

# Diel hypoxia in marsh creeks impairs the reproductive capacity of estuarine fish populations

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**ABSTRACT:** Seasonal hypoxia in estuaries and continuous hypoxia in the laboratory significantly reduce the reproductive capacity of estuarine fish, but the impacts of diel hypoxia are unknown. This field study quantifies reproductive capacity in populations of gulf killifish (*Fundulus grandis*) sampled from sites with different dissolved oxygen cycles. Condition factor, gonadosomatic index (GSI), steroid sex hormones, and vitellogenin were measured in *F. grandis* captured in Weeks Bay (WB), Alabama (July 2004 and 2005) and Pensacola Bay (PB), Florida (July 2005). Compared to a control site with little or no diel hypoxia, testes and ovaries were significantly smaller under both moderate (WB: 2.61 mg l<sup>-1</sup>, 0.6 h d<sup>-1</sup>; PB: 2.41 mg l<sup>-1</sup>, 1.5 h d<sup>-1</sup>) and severe (0.93 mg l<sup>-1</sup>, 3.4 h d<sup>-1</sup>) diel hypoxia. Male 11-ketotestosterone (11KT) concentrations were significantly lower under moderate hypoxia, while both testosterone (T) and 11KT concentrations were significantly reduced under severe diel hypoxia. In females, T concentrations were similar regardless of the occurrence or severity of hypoxia, but estradiol-17 $\beta$  (E2) concentrations were lower under severe diel hypoxia. Since T is unchanged, but its products 11KT and E2 are significantly reduced, hypoxia may affect reproduction by inhibiting specific steroidogenic enzymes in the gonad. The association between diel hypoxia and lower reproductive capacity was consistent across estuaries. Hypoxia-related changes in gulf killifish populations could affect trophic structure in marshes and bays since *F. grandis* move carbon from the upper marsh surface to the lower marsh, as increased biomass and as prey for juvenile piscivores.

**KEY WORDS:** Hypoxia · Estuary · Reproduction · *Fundulus grandis* · Estradiol-17 $\beta$  · Testosterone · 11-ketotestosterone · Vitellogenin

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## INTRODUCTION

Most estuaries have underlying cycles of dissolved oxygen (DO) that are driven by internal nutrient cycles, diel photosynthesis and respiration cycles, freshwater discharge, tidal incursion of high density saltwater, and meteorological conditions that allow persistent stratification and development of low oxygen bottom water. Human nutrient input has significantly altered DO cycles in coastal waters such that

summertime hypoxia (DO < 2 mg l<sup>-1</sup>) and anoxia of bottom water are now common worldwide (Diaz & Rosenberg 2008). Microtidal estuaries of the Gulf of Mexico are especially sensitive to the effects of eutrophication (Cloern 2001) and 23% of the estuarine area surveyed in National Estuary Program sites develops summer hypoxia (US EPA 2007). Oxygen dynamics in shallow estuaries and salt marshes occur on shorter time scales than in deep estuaries. Diel cycles of DO are common, especially in late summer,

with oscillations between 0 and as much as 20 mg l<sup>-1</sup> in salt marsh creeks (Gardner & Gorman 1984, D'Avanzo & Kremer 1994, Timmerman & Chapman 2004, Tyler & Targett 2007).

Hypoxia alters fish metabolism (Gracey et al. 2001, Nikinmaa & Rees 2005, Martinez et al. 2006) and has the potential to alter individual energy budgets. Overall energy expenditure may be suppressed or specific portions of the energy budget may be reduced, particularly growth and reproduction. In the laboratory, constant and cyclic hypoxia suppress juvenile and adult growth of estuarine fishes (Stierhoff et al. 2003, McNatt & Rice 2004, Stierhoff et al. 2006, Landry et al. 2007). In wild fish, intermittent estuarine hypoxia reduces juvenile growth of the Atlantic croaker *Micropogonias undulatus* and the summer flounder *Paralichthys dentatus* (Eby et al. 2005, Stierhoff et al. 2006). Hypoxia also impairs, but does not prevent reproduction. Continuous hypoxia severely depresses reproduction in the laboratory (Wu et al. 2003, Thomas et al. 2006, Landry et al. 2007) and seasonal estuarine hypoxia suppresses wild Atlantic croaker reproduction (Thomas et al. 2006, 2007). The effects of diel hypoxia on fish reproduction are currently unknown.

The purpose of the present field study was to determine whether diel cycles of hypoxia suppress reproductive capacity in a resident marsh species. We focused on the gulf killifish *Fundulus grandis* because it is abundant along the entire northern Gulf of Mexico coast (Pattillo et al. 1997), has well documented semilunar spawning cycles (Greeley & MacGregor 1983, Greeley et al. 1988), and well characterized behavioral and physiological responses to oxygen stress. *F. grandis* is an oxygen regulator, sustaining constant oxygen consumption at DO concentrations above ~1.5 mg l<sup>-1</sup>, but becoming an oxygen conformer at lower concentrations (Virani & Rees 2000). At environmental DO ≤1.5 mg l<sup>-1</sup>, blood lactate levels are elevated (Virani & Rees 2000) and anaerobic capacity increases in the liver, heart, and brain (Martinez et al. 2006), indicating the occurrence of systemic hypoxia. In the laboratory, long-term (30 d) hypoxia impairs gulf killifish growth and reproduction. Gonad size is reduced by 40 to 70% and sex hormones mediating gamete production are similarly reduced. Importantly, these morphological and hormonal changes are associated with significant reductions in fecundity (Landry et al. 2007).

To quantify reproductive variation in *Fundulus grandis* populations experiencing different diel oxygen cycles in their natural environment, condition factor, gonadosomatic index (GSI), steroid sex hormone concentrations, and vitellogenin (VTG) concentrations were measured. Condition factor is an indicator of general health and GSI is an integrative measure of reproductive status because gonad size and gamete

production are regulated by steroid sex hormones as well as pituitary and hypothalamic hormones. During gonadal steroid hormone biosynthesis, testosterone (T) is produced from cholesterol in males and females. In males, T is the precursor to 11-ketotestosterone (11KT), the androgen that is primarily responsible for spermatogenesis and expression of male secondary sex characteristics in fish (Devlin & Nagahama 2002). In females, T is the precursor to estradiol-17β (E2). E2 stimulates VTG production in the female liver. VTG is secreted from the liver into circulation, taken up by growing oocytes, and cleaved into smaller egg yolk proteins (Hiramatsu et al. 2005). T, 11KT, E2, and VTG were measured as indicators of preparation for reproduction.

