Biomarkers of hypoxia exposure and reproductive function in Atlantic croaker: A review with some preliminary findings from the northern Gulf of Mexico hypoxic zone

Peter Thomas *, Md. Saydur Rahman

Marine Science Institute, University of Texas at Austin, 750 Channel View Drive, Port Aransas, TX 78373, USA

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ABSTRACT

The long-term impacts on marine ecosystems and fisheries of the recent worldwide increase in coastal hypoxia cannot be accurately assessed at present due to our limited knowledge of the chronic sublethal effects of hypoxia on marine organisms. Moreover, it is unclear whether many marine fish and other motile species remain in hypoxic bottom waters long enough to trigger adaptive responses to low dissolved oxygen. Therefore, there is an urgent need to develop reliable and sensitive biomarkers of sublethal hypoxia exposure and its deleterious effects on critical functions for maintaining population size such as reproduction. In this paper the molecular and biochemical responses to hypoxia and the role of the hypothalamus–pituitary–gonadal axis in the control of the reproductive cycle in fish are briefly reviewed. The potential use of hypoxia-inducible factors (HIFs) as specific biomarkers of hypoxia exposure and changes in hormone levels and gonadal histology as biomarkers of reproductive function in fish are discussed. Recent field studies with a hypoxic-tolerant estuarine teleost, Atlantic croaker, have provided the first clear evidence in an aquatic species that reproduction and endocrine function are particularly susceptible to interference by environmental hypoxia exposure. Marked impairment of reproductive function and endocrine disruption was observed in individuals collected from hypoxic sites in East Bay, Florida and Mobile Bay, Alabama. The production of mature oocytes and sperm (gametogenesis), as well as sex steroid and vitellogenin levels in the blood, were significantly lower in croaker from the hypoxic sites in East Bay compared to the values in fish collected from the adjoining normoxic Pensacola Bay, whereas gonadal HIF-1α and HIF-2α mRNA expression was significantly elevated in fish from the hypoxic sites. Similar patterns of reproductive and endocrine disturbances and increased HIF-1α and HIF-2α mRNA expression were observed in controlled hypoxia laboratory studies with croaker. Preliminary findings suggest that reproductive and endocrine functions were also impaired in female croaker collected in 2006 from the hypoxic zone off the Louisiana coast. The production of mature oocytes (fecundity) was significantly decreased in fish collected from the hypoxic site compared to that observed at the normoxic site and this was associated with declines in circulating sex steroid and vitellogenin levels and gonadotropin releasing hormone mRNA expression in the hypothalamus. Moreover, tissue expression of HIF-2α mRNA and protein was significantly increased in croaker collected at the hypoxic site. It is concluded from these studies that assessment of HIF(s) expression and reproductive/endocrine functions are promising as biomarkers of exposure to hypoxia and its potential long-term impacts on fish populations, respectively.

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1. Introduction

Hypoxia (dissolved oxygen, DO < 2 mg L⁻¹) occurs naturally in many estuaries during the warmer summer months when oxygen utilization by marine organisms is greatest and the water column becomes stratified as a result of the formation of haloclines and thermoclines (Pihl et al., 1991; Engle et al., 1999). There has been a marked increase in the incidence of seasonal hypoxia in many coastal regions around the world over the last few decades as a result of eutrophication caused by increased nutrient inputs from intense agricultural practices and other anthropogenic sources (Diaz and Rosenberg, 1995, 2008; Rabalais and Turner, 2001). However, the long-term impacts of this environmental change on the sustainability of coastal ecosystems cannot be predicted accurately due to our current poor understanding of the sublethal effects of chronic exposure to low DO on marine organisms (Rabalais et al., 1999; Thetmeyer et al., 1999). For example, virtually no information was available until recently on the effects of hypoxia on reproduction in fish, although it is particularly susceptible to disturbance by environmental stressors and persistent impairment of reproduction could have a marked long-term negative influence on the size of fish populations (Billard et al., 1981; Wu, 2002).
The region of seasonal hypoxia in the bottom water of the northwestern Gulf of Mexico has doubled in size over the past 20 years from an average area of 8300 km² during the late 1980s, to over 16,000 km² in subsequent years, making it one of largest hypoxic regions in the world (Rabalais et al., 2002, 2007). This increase in the extent of the hypoxic zone has been largely attributed to a three-fold increase in nitrogen loading to this region from the Mississippi River (Goolsby et al., 2001). Mobile bottom-dwelling species such as Atlantic croaker could potentially escape from the hypoxic zone in this region because it is confined to the bottom few meters of the water column. Consequently, the duration and pattern of hypoxia exposure are unclear in these motile species. In addition, the long-term impact of widespread seasonal hypoxia on valuable fishes and fishery resources in the northwestern Gulf of Mexico is currently unknown. Therefore, to address these concerns it will be necessary to develop biological indicators (biomarkers) in individuals of a representative marine species inhabiting the northern Gulf of Mexico that can be used to assess the severity or duration of exposure to hypoxic conditions as well as the chronic sublethal effects that are of potential ecological significance.

The selection and evaluation of biomarkers of hypoxia exposure and sublethal responses to hypoxia in Atlantic croaker, a relatively hypoxia-tolerant marine and estuarine teleost species, are reviewed in this paper. Evidence will be presented, from both field and laboratory studies, that hypoxia inducible factor (HIF) expression, and indices of reproductive and endocrine function, are promising biomarkers of hypoxia exposure and sublethal hypoxia effects, respectively. In addition, some preliminary results are presented on these biomarker responses in croaker collected from hypoxic regions in the northwestern Gulf of Mexico.

