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# Effects of hypoxia exposure on apoptosis and expression of membrane steroid receptors, ZIP9, mPRα, and GPER in Atlantic croaker ovaries

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ABSTRACT

Hypoxia exposure causes endocrine disruption in croaker resulting in impairment of ovarian growth and oocyte development, but the effects of hypoxia on non-classical steroid actions in fish ovaries *in vivo* remain largely unknown. Membrane androgen receptor (ZIP9) enhances apoptosis of cultured Atlantic croaker ovarian follicle cells *via* upregulation of p53 and Bax, and increased caspase 3 activity through non-classical steroid signaling. Conversely, apoptosis is inhibited in these cells by non-classical steroid mechanisms through membrane progestin receptor alpha (mPRα) and G protein-coupled estrogen receptor (GPER). Apoptosis and the expression of ZIP9, mPRα, and GPER were investigated in ovaries of croakers that had been exposed to normoxia (7.0 mg DO/L) or hypoxia (2.0 mg DO/L) for 6 weeks during their period of gonadal recrudescence. The proportion of tertiary yolk stage oocytes was decreased, and the proportions of atretic and apoptotic ovarian follicles were increased in ovaries from hypoxia-exposed fish compared to controls. Ovarian expression of all three receptors was altered after hypoxia exposure. Expression of mPRα and GPER mRNA and protein levels were decreased after hypoxia exposure, consistent with a decline in anti-apoptosis. In contrast, ZIP9 mRNA and protein levels were upregulated 4-fold in hypoxia-exposed fish compared to normoxic controls. The enhanced apoptosis in ovaries of hypoxia-exposed fish was associated with increased caspase-3/7 activity and elevated expression of the pro-apoptotic genes, Bax and p53. The finding that ZIP9 expression was increased in ovaries of croaker exposed to hypoxia provides *in vivo* evidence supporting ZIP9's proposed function in mediating ovarian follicle cell apoptosis.

## 1. Introduction

Ovarian follicle atresia is tightly regulated in mammals and birds and considered vital for removing excess follicles and maintaining ovarian homeostasis (Chun and Hsueh, 1998). Atresia of mammalian and avian ovarian follicles not destined for ovulation is induced by apoptosis, or programed cell death, which typically begins in the granulosa cells. (Chun and Hsueh, 1998; Johnson, 2003). Follicular atresia in mammals is controlled by a variety of hormones and paracrine factors including androgens which are atretogenic, and estrogens and progesterone which inhibit atresia (Chun and Hsueh, 1998; Svensson, Markstrom, Andersson, and Billig, 2000; Markström, Svensson, Shao, Svanberg, and Billig, 2002). In contrast, the mechanisms controlling atresia in teleost fish ovarian follicles have received little attention and remain unclear (Wood and Van Der Kraak, 2001; Yamamoto, Luckenbach, Goetz, Young, and Swanson, 2011). Moreover, the fact that teleost fishes have much higher fecundities and different patterns of oocyte recruitment than other vertebrates suggests that our knowledge of atresia mechanisms in mammals may not be applicable to teleosts (Wood and Van Der Kraak, 2001).

Atresia of ovarian follicles in fish occurs both before and after ovulation and is accompanied by apoptosis and an increase in the activity of the proteolytic enzyme, caspase 3 (Santos et al., 2008). Atresia in teleosts prior to ovulation occurs primarily in vitellogenic follicles (Wood and Van Der Kraak, 2001). Although the role of atresia at this stage of oogenesis is not known, it has been proposed to optimize fecundity in marine teleosts according to the prevailing environmental conditions and to remove oocytes that cannot complete vitellogenesis during the reproductive season (Macer, 1974; Bromley, Ravier, and Witthames, 2000). Atresia in fish follicles does not appear to be initiated in the granulosa cells, unlike the situation in mammals, but instead proceeds first in the oocyte and is accompanied by yolk

*Abbreviations:* ZIP9, ZRT and Irt-like Protein 9 (SLC39A9); mPRα, membrane progestin receptor alpha (PAQR7); GPER, G protein-coupled estrogen receptor; p53, tumor protein 53; Bax, bcl-2-associated X protein

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proteolysis and resorption (Santos et al., 2008). Therefore, it is unclear whether apoptosis of teleost granulosa and theca cells is controlled by the same hormones and paracrine factors, such as the sex steroids, as in mammalian follicles.