## MATERIALS AND METHODS

**Sampling design.** To evaluate the consistency of a diel hypoxia effect on fish reproduction, we used 2 sampling approaches. (1) Fish were sampled from different sites with different DO conditions at the same time. (2) Fish were sampled from the same site under different DO conditions at the same time of year in different years. In the first case, spatial variation in factors other than DO could confound interpretation, while in the second case, temporal variation could be a confounding effect. To further investigate spatial variation in physiological responses to diel hypoxia, we sampled 2 estuaries on the northern Gulf of Mexico coast.

**Field sites.** Weeks Bay (Alabama) and Pensacola Bay (Florida) were chosen as study sites because they differ in surface area, depth, and nutrient input. Weeks Bay is a 7 km<sup>2</sup>, microtidal (0.4 m) sub-estuary of Mobile Bay with a mean depth of 1.4 m. The system is highly eutrophic (Murrell & Caffrey 2005) and has a freshwater mean residence time of 13 d (Adams et al. 2004). Pensacola Bay is a 370 km<sup>2</sup>, microtidal (0.4 m) system in northwestern Florida with a mean depth of 3.5 m. Freshwater sources include the Escambia, Blackwater and Yellow Rivers. Mean freshwater residence time is ~25 d (Solis & Powell 1999). Eutrophication of Pensacola Bay is moderately low (Bricker et al. 2007).

In each estuary, 2 sites were identified from preliminary datasonde deployments: a site which rarely developed diel hypoxia and a site which frequently developed diel hypoxia. In both estuaries, distance between sites exceeded the expected linear movement of *Fundulus grandis* and sites were separated by open bay area. Based on the estimated home range of *Fundulus heteroclitus* (15 ha; Teo & Able 2003), linear distance traveled could be 400 to 550 m. Weeks Bay sites were on opposite shores of the bay and were separated by ~640 m. Pensacola Bay sites were on the same

peninsula, but were separated by 12 km (shoreline distance).

At both sites in Weeks Bay, fish were collected within 100 m of datasondes that were deployed at 30° 22.52' N, 87° 49.95' W (creek) and at 30° 22.67' N, 87° 50.25' W (shoreline). In Pensacola Bay, fish were collected from 2 marshes on the peninsula dividing Escambia from East Bay. A datasonde was deployed at 30° 26.541' N, 87° 05.299' W, near the mouth of a secondary creek feeding a network of marsh pools at Gargon Point. A second datasonde was deployed at 30° 31.665' N, 87° 06.103' W, just inside the mouth of a secondary creek flowing into Indian Bayou.

**Water quality data.** Water quality data were recorded using YSI (Yellow Springs Instruments) 600 XLM, 6920, or 6600 datasondes. All instruments included sensors for DO, salinity, temperature, and depth. Sondes were secured in a PVC housing that was anchored or forced into the bottom. At all sites, probes protruded from the housing ~20 cm above the substrate. Instruments were deployed for 6 to 30 d intervals. Instruments deployed for longer than 1 wk were serviced and data were uploaded and archived at 4 to 10 d intervals. Sensors were calibrated both before and after deployment to evaluate sensor drift over the deployment period. DO concentration, salinity, and temperature were recorded every 15 min. DO cycle indices were calculated only for calendar dates on which the sonde was at the site from 00:00 to 23:45 h.

**Fish collection.** Scientific collecting permits were issued by the Alabama Department of Conservation and Natural Resources and the Florida Fish and Wildlife Conservation Commission. Collection and tissue sampling methods were approved by the Institutional Animal Care and Use Committee of Southeastern Louisiana University. A minimum of 20 males and 20 females were collected from each site. Because lunar phase significantly influences the reproductive status of the gulf killifish (Greeley & MacGregor 1983), collection from both sites within an estuary was completed over 2 to 3 d in the 5 d period before the July full moon. Weeks Bay sites were sampled on lunar days 10 to 12 (lunar day 0 = new moon) in 2004 and 2005. Pensacola Bay sites were sampled on lunar days 13 and 14 in 2005. Simultaneous sampling in both estuaries was not logistically feasible. Fish were caught in baited minnow traps that were deployed for 0.17 to 13 h intervals (mean  $\pm$  SD: 2.1  $\pm$  1.1 h). Several traps were retrieved simultaneously and fish were bled on site. Within 44  $\pm$  34 min of trap retrieval, fish  $\geq$ 60 mm standard length (SL) were anesthetized in 0.1 g l<sup>-1</sup> ethyl 3-aminobenzoate methanesulfonate (MS-222, Sigma Chemical) in seawater, measured and bled. The caudal vein was severed and blood was collected by capillary flow into heparinized microcapillary tubes (Drummond

Scientific), transferred to heparinized microcentrifuge tubes, and mixed with 3  $\mu$ l of aprotinin (0.084 trypsin inhibitory units (TIU)  $\mu$ l<sup>-1</sup>; Calbiochem). Whole blood was held on ice until transport to the laboratory, then centrifuged at 16 000  $\times$  *g* for 30 s at room temperature. Plasma was aspirated and stored on dry ice for transport, then stored at -80°C until analysis.

After blood collection, fish were euthanized by severing the spinal cord, and gonads were removed. Fish carcasses and gonads were placed on ice until transported to the laboratory, then weighed to calculate GSI (gonad weight/[body weight without gonad]  $\times$  100) and Fulton's condition factor ( $K = [100\,000 \times \text{weight}]/\text{length}^3$ ).

