxia (C), but exhibited cessation in spermatozoa movements with occasional vibration in cyclic hypoxia for more than 9 h (Table 1). However, 6 h cyclic hypoxia (T1) did not show any significant differences in sperm count as well as sperm motility as compared to the control (Table 1).

Discussion

Hypoxia-tolerant fish species generally cope with low DO by increasing oxygen delivery (Hochachka and Somero 2002; Saroglia et al. 2002), by activation of anaerobic glycolysis, and in most cases by metabolic depression and energy conservation (Thillart van den et al. 1989; van der Meer et al. 2005). Interestingly, the present study reveals for the first time that continuous hypoxia as well as diel cyclic hypoxia over 9 h daily for 60 days altered plasma lipid dynamics in goldfish during the late phase of gonadal development. However, exposure to 6 h hypoxia daily did not alter the said profile in both sexes. In teleosts, predominant lipoprotein in blood is HDL and most of the circulatory cholesterol remains in HDL fraction (Sharpe et al. 2006). Earlier studies on lipid dynamics in goldfish during a period of gonadal recrudescence showed that total cholesterol concentration remains high throughout the gonadal development, whereas TG concentration peaks during early recrudescence and subsequently attenuates in late gonadal development (Sharpe and MacLatchy 2007). In contrast, the current research found reduction in plasma CHL and HDL and concomitant increase in TG levels during the late gonadal development phase in male and female goldfish exposed to hypoxia for more than 9 h daily. It is well known that de novo biosynthesis of cholesterol requires production of its precursor molecule, acetyl Co-A, whereas, TG biosynthesis also utilizes acetyl Co-A as