Recent studies have shown that testosterone (T) promotes serum starvation-induced cell death in cultured Atlantic croaker (*Micropogonias undulatus*, croaker) ovarian follicle cells (co-cultured granulosa and theca cells) cells through a membrane androgen receptor, ZIP9, which is a member of the ZRT and Irt-like Protein (ZIP, SLC39A) zinc transporter family (Berg, Rice, Rahman, Dong, and Thomas, 2014). ZIP9 is localized on the cell membrane of croaker follicle cells and is coupled to a stimulatory G protein (Gs) through which T induces non-classical steroid second messenger signaling and apoptosis *via* the intrinsic pathway through upregulation of p53, Bax and increases in caspase 3 activity (Berg et al., 2014; Converse, Zhang, and Thomas, 2017). However, information is currently lacking on the potential role of ZIP9 in teleost ovarian physiology, including its involvement in follicular atresia and apoptosis in response to environmental stressors. Progestin activation of membrane progesterone receptor alpha (mPRα, or PAQR7), another transmembrane receptor mediating non-classical steroid signaling, causes an opposite response to that of ZIP9, promoting cell survival and anti-apoptosis of croaker follicle cells through activation of an inhibitory G protein (Dressing, Pang, Dong, and Thomas, 2010). Finally, a third steroid membrane receptor unrelated to nuclear steroid receptors, G protein-coupled estrogen receptor, GPER (previously known as GPR30), is expressed in croaker ovaries (Pang, Dong, and Thomas, 2008) and has been shown to mediate anti-apoptotic actions of estrogens in mammalian breast cancer cells through activation of Gs-dependent signaling pathways (Yu et al., 2014). Collectively, these results suggest the potential involvement of multiple novel steroid membrane receptors in the regulation of apoptosis in fish ovaries.

Because fish invest enormous amounts of energy in egg production, oogenesis is extremely susceptible to disruption by environmental stressors that increase energy demand and is frequently curtailed in adverse environments. This reproductive impairment results in atresia of ovarian follicles and yolk resorption. For example, stressors such as starvation and exposure to endocrine disrupting chemicals (EDCs) increase the proportion of atretic oocytes in teleost and mammalian ovaries (Yamamoto et al., 2011; Peretz, Craig, and Flaws, 2012). However, despite the fact that exposure to low oxygen concentrations dramatically increases the energy requirements of fish during the reproductive cycle, information is currently lacking on whether chronic hypoxia exposure increases oocyte atresia in fish. Increased oocyte atresia has been reported in a single teleost species, croaker, after hypoxia exposure (Thomas, Rahman, Khan, and Kummer, 2007; Thomas, Rahman, Picha, and Tan, 2015). Croaker collected from hypoxic (dissolved oxygen, DO, < 2.0 mg/L) sites in the northern Gulf of Mexico displayed impaired reproductive and endocrine functions, oogenesis, and ovarian growth and an increased percentage of oocytes that were atretic compared to croaker collected from normoxic reference sites (Thomas et al., 2015). However, the underlying mechanism(s) controlling oocyte atresia in croaker exposed to hypoxia is unknown.