**Hormone and VTG analysis.** E2, T, and 11KT were analyzed using acetylcholinesterase-based competitive enzyme-linked immunosorbent assay (ELISA) performed according to the manufacturer's instructions (Cayman Chemical) and validated for use with *Fundulus grandis* plasma (Landry et al. 2007). Prior to analysis, duplicate plasma aliquots (E2 and T, 3  $\mu$ l; 11KT, 5  $\mu$ l) were extracted with two 1.5 ml aliquots of a hexane-ethyl acetate mixture (70:30 v/v), evaporated to dryness, and reconstituted in assay buffer. Steroid recovery was >90%. Inter-assay variation was 17.4% for E2 (n = 3 different pools, each pool analyzed in quadruplicate in 5 to 7 assays), 22.0% for T (n = 2 different pools, each pool analyzed in quadruplicate in 9 to 10 assays), and 9.4% for 11KT (n = 2 different pools, each pool analyzed in quadruplicate in 4 to 6 assays).

Circulating VTG concentrations were analyzed using competitive ELISA with purified *Fundulus grandis* VTG as the standard, and immobilized competitor and anti-killifish (*Fundulus heteroclitus*) VTG antibody (Cayman Chemical) as the primary antibody (Landry et al. 2007). Recovery, intra-assay (n = 20 wells), and inter-assay (n = 2 different pools analyzed in quadruplicate in 3 to 6 assays) variations were 83, 5.6, and 11.7%, respectively.

**Statistics.** Differences in condition factor, relative gonad size, steroid sex hormones, and VTG between sites were analyzed using stepwise forward regression with site as a categorical independent variable and SL, lunar day, duration of trap deployment, holding time between trap collection and blood sampling, and time of day as covariates. The interaction between site and SL was also tested. The criterion for including or removing a covariate was  $\alpha = 0.10$ . SL was included in the model because relative gonad size and steroid sex hormones can vary with body size in fish. Lunar day was included because lunar phase significantly affects GSI and sex steroids in *Fundulus grandis* (Greeley & MacGregor 1983, Greeley et al. 1988), and sampling occurred over a 2 to 3 d period with samples being collected at both sites each day. Time of day was included

because *F. grandis* steroid hormone concentrations and GSI vary over a 24 h period (Emata et al. 1991). Duration of trap deployment and holding time are potential methodological confounding factors. In practice, trap deployment time was targeted at 2 h and holding time before processing was kept as short as possible, but fish were sampled at all times of day since the approach was to collect 80 fish in each estuary in the shortest possible period.

Reproductive status was classified based on GSI. The post-spawning GSI of each sex was measured in November in each estuary (A. O. Cheek unpubl. data). Fish sampled in July (the present study) were classified as active if the GSI exceeded the maximum post-spawning GSI or as regressed if the GSI was below the estuary-specific post-spawning GSI. Site differences in proportions of regressed vs. active fish were analyzed using Chi-square test. Males and females were analyzed separately for each year and for each estuary. Hormone and GSI data were respectively  $\log [10(y + 1)]$  and  $\arcsin(\sqrt{y})$  transformed to meet assumptions of normality and homoscedasticity. Analyses were conducted using Systat 11 (Systat Software). Differences were considered significant at  $p \leq 0.05$ .

## RESULTS

### Diel dissolved oxygen cycles

Within each estuary, sites that differed in DO regime but had similar temperature and salinity were identified (Table 1). The most severe hypoxia was recorded at the Weeks Bay creek site in July 2004, when a mean daily DO minimum of  $0.93 \text{ mg l}^{-1}$  occurred for  $\sim 3 \text{ h d}^{-1}$ , nearly every day. In contrast, the Weeks Bay shoreline site had a mean daily DO minimum of  $4.03 \text{ mg l}^{-1}$  and a mean daily period of hypoxia (defined as  $\text{DO} \leq 2 \text{ mg l}^{-1}$ ) of  $< 1 \text{ h}$  occurring  $\sim 1 \times \text{wk}^{-1}$  (Fig. 1). In July 2005,

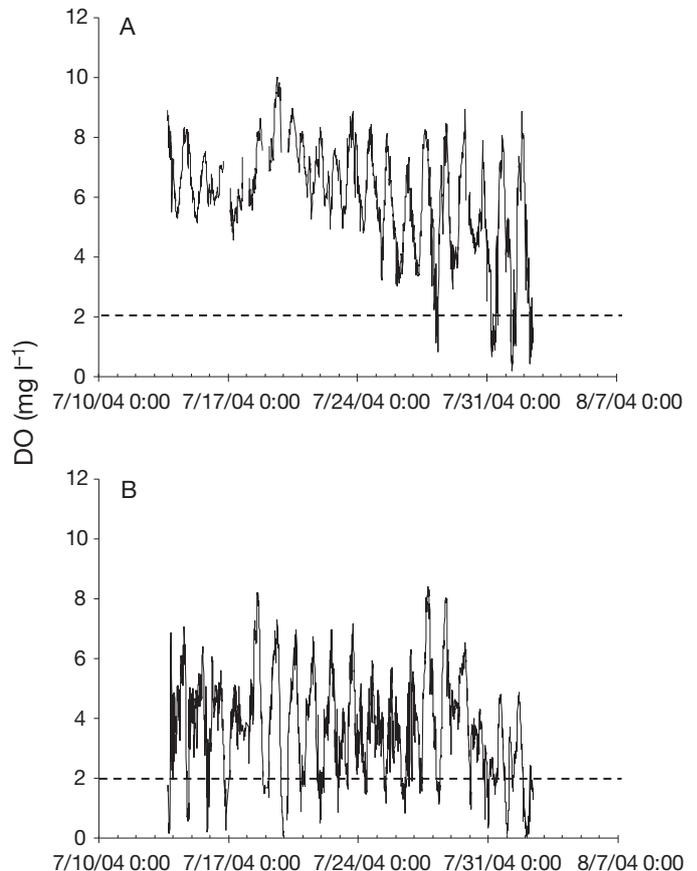


Fig. 1. Dissolved oxygen (DO) cycles at 2 sites in Weeks Bay (Alabama) in July 2004; dashed line indicates hypoxia ( $\leq 2 \text{ mg DO l}^{-1}$ ): (A) Weeks Bay shoreline, (B) Weeks Bay creek. DO ( $\text{mg l}^{-1}$ ) was recorded every 15 min at each site using YSI datasondes. Site descriptions and geographic coordinates are in 'Materials and methods'. Dates are mo/d/yr

diel hypoxia did not occur at the Weeks Bay shoreline site during the recording period, but occurred for  $\sim 0.6 \text{ h d}^{-1}$  at the creek site. In Pensacola Bay, DO regimes differed between the sampling sites in a simi-