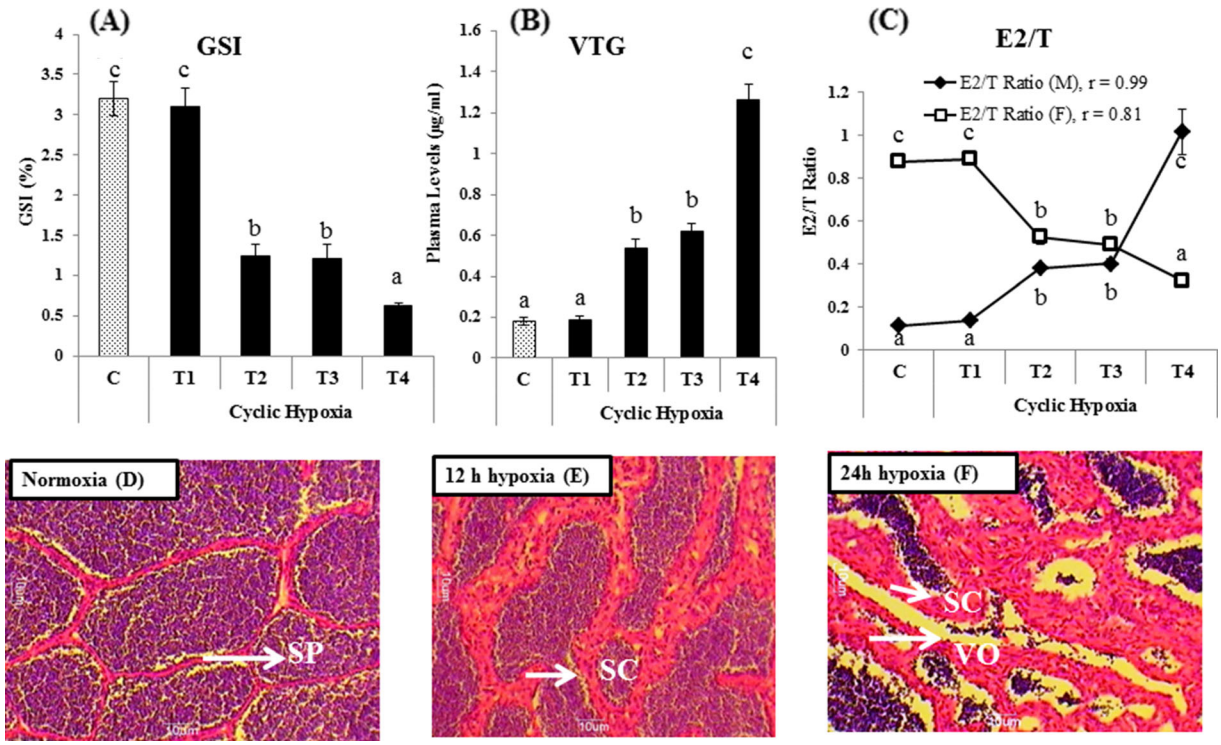

Fig. 2 Testicular development in goldfish under hypoxia. **a** GSI. **b** Male plasma vitellogenin level. **c** E2/T ratio. **d**, **e** Histological appearances of respective testis. *SC* spermatocytes, *SP* spermatozoa, *VO* vacuoles. *Different superscripts* indicate significant difference ($p < 0.05$) among different treatments and control. Values are expressed as mean ± SEM ($n = 18$)

a substrate, causing it to be potentially diverted from the de novo cholesterol synthesis pathway (Sharpe et al. 2006). This appears to be the case with continuous or cyclic hypoxia, which preferentially directs acetyl Co-A towards TG synthesis over cholesterol in late gametogenesis in both sexes. Moreover, ovarian tissue is found to be more sensitive as it preferentially uses lipoprotein-derived CHL as a steroidogenic substrate (Gwynne and Strauss 1982). Therefore, depletion in intracellular CHL availability is likely to occur at least partially owing to hypocholesterolemia and attenuation of plasma HDL levels. Such depletions in the plasma cholesterol and HDL levels may inhibit cholesterol supply to gonads. Again, cholesterol is crucially important for neural development in fish. It is conceivable that depletion of CHL level in growing oocytes may lead to embryonic abnormality, as yolk-derived circulating lipoproteins should be able to access the CNS prior to start of blood–brain barrier function in early embryo (Jeong et al. 2008). On the other hand, either suppression of lypolysis (a common response of hypoxia in fish) (Van Raaij et al. 1996) or poor incorporation of TG into the growing oocytes may be attributed to the increasing plasma TG levels under hypoxia. As TG is the primary metabolic energy storage molecule in fish (where excess of 80% of total body lipid composition is present in the form of TG) (Henderson and Tocher 1987), such adaptations of maintaining a high plasma TG level as a future source of metabolic energy cannot be ruled out. Steroidogenesis in fish is a multifaceted feedback system regulated by the BPG axis. Therefore, impairment of function can occur at multiple levels. 17-HP is an intermediary steroid in the conversion pathway of cholesterol to testosterone. The increase in plasma 17-HP level seen currently in the hypoxic females and its absence in hypoxia-treated males demonstrates that attenuation in lipoprotein-derived CHL alters 17-HP at least in females. However, decreased 17-HP levels did not alter T in females. This may be attributed to inhibition in conversion of T to E, which is supported by previous reports (Thomas et al. 2006, 2007; Landry et al. 2007). Further, decrease in the plasma estradiol concentration may be attributed to suppression of cyp 19a1b, which catalyzes an auto-limiting step in the synthesis of estradiol, as well as downregulation of the estradiol receptor, indicating a disruption of reproductive functions, likely