2. Selection of biomarkers of hypoxia exposure

Fish inhabiting many estuaries and coastal regions are exposed to periods of chronic or intermittent hypoxia during the summer months (Grantham et al., 2004; Craig and Crowder, 2005). Whereas some species show a strong avoidance response to hypoxic waters and attempt to migrate to normoxic regions, others such as Atlantic croaker can tolerate low DO and remain in hypoxic areas (Bell and Egglestone, 2005). These hypoxia-tolerant species initially respond to low DO by improving oxygen delivery by a variety of mechanisms, including increasing blood flow, red blood cell number and hemoglobin content (Hochachka and Somero, 2002). A second strategy is to conserve energy expenditure by decreasing aerobic metabolism and oxygen demand through reductions in ATP utilization (metabolic suppression) and by increasing the efficiency of ATP production (Hochachka and Somero, 2002). A variety of processes requiring ATP, such as the synthesis of proteins, glucose and urea, and membrane channel activity, especially Na⁺,K⁺ ATPase activity, are markedly suppressed under hypoxic conditions (Wu, 2002). Metabolic suppression is also an effective strategy for defense against other adverse environmental conditions in vertebrates, such as food limitation. The main benefit of this strategy is to slow down biological time, enabling the organism to survive until environmental conditions again become favorable (Hochachka and Somero, 2002). Induction of glycolytic gene expression is a near universal response in animals to hypoxia. Transcriptional upregulation of glycolytic genes in the liver and many other tissues during hypoxia exposure, as well as upregulation of a wide variety of other genes involved in adaptation to low oxygen levels, are mediated through a specific oxygen-sensitive transcription factor, hypoxia-inducible factor (HIF, Fig. 1; Ramirez-Bergeron and Simon, 2001; Bracken et al., 2003). Changes in the transcription rates of these genes are preceded by alterations in HIF mRNA and protein expression. Therefore, HIF is potentially useful as an early warning indicator of the induction of hypoxia defense mechanisms in organisms exposed to low environmental oxygen levels. Recent progress in our laboratory on the development and evaluation of HIF biomarkers of hypoxia exposure in Atlantic croaker is briefly reviewed in the following sections.

2.1. Hypoxia-inducible factors (HIFs) and their regulation of hypoxia defense mechanisms

Most of our knowledge of the structure, degradation pathway, and functions of hypoxia inducible factors has been obtained from studies in mammalian models. Hypoxia inducible factors are composed of two subunits, a hypoxia-regulated α subunit (HIF-1α, -2α, -3α), and an oxygen-insensitive β subunit (HIF-1β), also known as the aryl hydrocarbon receptor nuclear translocator, ARNT (Wang et al., 1995; Wenger and Gassmann, 1999). A fourth α subunit, HIF-4α has also been identified in teleosts (Law et al., 2006). Both HIF α and β subunits are members of the basic helix-loop-helix (bHLH) family of transcription factors containing the Per-ARNT-Sim (PAS) domains (Wang et al., 1995; Wenger, 2002). Extensive studies in mammalian cells have shown that the expression and activity of the α subunit controls the biological activity of HIF-1α (Jiang et al., 1996; Wiener et al., 1996; Pugh et al., 1997), and this can occur via a variety of mechanisms including changes in mRNA and protein expression (Jiang et al., 1996; Wiener et al., 1996; Pugh et al., 1997), and nuclear localization and transactivation (Jiang et al., 1996; Pugh et al., 1997; Kallio et al., 1998). Among these, the most intensively studied mechanism in mammalian cells has been the regulation of steady-state HIF-1α protein levels. The amount of the HIF-1α protein in the cell and its DNA-binding activity are regulated by the oxygen concentration and are increased as oxygen levels decline (Jiang et al., 1996). Hypoxia inducible factors are constitutively expressed and the HIF-1α protein is rapidly degraded under normoxic conditions through a pathway initiated by hydroxylation of two proline residues in the oxygen-dependent degradation (ODD) domain by prolyl hydroxylase enzymes (PHD, Fig. 1). These enzymes function as critical intracellular oxygen sensors, maintaining a low steady-state level of HIF-1α under normoxic conditions, thereby preventing it from activating target genes (Masson and Ratcliffe, 2003; Willam et al., 2004; Leite et al., 2008). Hydroxylation of the proline residues facilitates binding of von Hippel-Landau protein (pVHL) and activation of ubiquitin ligase resulting in polyubiquitination of HIF and its degradation through the ubiquitin-proteosome pathway (Jaakola et al., 2001; Bracken et al., 2003; Nikinmaa and Ress, 2005).

Under hypoxic conditions, the hydroxylation reaction is attenuated, allowing HIF-1α to escape degradation, form heterodimers with ARNT and recruit its co-activator p300/CBP (Fig. 1). The active HIF complex then binds to hypoxia response elements (HREs) containing the consensus sequence 5′-A(T/G)CGTG-3′ on the promoter or enhancer regions of genes, resulting in changes in their rates of transcription (Fig. 1; Bracken et al., 2003). Since ARNT is a dimerization partner of several transcription factors, it is possible that there is competition between HIF-α and other ARNT-dependent gene expression pathways under hypoxic conditions.

Protein levels of another HIF-α homolog in higher vertebrates, HIF-2α, also mediate hypoxia-dependent changes in gene expression in a similar manner to HIF-1α (Rakjukumar and Conrad, 2000). The third HIF α homolog in higher vertebrates, HIF-3α, appears to have weaker transcriptional activity than HIF-1α and -2α (Gu et al., 1998).

2.2. Hypoxia-inducible factors in fish

Three HIF-α homologs, HIF-1α, HIF-2α, and HIF-4α, have been identified in teleost fishes. HIF-1α has been cloned in rainbow trout (Oncorhynchus mykiss), grass carp (Ctenopharyngodon idellus), zebrafish (Danio rerio), Atlantic croaker (Micropogonias undulatus) and sea bass (Dicentrarchus labrax) (Soitamo et al., 2001; Law et al., 2006; Rahman and Thomas, 2007; Rojas et al., 2007; Terova et al., 2008). The HIF-1α protein is expressed in salmonid cells derived from liver, gonad, and embryonic tissues (Soitamo et al., 2001) and protein levels
increase in embryonic tissues of Baltic salmon during development (Vuori et al., 2004). HIF-2α has been identified in killifish (Fundulus heteroclitus), zebrafish and Atlantic croaker (Powell and Hahn, 2002; Rahman and Thomas, 2007; Rojas et al., 2007) and HIF-4α has been cloned from grass carp (Law et al., 2006). HIFs are expressed in a broad range of fish tissues, including the gonads, brain, liver, muscle, heart, spleen, intestine, gill, and kidney. The finding that HIF-1α and HIF-2α mRNAs are present in all the croaker tissues examined suggests that these genes are ubiquitously expressed in fish tissues (Rahman and Thomas, 2007). Although the molecular responses to hypoxia have not been characterized extensively in fishes, the results obtained to date indicate that fish HIFαs have a similar role in adaptation to hypoxia to that described in mammals (reviewed in Nikinmaa and Ress, 2005).