Table 1. Dissolved oxygen (DO) regime in July. % saturation: ratio of recorded DO to the possible DO at 100% air saturation of water at the stated salinity and temperature, hypoxic period: daily period with  $\text{DO} \leq 2 \text{ mg l}^{-1}$ , % of days: percentage of recording days in which hypoxic periods occurred, N: number of days with sufficient records ( $\geq 70$  of 96 possible recording intervals  $\text{d}^{-1}$ ) for analysis of DO parameters. Values for DO, period, temperature, and salinity are means  $\pm 1 \text{ SD}$ . Site descriptions and geographic coordinates are in 'Materials and methods'. WB: Weeks Bay; PB: Pensacola Bay

Year	Estuary	Site	Daily minimum		Hypoxic period (h)	% of days	N	Temp ( $^{\circ}\text{C}$ )	Salinity
			DO ( $\text{mg l}^{-1}$ )	% saturation					
2004	WB	Shoreline	$4.03 \pm 1.98$	56.2	$0.79 \pm 2.08$	17	18	$30.6 \pm 0.9$	$6.3 \pm 3.3$
		Creek	$0.93 \pm 0.67$	12.9	$3.36 \pm 2.12$	95	19	$30.8 \pm 1.3$	$5.6 \pm 3.4$
2005	WB	Shoreline	$4.65 \pm 0.59$	61.2	0	0	6	$29.2 \pm 0.9$	$1.8 \pm 0.7$
		Creek	$2.61 \pm 1.05$	33.1	$0.63 \pm 0.89$	50	6	$27.1 \pm 1.0$	$1.2 \pm 0.2$
2005	PB	Garcon Point	$4.39 \pm 1.00$	61.1	0	0	9	$31.1 \pm 1.7$	$5.9 \pm 2.1$
		Indian Bayou	$2.41 \pm 1.25$	33.0	$1.53 \pm 1.99$	50	8	$30.8 \pm 0.8$	$3.0 \pm 0.6$

Table 2. Values (mean and range) of covariates included in the general linear model for site differences in reproductive capacity of *Fundulus grandis*. SL: standard length; lunar day: day since new moon; trap time: duration of trap deployment; hold time: period of time between removal of fish from trap and blood sample collection; time of day: time at which the blood sample was drawn; N: number of fish sampled from each site (males and females combined). WB: Weeks Bay; PB: Pensacola Bay. \*\*\* $p < 0.001$  for differences between sites within the same estuary (1-way ANOVA)

	WB 2004		WB 2005		PB 2005	
	Shoreline	Creek	Shoreline	Creek	Garcon Point	Indian Bayou
SL (mm)	79.6 (62 – 112)	71.3 (60 – 100)***	71.7 (60 – 88)	73.6 (60 – 112)	77.2 (65 – 100)	77.9 (64 – 110)
Lunar day	11.8 (11 – 12)	10.4 (10 – 11)***	11.0 (10 – 12)	10.9 (10 – 12)	13.2 (13 – 14)	14.3 (14 – 15)***
Time of day	16:29 (14:56 – 18:48)	17:24*** (16:08 – 18:20)	14:27 (11:05 – 19:42)	13:58 (8:10 – 21:01)	12:42 (8:57 – 16:13)	13:38 (9:49 – 17:33)
Trap time (h)	1.3 (0.2 – 2.4)	2.4 (2.0 – 2.8)***	3.0 (1.5 – 4.7)	2.6 (1.8 – 4.1)	1.4 (0.4 – 4.3)	1.6 (1.0 – 3.3)
Hold time (min)	48.6 (0 – 100)	89.9 (27 – 143)***	16.4 (0 – 42)	19.3 (3 – 56)	39.3 (9 – 78)	37.2 (15 – 63)
N	49	61	45	45	54	40

lar manner: diel hypoxia did not occur at the Garcon Point site, but occurred for 1.5 h d<sup>-1</sup> at the Indian Bayou site (Table 1).

#### Consistency of sampling between sites

Mean size of sampled fish, timing of sampling, and handling time (trap time and holding time) were similar between sites within each estuary in 2005 with the exception that most fish at the Indian Bayou site in Pensacola Bay were sampled 1 d later than fish at the Garcon Point site (Table 2). In 2004, fish sampled at the Weeks Bay creek site were smaller, were sampled 1 d earlier, were in the trap 1 h longer, were held 41 min longer before sampling, and were sampled 1 h later in the day, on average, than those sampled at the shoreline site (Table 2).

#### Effect of different dissolved oxygen regimes

The reproductive capacity of *Fundulus grandis* experiencing different frequency and severity of diel

hypoxia was compared between the Weeks Bay creek and Weeks Bay shoreline sites in 2004. In both sexes, body condition was similar between sites (Fig. 2), suggesting that cyclic hypoxia did not impair the overall health of *F. grandis*. Body condition was not affected by lunar phase, time of day, duration of trap deployment, or holding time before sampling. In females, but not in males, condition increased with length, i.e. longer females were also more plump ( $F = 6.2$ ,  $p = 0.016$ ). There was no Site  $\times$  SL interaction for either sex, indicating that the relationship between condition and size was the same at both sites.

Male GSI was positively related to lunar phase ( $F = 8.0$ ,  $p = 0.007$ ), such that fish collected closer to the full moon had larger GSI at both sites. Mean male GSI neither varied significantly with other temporal variables or with body size, nor did it differ significantly between sites (Fig. 2). Female GSI was not affected by any of the temporal variables or by size. However, mean GSI was 43% lower in females at the site with severe diel hypoxia (Fig. 2;  $F = 19.0$ ,  $p < 0.001$ ).