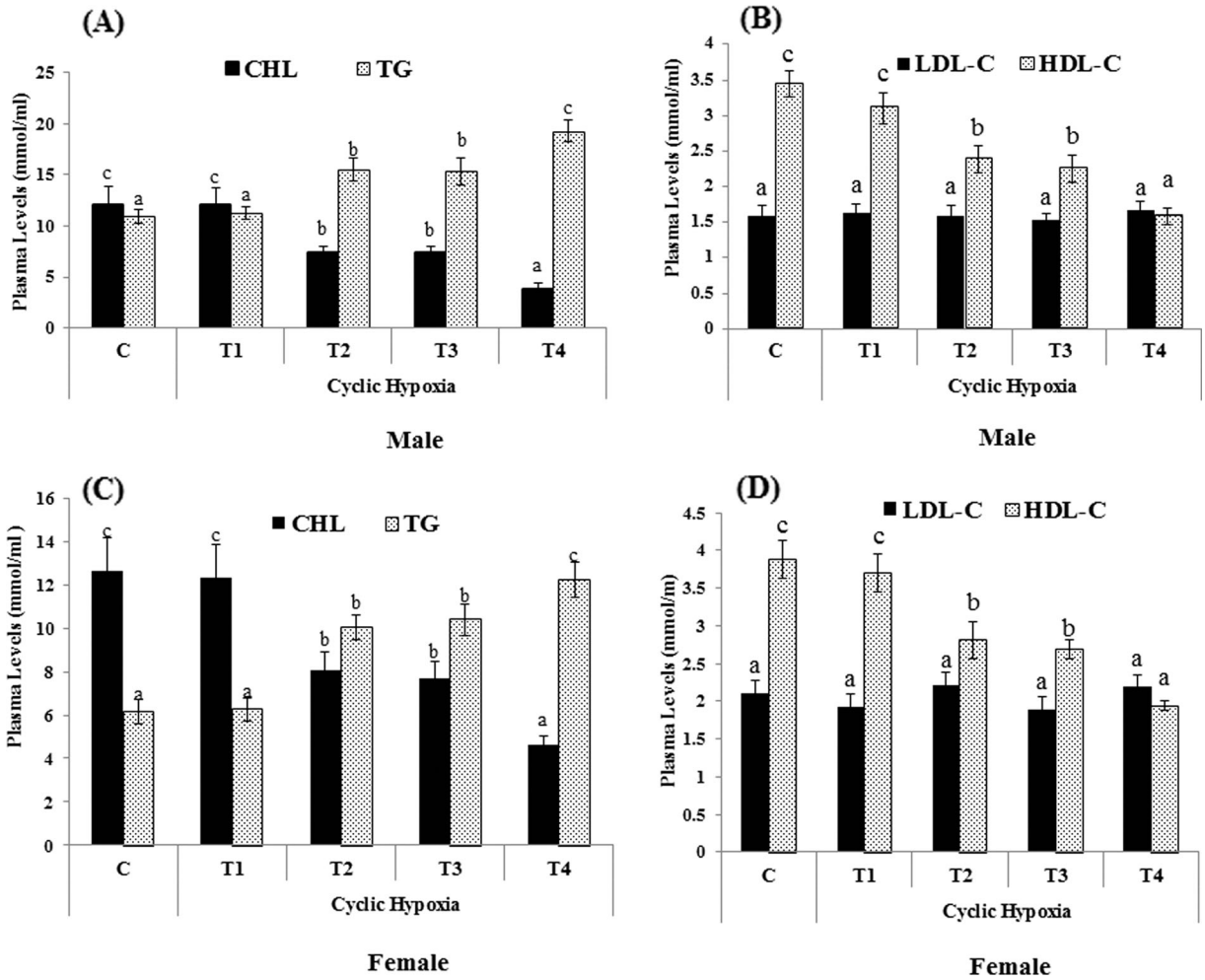

Fig. 3 Plasma lipid profiles under hypoxia. Plasma concentrations of total cholesterol (CHL) and triglycerides (TG) in males (**a**) and females (**c**). Plasma concentrations of high-density lipoprotein (HDL) and low-density lipoprotein (LDL) in males (**b**) and in females (**d**). *Different superscripts* indicate significant difference ($p < 0.05$) among different treatments and control. Values are expressed as mean ± SEM ($n = 18$)

to result in fertility loss in fish (Thomas et al. 2007). As a consequence, Vtg production was impaired in females exposed to cyclic hypoxia over 9 h per day. Such impaired estrogen-mediated Vtg synthesis associated with dramatic reduction in the development and growth of vitellogenic oocytes in hypoxic females responsible for the decreased relative GSI and ovarian development (Thomas et al. 2006, 2007). The impaired estrogen-mediated Vtg production is a very common endocrine response to hypoxic stress and may even induce gonadal masculinization in female fish (Thomas and Rahman 2009, 2011).