For example, activated HIF-1α protein capable of binding to HREs on erythropoietin in rainbow trout cells after hypoxia exposure (Soitamo et al., 2001). Moreover, as described in the next section, hypoxia has been shown to increase the expression of HIF-1α, -2α and -4α mRNAs and proteins in fish tissues (Law et al., 2006; Rahman and Thomas, 2007; Thomas et al., 2007a).

2.3. Transcriptional activity of hypoxia-inducible factors

Hypoxia inducible factors alter the expression of a wide variety of genes involved in adaptation to low oxygen levels including genes involved in erythropoiesis, angiogenesis, oxygen delivery, apoptosis and glycolysis (Wenger and Gassmann, 1999; Semenza, 2001; Bruick and McKnight, 2002; Nikinmaa, 2002; Nikinmaa and Ress, 2005). Altogether, over 120 genes are regulated by hypoxia in the estuarine teleost, Gillichthys mirabilis (Gracey et al., 2001). In addition to increasing cellular levels of HIF proteins, hypoxia exposure has also been shown to upregulate HIFα mRNA levels in the tissues of several hypoxia-tolerant mammalian species (Zhao et al., 2004; Wang et al., 2006; Dolt et al., 2007). Thus, both HIFα mRNA and protein levels are potentially useful as indicators of hypoxia exposure in mammalian and non-mammalian species.

2.4. Evidence for increased hypoxia-inducible factor expression in fish exposed to hypoxia

Despite the importance of HIF(s) as the major transcriptional regulator of molecular responses to chronic hypoxia, there are only a few reports on hypoxia regulation of HIF in fishes (Law et al., 2006; Rahman and Thomas, 2007; Thomas et al., 2007a). Our recent studies on Atlantic croaker collected from sites in a Florida estuary that experience persistent seasonal hypoxia have provided the first evidence for upregulation of HIF expression in fish environmentally exposed to hypoxia (Thomas et al., 2007a). On the basis of our extensive field and laboratory results, we proposed that HIFα expression in fish has potential...
as a specific biomarker in fish of environmental exposure to hypoxia. The field studies showed that HIF-1α mRNA levels were significantly elevated in the ovaries of croaker collected in October 2003 from the hypoxic sites compared to those in fish from the normoxic sites (Thomas et al., 2007a). Interestingly, expression of both HIF-1α and HIF-2α mRNAs was significantly increased in the ovaries of croaker collected from hypoxic sites H1–H3 1 month later, even though DO levels in the bottom water at these sites had increased to 2.2–3.5 mg L$^{-1}$ (Fig. 2). In contrast, expression of HIFs in croaker from formerly hypoxic site H4 (DO: 4.7 mg L$^{-1}$) and the transition site (TR, DO: 6.7 mg L$^{-1}$) was not significantly different from that obtained at the normoxic sites, which suggests that HIF mRNA expression returns to normoxic levels in this species when the DO concentration increases above a critical threshold level between 3.5 and 4.7 mg L$^{-1}$.

The time-course and DO concentration-response relationships of HIF-1α and HIF-2α expression in croaker ovaries were investigated in controlled laboratory hypoxia exposure experiments (Rahman and Thomas, 2007). HIF-1α and HIF-2α mRNA levels began to increase within 12 h of exposure to 1.7 mg L$^{-1}$ DO (~25% normoxic oxygen levels), and were significantly increased compared to controls after 3 and 7 days of hypoxia exposure, respectively. Expression of HIF-1α and HIF-2α mRNA was seven-fold and four-fold higher than control levels, respectively, after 7 days of hypoxia exposure, but had returned to basal values within 1 day of exposure of the fish to normoxic conditions. Transcript levels of HIF-1α had not further increased after 3 weeks exposure to 1.7 mg L$^{-1}$ DO, whereas HIF-2α mRNA levels continued to increase during this period. More moderate hypoxic conditions, 2.7 and 3.7 mg L$^{-1}$ (~38% and 52% normoxic oxygen levels, respectively), caused similar increases in the expression of both HIF mRNAs after 3 weeks exposure, suggesting that HIF-1α and HIF-2α mRNA expression in croaker may be altered under a wide range of DO conditions commonly observed in estuarine and marine environments. In contrast, HIF-1α mRNA expression was only upregulated in the kidney tissues of grass carp during the first few hours of hypoxia exposure and was not significantly different from control levels in any of the tissues examined after 4 days of hypoxia exposure (Law et al., 2006). On the other hand HIF-4α mRNA levels were upregulated in a wide variety of grass carp tissues after both acute and chronic hypoxia exposure (Law et al., 2006). These studies suggest that the HIF mRNA response to hypoxia varies among the α homologs and between different fish species.

An increase in the tissue concentrations of the HIFα proteins is a ubiquitous response of organisms to low oxygen levels. Ovarian levels of both HIF-1α and HIF-2α proteins were increased after continuous exposure of croaker in the laboratory to low DO (1.7 mg L$^{-1}$) for 2–4 weeks (Fig. 3). Concentrations of the HIF-1α protein in the ovaries of croaker exposed for 2 weeks to low DO were 3.0-fold higher than those in fish exposed to normoxic conditions (Fig. 3A), but were not significantly different after longer-term (4 weeks) exposure (results not shown). In contrast, ovarian levels of the HIF-2α protein rose more slowly in croaker under these DO conditions and were significantly higher than control values after 4 weeks exposure to 1.7 mg L$^{-1}$ DO (Fig. 3B). Additional experiments will be required to confirm that these two HIFα proteins have different temporal patterns of expression after hypoxia exposure. If the time-courses of increased tissue expression of these two HIFα proteins do indeed differ significantly, it may be possible to infer the duration of environmental exposure to hypoxia by determining the relative abundance of the HIF-1α and HIF-2α proteins in croaker tissues or by comparing their relative increases compared to values in fish from the normoxic sites.