At each site, some fish were reproductively regressed. Across sites, regressed fish were smaller than reproductively active fish (Table 3; males:  $F_{\text{status}} = 5.8$ ,

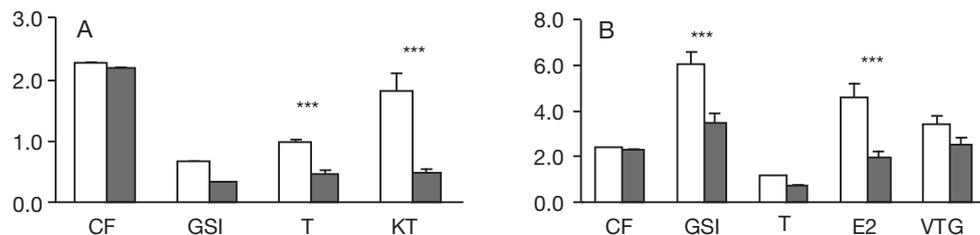


Fig. 2. *Fundulus grandis*. Effect of diel hypoxia on condition and reproduction: (A) males, (B) females. In 2004, diel hypoxia occurred infrequently and was brief at the Weeks Bay shoreline site (□). Severe, frequent diel hypoxia (12.9% saturation) occurred at the Weeks Bay creek site (■). CF: condition factor ( $[100\,000 \times \text{weight}]/\text{length}^3$ ); GSI: gonadosomatic index (gonad weight/[body weight – gonad weight]); T: testosterone (ng ml<sup>-1</sup>); KT: 11-ketotestosterone (ng ml<sup>-1</sup>); E2: estradiol-17 $\beta$  (ng ml<sup>-1</sup>); VTG: vitellogenin (mg ml<sup>-1</sup>). Values are means  $\pm$  1 SE. N = 22 to 31 ind. of each sex at each site. \*\*\* $p < 0.001$  for the site effect after accounting for temporal and body size covariates (defined in 'Materials and methods')

$p = 0.02$ ; females:  $F_{\text{status}} = 4.4$ ,  $p = 0.04$ ). Significantly more males were regressed at the site with severe diel hypoxia (Table 3;  $\chi^2 = 12.2$ ,  $p < 0.001$ ). The proportion of regressed females was low and similar between sites (Table 3;  $\chi^2 = 1.6$ ,  $p = 0.20$ ).

Steroid hormone concentrations did not vary with body size in either sex. Androgen concentrations were positively related to lunar phase in both sexes. Male T and 11KT increased as the moon waxed to full (T:  $F_{\text{lunar phase}} = 7.5$ ,  $p = 0.009$ ; 11KT:  $F_{\text{lunar phase}} = 4.9$ ,  $p = 0.031$ ) as did female T ( $F_{\text{lunar phase}} = 12.9$ ,  $p = 0.001$ ). Female E2 was unrelated to lunar phase. Male T and 11KT decreased with holding time before sampling (T:  $F_{\text{holding time}} = 12.9$ ,  $p = 0.001$ ; 11KT:  $F_{\text{holding time}} = 20.9$ ,  $p < 0.001$ ), emphasizing the importance of minimizing holding time as much as logistically possible. Male androgen concentrations were 54 to 74% lower at the site with frequent, severe diel hypoxia (Fig. 2; T:  $F_{\text{site}} = 16.5$ ,  $p < 0.001$ ; 11KT:  $F_{\text{site}} = 16.4$ ,  $p < 0.001$ ). Female T did not differ between sites, but female E2 was 56% lower at the site with frequent, severe diel hypoxia ( $F_{\text{site}} = 17.9$ ,  $p < 0.001$ ). Female VTG concentrations increased significantly with body size ( $F_{\text{size}} = 7.1$ ,  $p = 0.010$ ), but did not differ between sites.

### Consistency across years

To evaluate whether differences in frequency and intensity of diel hypoxia are consistently associated with lower reproductive capacity, we sampled *Fundulus grandis* from the same Weeks Bay sites in a second

year. Fish were collected at the same lunar phase as in the first year: lunar days 10 to 12 prior to the July full moon. In 2005, the DO regime differed between sites and also differed from that in July 2004 (Table 1). Diel hypoxia did not occur at the shoreline site during the recording period, and was less severe and less frequent at the creek site than in the previous year (Table 1).

As in the first year, diel hypoxia had no effect on condition factor in either sex (Fig. 3). Unlike 2004, however, male GSI did not vary with lunar phase, although female GSI increased as the full moon approached ( $F_{\text{lunar phase}} = 11.4$ ,  $p = 0.002$ ). In contrast to 2004, GSI increased significantly with body size in both sexes (male:  $F_{\text{size}} = 12.0$ ,  $p = 0.001$ ; female:  $F_{\text{size}} = 17.2$ ,  $p < 0.001$ ), i.e. larger fish invested a larger proportion of body mass in gonads than did smaller fish. Accounting for size and lunar phase effects, GSI was 57% lower in males and 41% lower in females at the site with moderate diel hypoxia (Fig. 3; males:  $F_{\text{site}} = 45.9$ ,  $p < 0.001$ ; females:  $F_{\text{site}} = 11.9$ ,  $p = 0.001$ ).

Similar to 2004, some fish were regressed at each site and regressed fish were smaller than reproductively active fish (Table 3; males:  $F_{\text{status}} = 6.0$ ,  $p = 0.02$ ; females:  $F_{\text{status}} = 5.9$ ,  $p = 0.02$ ). More fish were regressed at the site with diel hypoxia and the effect was more pronounced in males than in females (Table 3; male  $\chi^2 = 19.5$ ,  $p < 0.0001$ ; female  $\chi^2 = 3.7$ ,  $p = 0.05$ ).