The number of coastal hypoxic bodies of water, also known as “dead zones”, has approximately doubled worldwide each decade since the 1960s (Diaz and Rosenberg, 2008). In view of this exponential increase in the number of coastal dead zones, it is important to understand the effects of hypoxia on the physiology and reproduction of fishes, as decreased reproductive output can lead to long-term population declines (Rose et al., 2018). On the basis of previous results, we hypothesize that the increase in atresia in croaker ovaries after chronic hypoxia exposure is accompanied with an increased incidence of follicle cell apoptosis, and that this increase is associated with an increase in the ovarian expression of the pro-apoptotic regulator, ZIP9, and with decreases in the ovarian expression of the receptors promoting cell survival, mPRα, and GPER. Therefore, in the present study, the effects of chronic hypoxia exposure of female Atlantic croaker on oogenesis and apoptosis of ovarian follicles as well as the ovarian expression and functions of the membrane steroid receptors, ZIP9, mPRα, and GPER were investigated.

## 2. Materials and methods

### 2.1. Chemicals

All chemicals were purchased from Sigma Aldrich (St. Louis, MO) unless indicated otherwise. The Caspase-Glo 3/7 kit was purchased from Promega (Cat# G8090; Madison, WI). The ApopTag peroxidase *in situ* apoptosis detection Kit was purchased from EMD Millipore (Cat# S7100, Billerica, MA).

### 2.2. Experimental fish

Young-of-year croaker (length: 10-11 cm) were purchased from a local commercial fisherman in August 2014 and acclimated to laboratory conditions at the University of Texas Marine Science Institute in recirculating 5187 L seawater tanks (salinity 33 ppt, temperature 25 °C, photoperiod 12L:12D) for two months prior to experimentation at which time they had become sexually mature and had begun gonadal development.

### 2.3. Chronic hypoxia exposure

Croaker were exposed to hypoxia during the normal period of gonadal recrudescence, October through December, under fall environmental conditions (temperature 23 °C, photoperiod 9L:13D) to promote gonadal and gamete development (Thomas et al., 2007). Fish were moved into nine experimental tanks (2025 L capacity) in late September. Twenty-five mixed-sex fish were stocked into each experimental tank. Several fish were sampled prior to stocking, and the average weight of females was 33.65 ± 2.16 g (mean ± SEM) and the average gonadosomatic index (GSI) of females was 0.848 ± 0.028% (mean ± SEM). Following a two-week acclimation period in the experimental tanks, four tanks were randomly assigned the normoxia (control) treatment, and five tanks were randomly assigned the hypoxia treatment. The flow of air into the hypoxia experimental tank was gradually reduced over a one-week period using an air flow meter (SCFH AIR, Key Instruments, Trevose, PA) and water recirculation was reduced to decrease the dissolved oxygen (DO) concentration gradually until the target level of 2.0 mg DO/L was reached. A YSI multiprobe (YSI 55g Multiprobe System, YSI Incorporated, Yellow Springs, OH) was used to monitor DO, pH, and temperature three times daily (morning, afternoon, and night). The actual average DO concentration in the hypoxic tanks during the experimental period was 2.17 mg DO/L with a standard deviation of 0.66. The DO level in the normoxia tanks was held at as close to 100% saturation as possible. The actual average DO concentration in the normoxic tanks during the experimental period was 6.36 mg DO/L with a standard deviation of 0.40. The pH was adjusted using sodium bicarbonate buffer when necessary to maintain the pH between 7.7 and 7.9 during the experiment. A single parameter water quality kit (HACH, Loveland, CO) was used to measure ammonia and nitrate daily. There were no major changes in water quality parameters (ammonia 0.1–0.2 mg/L, nitrate 0.01–0.02 mg/L) during the experiment. Fish were fed the same amount of a mixture of shrimp and pellets (Rangen) daily (5% BW/day).

Following 6 weeks of hypoxia exposure, approximately 50 fish (comprising 11 females from hypoxia tanks, and 14 females from normoxic tanks) were randomly sampled from the experimental tanks and anesthetized in an immersion bath of quinaldine (20 mg/L) before being humanely sacrificed by decapitation. Ovarian tissues were rapidly excised, placed in fixative for histological examination, or frozen in liquid nitrogen and stored at −80 °C prior to analysis. All