3. Selection of reproductive biomarkers as indicators of potential population and ecological impacts of hypoxia

Biomarkers of reproductive processes are considered to be the most useful indicators in individuals of potential ecological impacts in monitoring programs (Jackson et al., 2000), because even slight decreases in the reproductive success of individuals can eventually have ramifications at higher levels of organization, leading to a population decline and community disturbance (Cushing, 1979). Numerous field and laboratory studies have shown that reproduction is one of the most sensitive stages of the fish’s life cycle to interference by a wide variety of environmental stressors in the marine environment, including contaminants and salinity stress, many of which exert their effects by disrupting the reproductive endocrine system (Billard et al., 1981; Donaldson, 1990; Thomas, 1990; Spies and Thomas, 1997; Khan and Thomas, 2001; Thomas and Khan, 2005). Consequently, indices of reproductive function could potentially integrate the reproductive effects of the multiple stressors often present in degraded environments. Moreover, modeling has been used to scale reproductive biomarker responses in individuals to higher levels of biological organization, such as croaker population dynamics (Rose et al., 2003). The maintenance of estuarine populations of commercially and recreationally important fish species is a management priority and an essential component of ecological condition and sustainability. Our recent studies show that hypoxia exposure dramatically impairs reproduction in fishes. Therefore, we are currently evaluating the utility of reproductive biomarkers to assess the potential population and ecological impacts of environmental hypoxia exposure. A variety of endocrine indicators of reproductive status are being examined because, as discussed in the following section, they control nearly all the complex processes that occur in the gonads and other reproductive tissues during the reproductive cycle.
3. Hypothalamus–pituitary–gonadal axis and endocrine control of the reproductive cycle

The onset of puberty and the annual reproductive cycle in teleost fish are controlled by hormones secreted by the hypothalamus–pituitary–gonadal (HPG) axis which is shown schematically in Fig. 4. Environmental stimuli that exert positive influences on reproduction, as well as the fish’s physiological status and nutritional state, are detected by sensory systems and this information is relayed via a variety of neural pathways to the hypothalamus. The information is integrated there and results in the synthesis and secretion of gonadotropin releasing hormone (GnRH), the primary neuropeptide that controls reproduction, as well as other stimulatory neuropeptides and neurotransmitters. There are multiple forms of GnRH in teleosts (Gothilf et al., 1996) and the primary form regulating gonadotropin secretion varies amongst the different teleost orders (Lethimonier et al., 2004; Mohamed et al., 2005). In teleosts GnRH neurons directly innervate the gonadotropin producing cells in the anterior pituitary (gonadotropes) and release GnRH which binds to specific receptors to regulate the synthesis and secretion of two glycoprotein hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH), previously named GTH I and GTH II, respectively.

During puberty GnRH expression in the hypothalamus is upregulated and this increase is associated with increased expression of the Kiss1 receptor (previously called GPR54; Mohamed et al., 2007; Nocillado et al., 2007; Filby et al., 2008), which in mammals has been shown to initiate puberty through its ligand, kisspeptin. The hypothalamic and pituitary concentrations of GnRH and the number of GnRH receptors on gonadotropes also increase during the annual reproductive cycle, which potentiates their responsiveness to subsequent GnRH stimulation, enabling the preovulatory surge in LH secretion to occur (Habibi et al., 1989; Holland et al., 1998; Khan et al., 2001).

Neurotransmitters such as serotonin and dopamine also act at the pituitary to modulate the secretion of GnRH and gonadotropins. Serotonin augments the action of GnRH on LH secretion during certain stages of the reproductive cycle in Atlantic croaker and goldfish (Somoza et al., 1988; Somoza and Peter, 1991; Khan and Thomas, 1992, 1994). The two gonadotropins, FSH and LH, are thought to regulate different phases of the seasonal reproductive cycle in teleosts, like they do in higher vertebrates, although different secretory patterns during the reproductive cycle of the two gonadotropins have only been demonstrated to date in salmonids (Gomez et al., 1999; Swanson et al., 2003). In salmonid fish FSH (GTH I) has been shown to have important roles during early gonadal development and vitellogenesis, oogenesis or spermatogenesis, whereas LH (GTH II) regulates the final stages of the reproductive cycle including oocyte maturation, ovulation and spermatization. However, in other species such as Atlantic croaker, LH secretion is regulated early in the gonadal cycle and changes in LH secretion have been associated with alterations in gametogenesis under a wide variety of experimental conditions (Khan et al., 1999) which suggests that LH is also involved in regulating this stage of the reproductive cycle. The gonadotropins bind to specific receptors on granulosa and theca cells in the ovarian follicle and Sertoli and Leydig cells in the testis to regulate the synthesis and secretion of sex steroids, growth factors and regulatory peptides.

Steroid hormones are synthesized from cholesterol via a series of biosynthetic steps catalyzed by different steroidalogenic enzymes. Gonadotropin regulates the production of steroidalogenic acute regulatory protein (StAR), which controls the transfer of cholesterol into the inner mitochondrial membrane, a key rate-limiting step in steroid synthesis. The side chain of cholesterol is cleaved by a P450 enzyme (P450scct) to produce a 21 carbon (C-21) steroid, pregnenolone, which is converted to C-21 steroid hormones, glucocorticoids and the two principal progestin hormones produced in fish, 17, 20α-dihydroxy-4-pregnen-3-one (17, 20α-P) and 17, 20β,21-trihydroxy-4-pregnen-3-one (20β-S), by a suite of steroidalogenic enzymes (Nagahama, 2000). The side chain of a C-21 steroid, 17α-hydroxyprogesterone is removed to produce androstenedione, an androgen (C-19 steroid), by the enzyme P450c17,
Fig. 4. Schematic representation of the hypothalamus–pituitary–gonadal axis controlling reproduction in teleost fish. An environmental stressor such as hypoxia could potentially alter reproductive endocrine function by different mechanisms at several sites on the HPG axis (indicated by capital letters). A: down-regulation of the activity of tryptophan hydroxylase, the rate-limiting enzyme in serotonin synthesis, resulting in decreased serotonergic activity, thereby decreasing a stimulatory neuroendocrine pathway within the HPG axis (indicated by capital letters). A: down-regulation of the activity of tryptophan hydroxylase, the rate-limiting enzyme in serotonin synthesis, resulting in decreased serotonergic activity, thereby decreasing a stimulatory neuroendocrine pathway within the HPG axis. B: possible alteration of the secretion of growth hormone, insulin-like growth factor 1 (IGF-1), prolanatin, thyroid and corticosteroid hormones, all of which can modulate the activity of the HPG axis, resulting in decreased reproductive function. C: decreased activity of steroid hormone biosynthetic enzymes requiring oxygen, such as aromatase and other steroidalogenic cytochrome P450 enzymes (Shang et al., 2006). D: likely alteration of the synthesis of proteins such as vitellogenin, which is synthesized in large amounts by the liver, as a result of decreased metabolic activity. Modified from a figure in Thomas (2008) with permission.

which in turn is converted to the major teleost androgens, testosterone and 11-ketotestosterone by the enzymes 17 keto reductase and 11β-hydroxysteroid dehydrogenase. Testosterone is subsequently converted to estradiol-17β, the major estrogen (C-18 steroid) hormone in teleosts, by the aromatase enzyme (P450arom). Progestin, androgen and estrogen hormones have both endocrine and paracrine effects mediated by binding to specific receptors on distant target tissues such as the liver and hypothalamus, and within the gonads themselves. The pattern of steriodogenesis changes during the reproductive cycle in both males and females from the production of estrogens and androgens during the period of gamete production (gametogenesis) to the production of progesterins during gamete maturation and spawning.