In 2005, lunar phase had no significant effect on male androgens. However, concentrations of T and 11KT increased with body size (T:  $F_{\text{size}} = 2.6$ ,  $p = 0.013$ ; 11KT:  $F_{\text{size}} = 14.0$ ,  $p = 0.001$ ). Mean male T concentrations were not affected by moderate diel hypoxia (Fig. 3; 2005) as they were by severe diel hypoxia (2004). There was a Site  $\times$  SL interaction for 11KT such that the size-related increase was dampened by moderate diel hypoxia ( $F_{\text{site} \times \text{SL}} = 10.2$ ,  $p = 0.003$ ) and 11KT concentrations were 42% lower (Fig. 3). Under less severe hypoxia, a significant influence of lunar phase on female sex steroids was detectable. E2 and T increased as the moon waxed (E2:  $F_{\text{lunar phase}} = 38.5$ ,  $p < 0.001$ ; T:  $F_{\text{lunar phase}} = 4.6$ ,  $p < 0.001$ ). After accounting for temporal and body size variables, mean female T concentrations were unaffected by diel hypoxia, just as in 2004. Moderate diel hypoxia was not associated with a reduction in E2 concentrations, unlike the 56% reduction observed under severe diel hypoxia. VTG concentrations increased significantly with body size ( $F_{\text{size}} = 8.8$ ,  $p = 0.005$ ), but

Table 3. *Fundulus grandis*. Reproductive status and size at sites with differing severity of diel hypoxia. Site descriptions and geographic coordinates are in 'Materials and methods'; dissolved oxygen (DO) regime is described in Table 1. WB: Weeks Bay; PB: Pensacola Bay; SL: standard length (mm). 1-way ANOVA between sites within the same estuary: \* $p \leq 0.05$ , \*\*\*  $p \leq 0.001$ . 1-way ANOVA between reproductive status (active or regressed) within the same estuary in the same year: ( $\dagger$ )  $p \leq 0.05$ . No Site or Site  $\times$  Status main effects on size were detected in any sample

	— WB 2004 —		— WB 2005 —		— PB 2005 —	
	Shoreline Creek		Shoreline Creek		Garcon Point	Indian Bayou
<b>Female</b>						
% regressed	4	14	5	26*	33	95***
SL regressed	78	64 $\pm$ 2	60	65 $\pm$ 2	72 $\pm$ 2	79 $\pm$ 2
SL active	82 $\pm$ 2	74 $\pm$ 2 $\dagger$	73 $\pm$ 2	78 $\pm$ 3 $\dagger$	81 $\pm$ 2	84
N	26	29	21	23	24	20
<b>Male</b>						
% regressed	0	41***	5	70***	48	100***
SL regressed	—	69 $\pm$ 2	64	68 $\pm$ 2	75 $\pm$ 2	76 $\pm$ 3
SL active	78 $\pm$ 2	71 $\pm$ 1 $\dagger$	72 $\pm$ 2	85 $\pm$ 4 $\dagger$	79 $\pm$ 2	—
N	23	32	22	20	29	20

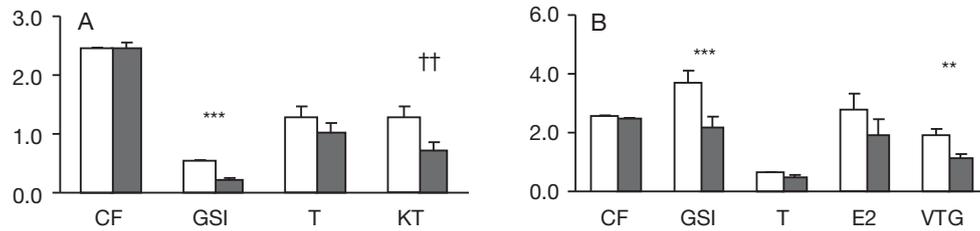


Fig. 3. *Fundulus grandis*. Consistency of hypoxia effects during a second year at Weeks Bay sites: (A) males, (B) females. In July 2005, diel hypoxia did not occur at the Weeks Bay shoreline site (□). At the creek site (■), moderate diel hypoxia (33.1% saturation) occurred frequently. CF: condition factor ( $[100\,000 \times \text{weight}]/\text{length}^3$ ); GSI: gonadosomatic index (gonad weight/[body weight – gonad weight]); T: testosterone ( $\text{ng ml}^{-1}$ ); KT: 11-ketotestosterone ( $\text{ng ml}^{-1}$ ); E2: estradiol-17 $\beta$  ( $\text{ng ml}^{-1}$ ); VTG: vitellogenin ( $\text{mg ml}^{-1}$ ). Values are means  $\pm$  1 SE. N = 19 to 23 ind. of each sex at each site. \*\*\* $p \leq 0.001$  for the site effect, †† $p \leq 0.01$  for Site  $\times$  Standard length (SL) interaction, after accounting for temporal and body size covariates (defined in 'Materials and methods')

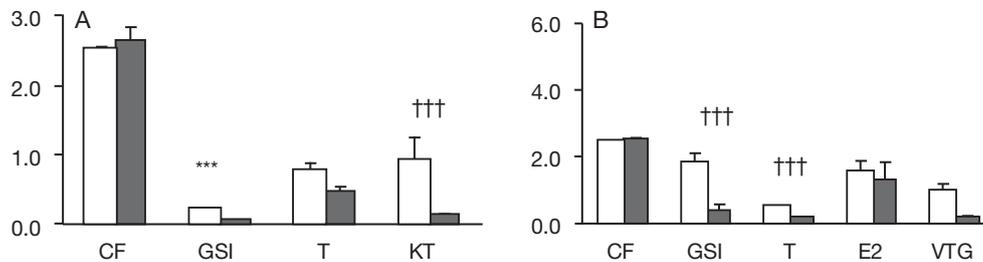


Fig. 4. *Fundulus grandis*. Consistency of hypoxia effects in another estuary: (A) males, (B) females. In July 2005, dissolved oxygen (DO) regimes at Pensacola Bay (FL) sites were similar to those at Weeks Bay (AL) sites (see Figs. 1 & 2). Diel hypoxia did not occur at the Garcon Point creek (□), but moderate diel hypoxia (33% saturation) occurred frequently at the Indian Bayou creek (■). CF: condition factor ( $[100\,000 \times \text{weight}]/\text{length}^3$ ); GSI: gonadosomatic index (gonad weight/[body weight – gonad weight]); T: testosterone ( $\text{ng ml}^{-1}$ ); KT: 11-ketotestosterone ( $\text{ng ml}^{-1}$ ); E2: estradiol-17 $\beta$  ( $\text{ng ml}^{-1}$ ); VTG: vitellogenin ( $\text{mg ml}^{-1}$ ). Values are means  $\pm$  1 SE. N = 20 to 29 ind. of each sex at each site. \*\*\* $p \leq 0.001$  for the site effect, ††† $p \leq 0.001$  for the site  $\times$  standard length (SL) interaction, after accounting for temporal and body size covariates (defined in 'Materials and methods')

were 39% lower under moderate diel hypoxia ( $F_{\text{site}} = 6.4$ ,  $p = 0.015$ ).