Hypoxia-induced reduction of plasma T and 11-KT levels suppressed the spermatogenesis process, causing a significant reduction in GSI, sperm count, and motility ($p < 0.05$) in males exposed either to cyclic or chronic hypoxia. In spite of de novo cholesterol synthesis in testis and unaltered plasma 17-HP in hypoxic males, a marked reduction in plasma testosterone level may be attributed to the inhibition of 17β-HSD enzymes which convert androstenedione to testosterone. A microarray study on hypoxia for 14 days in male zebrafish clearly demonstrated the downregulation of 17β-HSD gene (Martinovic et al. 2009). Not surprisingly, cyclic hypoxia demonstrated a similar increase in the plasma estradiol level in male goldfish as reported in male *C. carpio* (Wu et al. 2003) under long-term chronic hypoxia. The effect was further confirmed by an

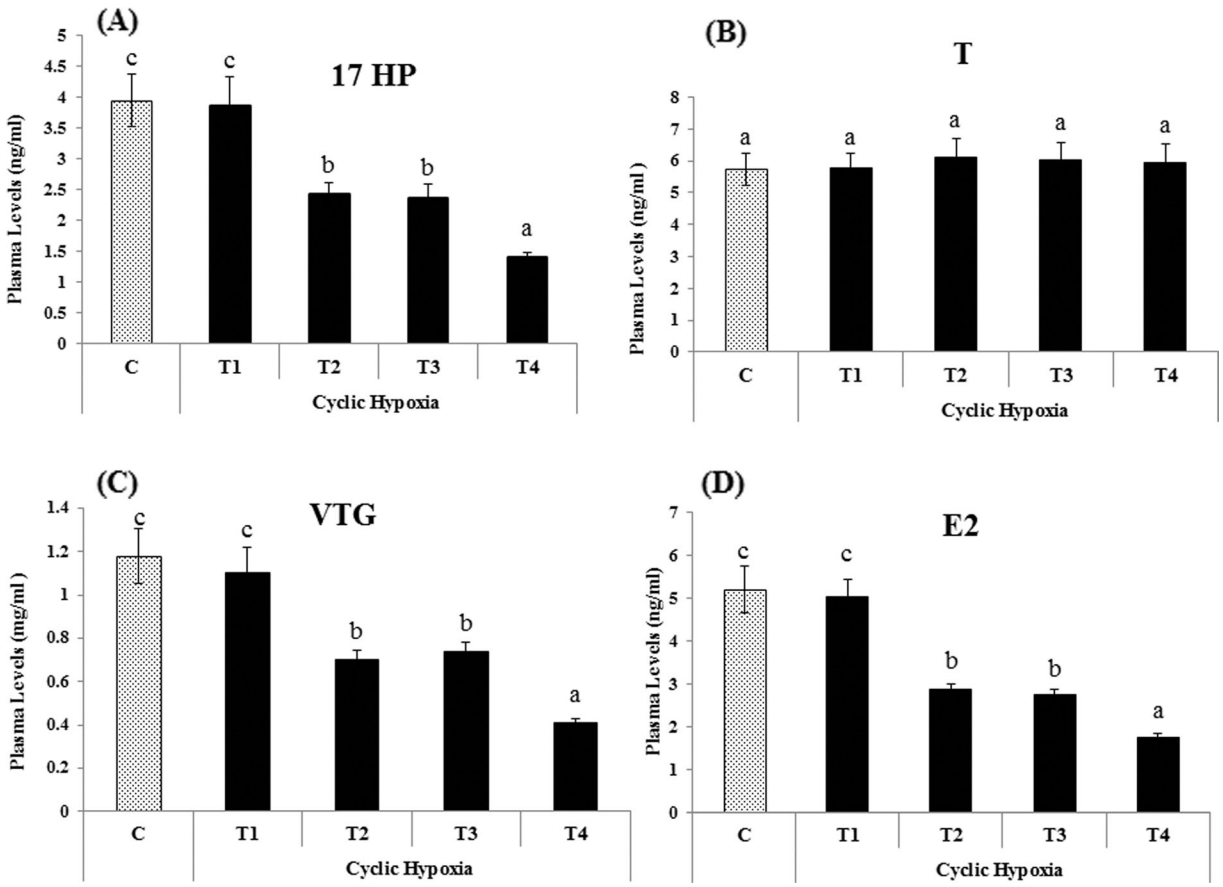

Fig. 4 Plasma sex steroid and vitellogenin levels in females under hypoxia. **a** 17α-Hydroxyprogesterone (17-HP). **b** Testosterone (T). **c** Vitellogenin (VTG). **d** Estradiol (e2). *Different superscripts* indicate significant difference ($p < 0.05$) among different treatments and control. Values are expressed as mean ± SEM ($n = 18$)

increase in the E/T ratio, which suggests stimulatory effects of hypoxia on aromatase activity, increasing the conversion of T to E (Wu et al. 2003). Increased plasma estradiol levels in sexually active male goldfish were reported by Spanò et al. (2004), in a time-dependent manner without concomitant increase in plasma Vtg when exposed to a herbicide, atrazine. In the current study, however, the plasma