Although the initial oogenesis stages, oogonial proliferation and primary oocyte growth, are not regulated by gonadotropins, they have essential roles in both sexes during later phases of gametogenesis, particularly in the regulation of gonadal steroid production. During the secondary oocyte growth phase in females, gonadotropins regulate the synthesis and secretion of estradiol-17β in the granulosa cells and its precursor, testosterone, in the theca cells. Estradiol-17β regulates the production of the egg yolk precursor proteins, vitellogenins, and vitelline envelope (zona radiata) proteins in the liver (Hiramatsu et al., 2002) through binding and activation of a specific estrogen receptor, ERα, which is upregulated by estrogens during this phase of the reproductive cycle (Smith and Thomas, 1991; Flouriot et al., 1996; Thomas et al., 2007b). Large amounts of vitellogenins are produced by fish livers and released into the circulation during the secondary oocyte growth stage and are incorporated into the growing oocytes by a gonadotropin-dependent mechanism, resulting in dramatic increases in the size of the oocytes and the ovaries (Smith and Thomas, 1991). Female fish also have high circulating levels of testosterone during this period which may be involved in feedback control of gonadotropin secretion by its aromatization to estradiol-17β and also in the regulation of ovarian steroid production, possibly through binding to specific androgen receptors which have been identified in teleost ovaries (Sperry and Thomas, 2000; Thomas et al., 2007b). The feedback effects on gonadotropin secretion of estradiol-17β and testosterone change from being stimulatory on GnRH-induced LH secretion in immature and early recrudescing individuals to becoming inhibitory at the end of the reproductive cycle (Trudeau and Peter, 1995; Khan et al., 1999; Mathews et al., 2002). In addition, fish ovarian follicles synthesize inhibin and activin and growth factors which influence steroidogenesis and follicular growth as well as exerting feedback effects on gonadotropin secretion.

All three phases of spermatogenesis, mitotic proliferation of the spermatogonia, meiosis of spermatocytes, and transformation of spermaticids into flagellated spermatozoa, are controlled by gonadotropins (Schulz and Miura, 2002). Development of male germ cells is regulated by growth factors and activin secreted by the surrounding Sertoli cells which also provide nutrients. Sertoli cell function is in turn regulated by steroid hormones secreted by Leydig cells in response to FSH stimulation. All stages of spermatogenesis and the development of male secondary characters are regulated by the teleost androgens, 11-keto-testosterone and testosterone, through regulation of Sertoli cell production of IGF and activin B (Schulz and Miura, 2002). During meiosis of the germ cells, androgen production is mainly controlled by LH. Trace amounts of estradiol-17β are also produced in the testes and estrogen receptors have been identified in croaker testes (Loomis and Thomas, 1999). One function of estrogens in the testis may be to stimulate stem cell division (Schulz and Miura, 2002).

A surge in LH secretion initiates the final stages of gamete maturation and release by stimulating the synthesis of maturation-inducing steroids (MISs), the progestins 17β-P and 20β-S in the majority of species investigated (Scott and Canario, 1987; Thomas, 1994). The MIS activates a novel membrane receptor, mPRα, on fish oocytes to induce oocyte maturation (OM) by a nongenomic mechanism during which meiosis resumes, the germinal vesicle (nucleus) migrates to the animal pole (GVM) and breaks down (GVBD), and the ooplasm becomes clear due to lipid coalescence and hydration (Nagahama et al., 1993; Patiño et al., 2001; Thomas et al., 2002; Zhu et al., 2003; Thomas, 2004). The MIS induces ovulation by a genomic mechanism soon after completion of OM through binding to a nuclear progesterin receptor in the ovarian follicle wall resulting in the synthesis of arachidonic acid and prosta-glandins which cause smooth muscle contraction (Goetz et al., 1991; Pinter and Thomas, 1997, 1999; Patiño et al., 2003). Conjugates of the MIS released into the environment by ovulating females can also act as priming pheromones for conspecific males, triggering a surge in LH secretion which in turn stimulates milt production and MIS synthesis. Maturation of spermatozoa resulting in increased sperm motility is also regulated by the MIS through two different mechanisms, indirectly by increasing the pH of the seminal fluid through a genomic action mediated by the nuclear progesterin receptor, and a direct nongenomic action on sperm through activation of mPRα on the mid-pieces resulting in rapid increases in intrasperm concentrations of cAMP and free calcium (Pinter and Thomas, 1997; Thomas et al., 1997, 2004, 2007b; Schulz and Miura, 2002).
Extensive research has shown that all stages of the reproductive life history cycle are sensitive to interference by endocrine disrupting chemicals and other environmental stressors (reviewed in Thomas, 2008). Therefore, monitoring reproductive and endocrine functions at any of these stages could most likely be used to demonstrate adverse effects of hypoxia on reproduction. However, in our experience it has been easiest to detect stressor-induced impairment of reproductive function in wild populations of Atlantic croaker and several other estuarine and marine teleost species during gonadal crudescence and gametogenesis (Spies and Thomas, 1997; Thomas et al., 2006, 2007a).

Fish can be repeatedly sampled from control and degraded sites during the prolonged period of gonadal crudescence, and collection of fish during their first gonadal cycle will also permit detection of stressor effects on puberty and gonadal differentiation.