### Consistency across estuaries

To evaluate whether diel hypoxia is consistently associated with reproductive impairment across populations, we sampled *Fundulus grandis* in Pensacola Bay in July 2005. Fish were collected in Pensacola Bay during the same week of the lunar cycle as the collection in Weeks Bay. Diel hypoxia did not occur at the Garcon Point site during the recording period. At the Indian Bayou site, the severity of hypoxia was similar to the Weeks Bay creek site in 2005 and occurred with similar frequency (Table 1).

In Pensacola Bay, condition factor was unaffected by diel hypoxia (Fig. 4). GSI was unrelated to lunar phase in either sex, but increased significantly with body size in both sexes (male:  $F_{\text{size}} = 6.1$ ,  $p = 0.018$ ; female:  $F_{\text{size}} = 7.1$ ,  $p = 0.011$ ). Even so, mean male GSI was 79% lower

at the site with diel hypoxia than at the normoxic site ( $F_{\text{site}} = 47.1$ ,  $p < 0.001$ ). The size-related increase in female GSI differed between sites ( $F_{\text{site} \times \text{SL}} = 25.5$ ,  $p < 0.001$ ): under normoxia, female GSI increased with body size, but under moderate diel hypoxia, female GSI was similar for all sizes of fish. In fact, 95% of these females had regressed ovaries that averaged only 0.4% of body weight (Fig. 4, Table 3). At both sites, all sizes and both sexes of fish were terminating spawning (Table 3; male  $F_{\text{status}} = 0.5$ ,  $p = 0.61$ ; female  $F_{\text{status}} = 2.3$ ,  $p = 0.14$ ). However, less than half of fish at the normoxic site were regressed, while nearly all fish at the site with diel hypoxia were regressed (Table 3; male  $\chi^2 = 14.9$ ,  $p = 0.0001$ ; female  $\chi^2 = 17.5$ ,  $p < 0.0001$ ).

The relationship between moderate diel hypoxia and male sex steroids was the same in Pensacola Bay as in Weeks Bay: T concentrations increased with body size ( $F_{\text{size}} = 6.8$ ,  $p = 0.012$ ) and lunar phase ( $F_{\text{lunar phase}} = 7.0$ ,  $p = 0.011$ ), but did not differ between sites. The size-related increase in 11KT was suppressed at the site with diel hypoxia, resulting in 84% lower 11KT ( $F_{\text{site} \times \text{SL}}$

= 15.1,  $p < 0.001$ ; Fig. 4). T concentrations were significantly lower in large females at the site with diel hypoxia ( $F_{\text{site} \times \text{SL}} = 47.0$ ,  $p < 0.001$ ), but E2 and VTG concentrations did not differ with body size or between sites.

## DISCUSSION

Long-term, continuous hypoxia in the laboratory significantly reduces fecundity and delays spawning of *Fundulus grandis* collected in estuarine tributaries of Pascagoula Bay, MS (Landry et al. 2007). Our present field observations of populations in Weeks Bay and Pensacola Bay are the first to show that exposure to repeated short periods of hypoxia is associated with significantly impaired reproductive capacity in a marsh resident.

Inferring cause-effect relationships in wild populations is difficult. An eco-epidemiological approach applies causal criteria to evaluate observational and experimental evidence that a particular environmental condition causes a specific physiological effect (Fox 1991). Widely accepted causal criteria include (1) strength of association between stressor and effect, (2) consistency of association across populations and time, (3) existence of a dose-response relationship, and (4) evidence of a plausible physiological mechanism by which the stressor elicits the effect (Fox 1991).

The present study found strong temporal and spatial associations between diel hypoxia and significantly smaller gonad size as well as lower steroid hormone concentrations in *Fundulus grandis*. These associations were consistent across populations in Alabama and Florida and across years. The difference in DO regimes between years in Weeks Bay and between estuaries in 2005 provides an opportunity for qualitative analysis of a dose-response relationship between diel hypoxia and reproductive responses. In evaluating the potential for a dose-response, 2 criteria were applied: (1) the reduction in the reproductive variable at the hypoxic site had to be qualitatively greater under more severe or more prolonged hypoxia; and (2) the reduction in the reproductive variable had to be statistically significant under more severe or prolonged hypoxia. At the Weeks Bay hypoxic site, male androgen and female E2 concentrations were much lower relative to the control site when hypoxia was more severe (Fig. 5), suggesting that more severe hypoxia suppresses steroidogenic capacity. However, gonad size reductions were qualitatively similar regardless of the severity of hypoxia, suggesting that the presence rather than the intensity of hypoxia influences gonad size. The intensity of diel hypoxia was similar between the Weeks Bay creek site and the Pensacola Bay Indian Bayou site in 2005, but