estradiol level was very low in normoxic male fishes, as compared to the earlier report. Nevertheless, a 2.0-fold increase in plasma estradiol level was induced by 9 h daily hypoxia, which in turn enhanced plasma Vtg levels in male goldfish. Enhanced Vtg expression can be induced in males by injection of 17β-estradiol or exposure to chemicals that mimic estrogens (Byrne et al. 1989; Tong et al. 2004). It is difficult to understand why estradiol remains high in hypoxic males, where estrogens are not thought to play a direct role in late spermatogenesis. Besides its reproductive role, estrogen has profound effects on plasticity and cell survival of the adult brain. Estrogen is capable of exerting neuroprotective effects through several mechanisms acting through estrogen receptor (ER)-dependent and (ER)-independent and genomic and non-genomic means to attenuate neural injury and cell death (Wise 2002; Luzio et al. 2016). Physiological levels of estradiol can enhance synaptic plasticity, regulate the expression of neurotrophins and cognate receptors, and elevate the expression of cell survival factors. It is possible that enhanced estradiol in male goldfish might have helped it to survive under hypoxic stress via preventing brain cell death.

Semen quality parameters, such as spermatozoa count and motility, decides the success of fertilization in teleosts. Cyclic hypoxia clearly reduced the semen quality as evident by low sperm count and motility. The reduced T and 11-KT are directly related to regulation of the late spermatogenesis process in teleosts (Schulz et al. 2010). Hypoxia-induced hypocholesterolemia in males