3.2. Evidence for impairment of endocrine and reproductive functions in fish exposed to hypoxia

Surprisingly, despite the susceptibility of reproduction to interference by environmental stressors and its importance for the maintenance of population abundance, the reproductive effects of hypoxia exposure in fishes and other vertebrates have received little attention (Wu, 2002). However, the results of our recent studies show that reproductive function in croaker is extremely susceptible to disturbance by hypoxia exposure. Extensive field studies with Atlantic croaker collected from a Florida estuary provided the first clear evidence for impairment of reproductive and endocrine functions in a vertebrate species exposed to chronic hypoxia in its natural environment (Thomas et al., 2007a; Table 1). Persistent hypoxia in East Bay in the Florida panhandle in 2003 caused a dramatic decrease in several indicators of reproductive function and egg production in female Atlantic croaker, including significant impairment of ovarian growth (gonadosomatic index, GSI), oocyte development (gametogenesis) and decreased fecundity, whereas fish collected from adjoining normoxic sites showed normal seasonal reproductive development (Thomas et al., 2007a; Table 1). The decline in egg production was associated with reduced estrogen signaling due to a decrease in plasma estradiol-17β levels, resulting in declines in hepatic estrogen receptor (ER) mRNA levels and vitellogenin production. Thus, the results suggest that the decrease in ovarian and oocyte growth was due to reduced sequestration of vitellogenin by the yolk stage oocytes which was at least partially due to the dramatically reduced concentrations of vitellogenin in the blood. Disruption of endocrine function was also observed in males. Plasma levels of androgens (testosterone and 11-ketotestosterone) were reduced in hypoxia-exposed males and this was associated with a marked impairment of sperm production and testicular growth (Thomas et al., 2007a; Table 1). The histological appearance of the ovaries and testes of low DO-exposed fish indicated that the production of fully developed gametes (gametogenesis) was markedly inhibited. Moreover, gametogenesis had not recovered by the end of the normal period of gonadal growth at the end of October, immediately prior to their migration to spawning grounds offshore. These findings indicate that croaker from East Bay in 2003 showed almost complete reproductive failure and did not contribute significantly to the spawning population in that year. In contrast, normal reproductive development was observed in normoxic sites in the adjoining bay, Pensacola Bay. A very similar pattern of dramatic impairment of gametogenesis, gonadal growth, and endocrine function was observed in both male and female croaker after chronic exposure to low DO (2.7 and 1.7 mg L⁻¹ DO) at the end of the period of gonadal crudescence in controlled laboratory studies (Thomas et al., 2007a; Table 1). Female croaker collected from hypoxic field sites in Mobile Bay, Alabama, in October 2004, also showed significant reductions in ovarian growth and the development of fully grown oocytes, which was accompanied by decreased hepatic estrogen receptor mRNA and plasma vitellogenin levels compared to values in fish collected from the normoxic sites (Thomas et al., 2006). In both the field and laboratory studies the reproductive impairment observed in the hypoxia-exposed fish was not accompanied by any changes in their condition factor or in other gross morphometric indices of growth. It is concluded from these studies that the period of gametogenesis and gonadal crudescence in Atlantic croaker and its endocrine control are very susceptible to disruption by environmental exposure to hypoxia. Similar effects of hypoxia have been reported in Fundulus grandis and common carp (Cyprinus carpio) both of which showed reproductive and endocrine dysfunction after exposure to hypoxia during this period of the reproductive cycle (Wu et al., 2003; Landry et al., 2007).

3.3. Sites and mechanisms of hypoxia-induced impairment of endocrine function on the HPG axis

Comprehensive studies on the effects of endocrine disrupting chemicals (EDCs) over the past decade have identified a broad range of endocrine mechanisms and reproductive functions that can be disrupted at every level of the HPG axis during reproductive life history cycle in fish (reviewed in Thomas, 2008). Equivalent information on the endocrine effects of hypoxia is currently lacking, although preliminary results indicate that this environmental stressor can also act via several mechanisms at multiple sites on the HPG axis to interfere with teleost reproduction (Shang et al., 2006; Thomas et al., 2007a). Clear evidence has been obtained in controlled laboratory studies that exposure to low DO causes impairment of neuroendocrine and reproductive functions in Atlantic croaker through down-regulation of tryptophan hydroxylase (TPH) activity in the preoptic anterior hypothalamus (Thomas et al., 2007a; Fig. 4, site A). The TPH enzyme catalyzes the

<table>
<thead>
<tr>
<th>Reproductive indicators</th>
<th>Sex</th>
<th>Field sites</th>
<th>Laboratory studies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hypoxic (&lt;1.7 mg/L DO)</td>
<td>Hypoxic (1.7 mg/L DO)</td>
</tr>
<tr>
<td>Estradiol-17β (ng/ml)</td>
<td>Female</td>
<td>2.71 ± 0.49</td>
<td>1.19 ± 0.1*</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>6.37 ± 2.3</td>
<td>23.4 ± 16*</td>
</tr>
<tr>
<td>Estradiol-17β (ng/ml)</td>
<td>Female</td>
<td>1.55 ± 0.25</td>
<td>0.02 ± 0.11*</td>
</tr>
<tr>
<td>GSI (%)</td>
<td>Female</td>
<td>7.63 ± 1.32</td>
<td>1.05 ± 27**</td>
</tr>
<tr>
<td>Fecundity (10^6 eggs/ fish)</td>
<td>Female</td>
<td>26.5 ± 3.19</td>
<td>0.92 ± 0.55**</td>
</tr>
<tr>
<td>Relative sperm production</td>
<td>Male</td>
<td>3.72 ± 0.34</td>
<td>0.54 ± 0.15**</td>
</tr>
<tr>
<td>11-Ketotestosterone (ng/ml)</td>
<td>Male</td>
<td>59.55 ± 10.5</td>
<td>0.44 ± 0.11***</td>
</tr>
</tbody>
</table>

Note: endocrine function and gametogenesis were assessed at a later stage of gonadal crudescence in the laboratory study in which hypoxia exposures were continued until the end of the period of gonadal growth.

⁎ p<0.05, ⁎⁎ p<0.01, ⁎⁎⁎ p<0.001 compared to normoxic conditions (Student t-test). All measurements are mean ± SEM. N=7–20. Reproduced from Thomas et al. (2007a) with permission.

⁎ GSI = (gonad weight/body weight − gonad weight) × 100.

Arbitrary units.
rate-limiting step in serotonin synthesis, and decreased TPH activity after hypoxia exposure is associated with decreased hypothalamic levels of the neurotransmitter serotonin. Serotonin neurons exert a positive stimulatory influence on the neuroendocrine system controlling reproduction in croaker and other teleosts (Khan and Thomas, 1994), and the decline in hypothalamic serotonin content in hypoxia-exposed fish is accompanied by decreased expression of GnRH mRNA in the hypothalamus and a reduced LH response to GnRH stimulation (Thomas et al., 2007a). Interestingly, these hypoxia-induced decreases in hypothalamic serotonin content and GnRH mRNA expression were reversed by treatment with the immediate precursor of serotonin, 5-hydroxytryptophan, which bypasses the biosynthetic step catalyzed by TPH. Hypoxia-induced changes in the secretion of pituitary hormones involved in the control of metabolism and growth such as growth hormone and thyroid stimulating hormone (Fig. 4, site B), as well as pancreatic and corticosteroid (stress) hormones, could also modulate the activity of the HPG axis, although direct evidence is currently lacking. In contrast, clear evidence has been obtained that the activities of certain cytochrome P450 enzymes in the steroid hormone biosynthetic pathway, such as aromatase (C19 P450), are down-regulated in fish ovaries after hypoxia exposure (Fig. 4, site C), leading to an impairment of estrogen signaling and an increase in the proportion of fish that develop testes (Shang et al., 2006). The liver is another probable site of hypoxia interference with reproductive function in females during the period of oocyte and ovarian growth when a major portion of the lipid stores as well the energy derived from the diet is converted by the liver to vitellogenin (Fig. 4, site D). The energetically demanding process of vitellogenesis is likely to be severely compromised as a result of the metabolic suppression induced by hypoxia exposure. The liver is also a potential site of interactions between hypoxia and other environmental stressors that act through ARNT-dependent pathways, such as polyhalogenated aromatic hydrocarbons (PHAH, e.g. polychlorinated biphenyls) that act through the aryl hydrocarbon receptor (AhR, Prasch et al., 2004).

3.4. Preliminary assessment of biomarker responses to hypoxia in female Atlantic croaker collected in the northwestern Gulf of Mexico hypoxic zone

The effects of widespread seasonal hypoxia in the northwestern Gulf of Mexico on biomarkers of hypoxia exposure and reproductive function in Atlantic croaker are currently being investigated. Croaker samples collected during the first year of the project in October 3–5, 2006 have been analyzed. The distribution of hypoxic water during the summer and early fall in this region varies and is influenced by the weather (Rabalais et al., 2007). Strong winds for approximately a two-week period prior to the October sampling date had altered the spatial pattern of hypoxia somewhat in that region compared to that recorded on earlier cruises in the summer of 2006 (National Marine Fisheries SEAMAP cruise, July 2006, unpubl. data; N. Rabalais, pers. comm.), thereby complicating the characterization of the sampling sites. In spite of this confounding factor, on the basis of all of the available bottom DO data collected for this region during the summer and fall, it was still possible to characterize these sites into the following three broad categories of 1) normoxic most of the time (NOR; bottom DO: 6 mg L\(^{-1}\); water depth: 20 m; 29°12.785′N, 93°02.932′W, the most westerly site offshore from Calcasieu Pass), 2) a site further east that is hypoxic most of the summer time and was currently hypoxic (CUR-HYP; bottom DO: 2.1 mg L\(^{-1}\); water depth: 18 m; 29°10.788′N, 92°30.242′W, 46.6 km from the normoxic site), and 3) a site further east, 217 km from the normoxic site, that had been hypoxic previously (latest DO recording in area in mid-August) and bottom DO had likely increased within the last two and a half weeks as a result of strong winds during that period, hypoxia transition (HYP-TRN, bottom DO: 4.5–5.8 mg L\(^{-1}\); water depth: 16.5–18 m; 28°45.264′N, 90°32.315′W).

Adult female Atlantic croaker (size range: 12–14 cm), were collected with a 40′ otter trawl (duration of trawl: 15–20 min) and processed on board the RV Longhorn immediately. Blood and tissues were removed within 10–12 min of retrieval of the net, and frozen for hormone or mRNA analyses, respectively. Ovarian tissues were stored in 4% formalin for subsequent histological and fecundity analyses. Transcript levels in brain tissue of one of the potential biomarkers of hypoxia exposure, HIF-2α, displayed a clear positive relationship to exposure to low DO, whereas levels of the other biomarker, HIF-1α, were unchanged (Fig. 5). HIF-2α mRNA levels in fish collected from the hypoxic site were more than two-fold those at the normoxic site (p < 0.05). HIF-2α expression in female fish collected from the site that was normoxic at the time of collection, but had been hypoxic previously (HYP-TRN), was also significantly higher than that in fish from the normoxic site and intermediate between the expression levels in fish from the normoxic and hypoxic sites (Fig. 5). There was also a significant difference between the transition and hypoxic sites in ovarian HIF-2α protein levels as shown by Western blot analysis (Fig. 6, normoxic samples were lost during the analysis).

Reproductive and endocrine functions in female croaker collected from the hypoxic site (CUR-HYP) were impaired compared to those in fish collected from the normoxic site (NOR, Figs. 7–10). Similarly, a preliminary analysis of male fish collected from these sites showed impaired gametogenesis and decreased sperm production (results not shown). These findings are similar to those obtained with croaker collected from hypoxic sites in Pensacola Bay in 2003, and with the results of controlled laboratory hypoxia studies (Thomas et al., 2007a). The

\[\text{(A)}\]

\[\begin{array}{c}
\text{HIF-1α mRNA levels (copies x 10^4)}
\end{array}\]

\[\text{NOR} \quad \text{HYP-TRN} \quad \text{CUR-HYP}\]

\[\begin{array}{c}
\text{a} \quad \text{a} \quad \text{a}
\end{array}\]

\[\text{(B)}\]

\[\begin{array}{c}
\text{HIF-2α mRNA levels (copies x 10^4)}
\end{array}\]

\[\text{NOR} \quad \text{HYP-TRN} \quad \text{CUR-HYP}\]

\[\begin{array}{c}
\text{a} \quad \text{b} \quad \text{c}
\end{array}\]