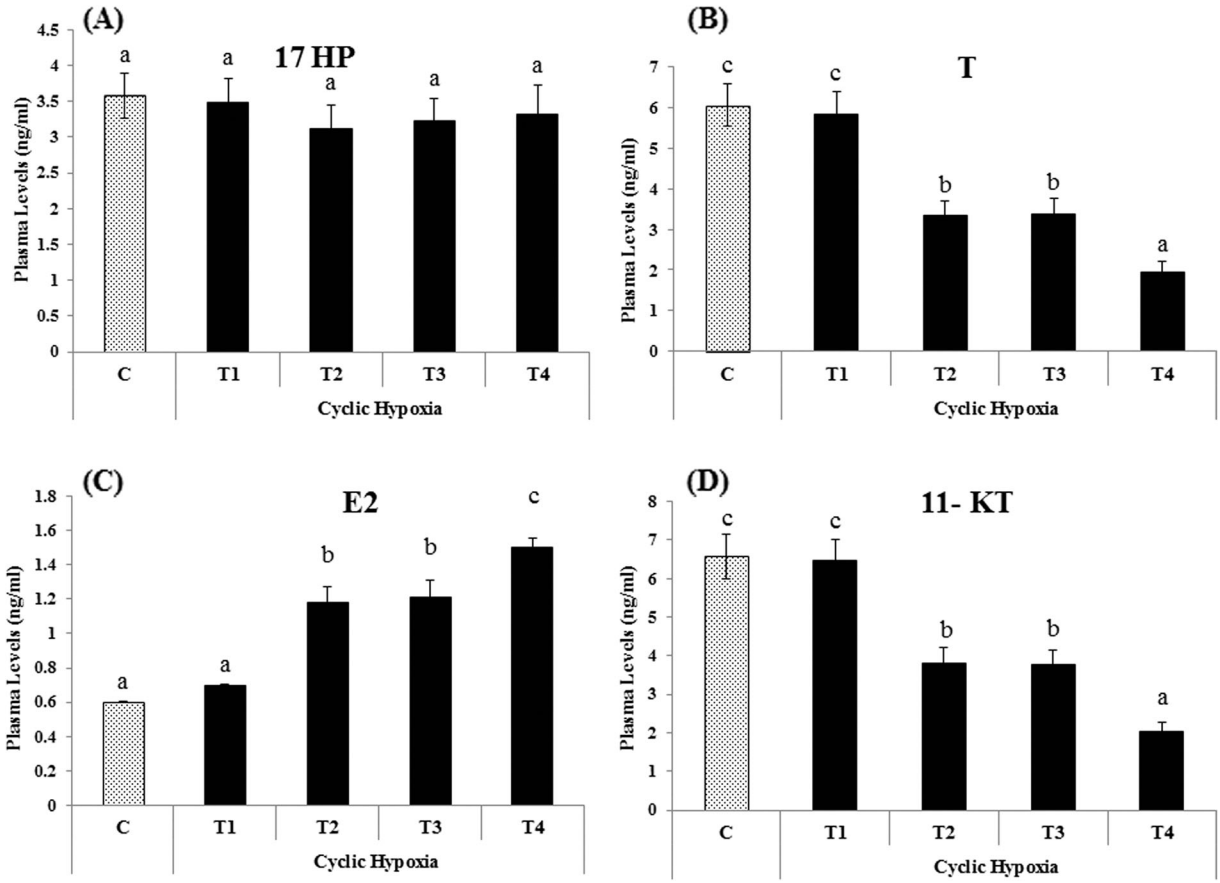

Fig. 5 Plasma sex steroid levels in males under hypoxia. **a** 17α-Hydroxyprogesterone (17-HP). **b** Testosterone (T). **c** 11-Ketotestosterone (11-KT). **d** Estradiol (e2). *Different superscripts* indicate significant difference ($p < 0.05$) among different treatments and control. Values are expressed as mean ± SEM ($n = 18$)

Table 1 Sperm count and relative sperm motility in goldfish under cyclic or chronic hypoxia

Treatments	Sperm count ($\times 10^9$)	Sperm motility (motility score of 5)
T_1 (normal)	3.17 ± .27c	4.20 ± .26c
T_2 (6 h hypoxia)	3.12 ± .25c	4.16 ± .30c
T_3 (9 h hypoxia)	2.00 ± .22b	2.67 ± .36b
T_4 (12 h hypoxia)	1.90 ± .22b	2.62 ± .37b
T_5 (24 h hypoxia)	0.98 ± .11a	1.63 ± .26a

Different lowercase letters indicate significant difference ($p < 0.05$) among different treatments and control. Sperm count values are expressed as mean ± SEM ($n = 18$), and relative sperm motility values are expressed in score of 5 ($n = 18$)

may deteriorate the sperm quality, since cholesterol is an important component of the lipid fraction in the sperm plasma membrane, playing an important role in promoting sperm membrane permeability and fluidity in mammals (Glazar et al. 2009 Ljubić et al. 2009; Mocé et al. 2010). Moreover, fluidity and permeability of sperm plasma membranes contribute to the maintenance of their motility and viability (Ljubić et al. 2009; Mocé et al. 2010), which support our results depicting the low motility sperm in hypocholesterolemic fish exposed to cyclic or chronic hypoxia.

Hypoxia impairs reproduction in a dose-dependent manner in fish (Thomas et al. 2007), and the responses vary with the period of hypoxia exposure (Wu et al. 2003). Being an anoxia-tolerant species (Lutz et al. 1996), it may assume that moderate hypoxia for 6 h failed to elicit significant metabolic depression in the fish. In addition, 18 h recovery under normoxia must be

sufficient to come out of such metabolic depression. If this is true, then the question arises as to why fish exposed to hypoxia for 9 h or more elicit significant responses. An earlier study showed that metabolic depression owing to severe hypoxia for 10 h recovered within 4 to 8 h of normoxia (Mandic et al. 2008). Although effects of diel hypoxia followed by normoxia on metabolic depression and recovery have not been elucidated in the fish, it may be assumed that 9 to 12 h diel hypoxia caused metabolic depression beyond recovery by 15 to 12 h diel normoxia. Consequently, such diel hypoxia altered plasma lipid dynamics, which in turn impaired gametogenesis. In addition, it is known that plasma endocrine profiles are variable and influenced by circadian endocrine rhythm in fish. The gonadotropin level, a key regulator of gonadal steroidogenesis, may vary over a 24-h period, but variations seem to depend on season, photoperiod, and even sex. The photoperiod regime of 16L/8D elicited two distinct peaks of GtH in fish, one at the onset of photoperiod and the other at 8 h of continuous light hour in females kept at 30 °C, whereas the males showed a distinct peak of GtH at 8 h of photoperiod under a 15L/9D regime at 18 °C (Gillet et al. 1978). Thus, exposure to hypoxia for more than 9 h during light hour seems to alter daily GtH rhythm which, at downstream, interrupted sex steroid production, thus delaying gamete growth.

Conclusion

The present study therefore provides the first clear evidence that diel cyclic hypoxia above 9 h impairs reproduction via multiple mechanisms. We propose for the first time that downregulation of lipoprotein-derived cholesterol biosynthesis and plasma HDL levels may lead to hypocholesterolemia and ultimately inhibit steroidogenesis, particularly in females. Specifically, we hypothesized that hypoxia either of chronic or cyclic nature facilitated utilization of acetyl-coA for TG synthesis over cholesterol biosynthesis. Further studies shall unearth the strategy of such shift in lipid dynamics to cope up under hypoxic conditions. Goldfish diverts a major portion of total energy towards ovarian growth (approximately 10–15%) during reproductive seasons. Therefore, we further propose that inhibition of lipid-derived cholesterol biosynthesis and transport during hypoxia could be a highly effective strategy to dramatically reduce energy and oxygen requirements in this freshwater fish, to survive until DO concentration becomes favorable. The current study supports the inference reported by other researchers that diel hypoxia inhibits gonadal steroidogenesis under continuous hypoxia. In addition, cyclic hypoxia-induced enhancement of plasma vitellogenin levels in male fish could have serious consequences for sustaining male fish population in open water bodies, where cyclic hypoxia occurs naturally and can be exacerbated by increased nutrient loading. The alteration in plasma lipid and steroid profiles associated with impairment in reproduction was not found to be dependent on duration of hypoxic exposure in the diel cycle. This appears to be caused by differential metabolic responses under variable durations of diel hypoxia followed by recovery and interrupted photoperiod-regulated circadian endocrine rhythm.

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