Fig. 5. Effects of environmental exposure to hypoxia in the northern Gulf of Mexico on expression of HIF-1α and HIF-2α mRNAs in croaker brains. Expression of HIF-1α and HIF-2α mRNAs was measured in the brains of croaker collected from normoxic (NOR), hypoxic transition (HYP-TRN), and currently hypoxic (CUR-HYP) sites in October 3–5, 2006. Each bar represents the mean ± SEM, N = 8–12. Significant differences identified with a multiple range test, Fisher’s PLSD, are indicated with different letters (p < 0.05). Copy number of the target mRNA level in the sample was determined by relating average threshold cycle (C\(_T\)) values to a gene-specific standard curve. The primers used for HIF-1α and HIF-2α mRNA quantification were obtained from the nucleotide sequences of croaker HIFs as described previously (Rahman and Thomas, 2007).
sublethal biological effects have been developed and proven to be useful tools for environmental monitoring and also as a basis for making sound regulatory and resource management decisions. In comparison, environmental exposure of marine organisms to hypoxia and the chronic sublethal effects of low DO have received relatively little attention until recently. Consequently, biomarkers of hypoxia exposure and its sublethal effects are still at an early stage of development. Recent results suggest that two responses to hypoxia exposure, increased HIFx mRNA and protein expression, and indices of hypoxia-induced impairment of reproductive and endocrine functions, are potentially useful as biomarkers of hypoxia exposure and its potential chronic population effects, respectively. However, the specificity of

reproductive biomarker responses of croaker collected from the hypoxia transition site were either similar to those at the hypoxic site or intermediate between those at the two DO extremes. The mean gonadosomatic index (gonad weight/body weight × 100) of female fish collected at the normoxic site (~6) indicated that the fish were at a midpoint of ovarian crudescence, whereas gonadal growth was significantly lower in croaker collected from the hypoxic and hypoxic transition sites (Fig. 7A). Histological assessment of oocyte development showed that the percentage of tertiary yolk (TYS) stage oocytes (i.e., fully grown oocytes capable of undergoing maturation and fertilization to produce viable offspring) in the ovaries of croaker from the hypoxic site was only about 50% of that observed at the normoxic site (Fig. 7B). Similarly, fecundity calculations revealed that the number of large oocytes in the ovaries of hypoxia-exposed fish was approximately 50% of that at the normoxic site (Fig. 7C). Histological examination of ovarian tissue from the three sites showed that the majority of oocytes had not progressed beyond the perinuclear stage in croaker at the hypoxic site and many were at the primary yolk stage at the transition site, whereas ovarian tissue collected from fish at the normoxic site typically had large numbers of tertiary yolk stage (full-grown) and secondary yolk stage oocytes (Fig. 8). The impairment of gametogenesis in the hypoxia-exposed fish was accompanied by declines in reproductive endocrine function. Plasma levels of the female sex steroid hormones, estradiol-17β and testosterone, and the yolk precursor protein, vitellogenin, were significantly decreased in females collected from the hypoxic site (Fig. 9). Finally, a biomarker of reproductive neuroendocrine function, GnRH mRNA expression, was also decreased in the hypothalamus of fish collected at the hypoxic site (Fig. 10).

4. Concluding remarks

Environmental degradation of marine and estuarine habitats due to contamination with anthropogenic chemicals, as well as exposure of marine organisms to these toxic chemicals and their sublethal effects, has been investigated extensively over the past 40 years. A wide variety of biomarkers of exposure to specific chemicals and their

![Fig. 6. Effects of environmental exposure to hypoxia in the northern Gulf of Mexico on the expression of HIF-2α protein in croaker ovaries. HIF-2α protein levels were measured in croaker ovaries collected from hypoxic transition (HYP-TRN) and currently hypoxic (CUR-HYP) sites in October 3–5, 2006. Each bar represents the mean ± SEM, N = 4. Asterisk indicates significant difference from HYP-TRN (Student’s t-test, p < 0.05).](image)

![Fig. 7. Effects of environmental exposure to hypoxia in the northern Gulf of Mexico on oocyte and ovarian development in female croaker. Fish were collected from normoxic (NOR), hypoxic transition (HYP-TRN), and currently hypoxic (CUR-HYP) sites in October 3–5, 2006. (A) Gonadosomatic index (GSI, a measure of ovarian growth); (B) percentage of oocytes at each development stage; and (C) fecundity. Each bar represents the mean ± SEM, N = 8–14. PNS, perinuclear stage; OD, oil-droplet stage; PYS, primary yolk stage; SY5, secondary yolk stage; TYS, tertiary yolk stage. Significant differences identified with a multiple range test, Fisher’s PLSD, are indicated with different letters (p < 0.05). For preparation of histological samples, ovaries were fixed in formalin, embedded in paraffin, sectioned at 7 μm, stained with haematoxylin-eosin, and analyzed histologically according to the method of Rahman et al. (2000).](image)
the HIF\(\alpha\) response to hypoxia has not been confirmed to date and the temporal pattern of HIF\(\alpha\) expression and its DO concentration-response relationship remain unclear. Moreover, field validation of HIF\(\alpha\) as a biomarker in fish of hypoxia exposure in many coastal regions such as the Louisiana shelf is complicated by the dynamic temporal and spatial fluctuations in the extent of hypoxia, and its restriction to the bottom few meters of the water column, thereby preventing an accurate assessment of the extent of a fish’s exposure to low DO. Despite these current problems in the interpretation of the HIF\(\alpha\) expression data obtained from field samples, the finding that HIF\(\alpha\) expression is consistently upregulated in croaker collected from both estuarine and coastal hypoxic sites suggests that in all of these instances the extent of low DO exposure was sufficient to initiate a physiological response that would lead to marked changes in metabolism and energy utilization. Measurement of HIF\(\alpha\) may be more useful, therefore, as a biomarker of exposure to environmental hypoxia conditions that trigger a physiological defense mechanism in an organism, than as a biomarker of exposure to a particular hypoxia regime.

Our results clearly show that reproduction and its endocrine control in croaker inhabiting estuaries and coastal regions in the northern Gulf of Mexico are very susceptible to disruption by environmental exposure to hypoxia. However, it is not known which reproductive stages in croaker are most susceptible to disturbance by hypoxia, and the mechanisms of hypoxia disruption of endocrine function. In addition, the percent of the croaker population affected in this manner by hypoxia exposure in these regions remains unclear. Similar studies on other estuarine species and on croaker populations in different geographical regions will be required to determine the broad applicability.
of these findings. Finally, there is an urgent need to assess the long- 

term population hazards of the hypoxia-induced impairment of repro-

duction in fishes inhabiting the extensive coastal hypoxic regions in 

the northern Gulf of Mexico and other regions of the world. A goal of 

a current collaborative project involving fish physiologists, ecologists 

and population modelers is to predict the population impacts of these 

changes in reproductive and endocrine biomarkers in croaker, using 

physiological and individual based models developed for this species 

(Rose et al., 2003; Murphy et al., 2005).

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