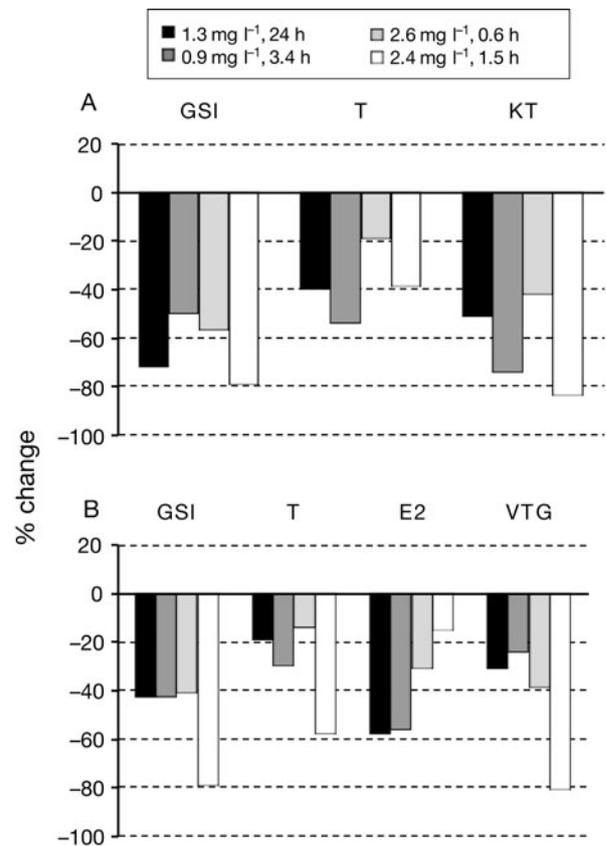


Fig. 5. *Fundulus grandis*. Magnitude of reproductive responses to hypoxia: (A) males, (B) females. Bars are the percentage reduction in the mean under the hypoxic treatment relative to the control (■) or the percentage reduction at the site with diel hypoxia relative to the site without hypoxia (□, □). (■) 1.3 mg l<sup>-1</sup>, 24 h continuous exposure in the laboratory; (■) 0.9 mg l<sup>-1</sup>, 3.4 h exposure at Weeks Bay creek in 2004; (□) 2.6 mg l<sup>-1</sup>, 0.6 h exposure at Weeks Bay creek in 2005; (□) 2.4 mg l<sup>-1</sup>, 1.5 h exposure at Pensacola Bay, Indian Bayou in 2005. GSI: gonadosomatic index (gonad weight/[body weight - gonad weight]); T: testosterone (ng ml<sup>-1</sup>); KT: 11-ketotestosterone (ng ml<sup>-1</sup>); E2: estradiol-17β (ng ml<sup>-1</sup>); VTG: vitellogenin (mg ml<sup>-1</sup>)

hypoxic periods occurred in the Indian Bayou marsh creek as early as April (A. O. Cheek unpubl. data). Male and female androgens and gonad size were markedly lower under prolonged moderate hypoxia (Fig. 5), which is unsurprising since 95% of females and 100% of males had regressed gonads. Perhaps, prolonged exposure to diel hypoxia has a cumulative impact resulting in earlier cessation of the spawning season.

The effect of environmental hypoxia on reproduction is not unique to *Fundulus grandis*, but also occurs in other estuarine fishes. Atlantic croaker (*Micropogonias undulatus*) captured in areas of persistent bottom water hypoxia (DO < 2.5 mg l<sup>-1</sup>) in Pensacola Bay and

Mobile Bay (Alabama) had severely suppressed egg and sperm production compared to 1 yr old fish captured in normoxic regions of each bay (Thomas et al. 2006, Thomas et al. 2007). Hypoxia appears to suppress gonadal function in Atlantic croaker via a central neuroendocrine mechanism. Laboratory exposure to both severe ( $1.7 \text{ mg l}^{-1}$ ) and moderate ( $3 \text{ mg l}^{-1}$ ) continuous hypoxia diminishes serotonin concentrations in the preoptic area of the brain, which is the region regulating activity of the reproductive endocrine system. Consequently, activity of the entire reproductive axis is reduced, including concentrations of hypothalamic, pituitary, and gonadal hormones. Ultimately, egg and sperm production are severely curtailed and GSI is dramatically decreased (Thomas et al. 2007).

Central neuroendocrine suppression of the reproductive axis is unlikely in *Fundulus grandis*. After laboratory exposure to continuous hypoxia or field exposure to diel hypoxia, circulating T concentrations are similar in both males and females, indicating that hypothalamic and pituitary stimulation of gonadal steroidogenesis is unimpaired. However, the testicular and ovarian products of T (11KT and E2) are significantly reduced, suggesting that hypoxia specifically inhibits enzymatic conversion of T in the gonads.

Steroidogenesis is orchestrated by oxygen-requiring enzymes in all vertebrates. In humans, hypoxia down-regulates aromatase, which is the enzyme responsible for converting T to E2 (Jiang et al. 2000) and 11 $\beta$ -hydroxysteroid dehydrogenase type 2 (11 $\beta$ -HSD2) (Hardy & Yang 2002, Heiniger et al. 2003). 11 $\beta$ -HSD in fish testis converts 11 $\beta$ -hydroxytestosterone (derived from T by the activity of 11 $\beta$ -hydroxylase, CYP11B1) to its keto form, 11KT (Kime 1998). Long-term (40 d) continuous hypoxia ( $0.8 \text{ mg l}^{-1}$ ) reduced the expression of steroidogenic enzyme-encoding genes during zebrafish sexual development (Shang et al. 2006), further supporting the hypothesis that hypoxia suppresses the activity of specific steroidogenic enzymes in the gonads.

Episodic hypoxia can occur at any time of the year in Gulf of Mexico marsh systems, but diel periods of hypoxia ( $\text{DO} < 2.0 \text{ mg l}^{-1}$ ) are more likely to occur from June to August, during the latter half of the gulf killifish spawning season. Rather than completely shutting down gamete production by centrally suppressing the reproductive endocrine system, environmental hypoxia perhaps specifically interrupts sex steroid production, thereby delaying gamete growth.

Demonstrating a causal relationship between diel hypoxia and impaired reproductive capacity in the natural environment is complicated by variation in other physical, biological, and methodological factors between sites. Temperature and salinity differences are unlikely to alter reproductive capacity either indirectly by influencing metabolic rate or directly by

changing reproductive function. The maximum temperature difference of  $1.1^\circ\text{C}$  would have minimal effect on metabolic rate, since the metabolic rate of most ectotherms increases by a factor of 2 to 3 in response to a  $10^\circ\text{C}$  increase in temperature (Randall et al. 2002). Metabolic rate is unchanged by salinity in the congener *Fundulus heteroclitus*, even upon transfer from a salinity of 10 to 30 (Kidder et al. 2006). In addition, spawning cycle length in *F. grandis* is not altered by temperature. Hsiao & Meier (1992) calculated a  $Q_{10}$  value of  $1.13 \pm 0.14$ , a value not significantly different from 1.0. The hatching success of *Fundulus grandis* across a wide range of salinities (0 to 35) is 80 to 90% (Perschbacher et al. 1990), strongly suggesting that small variations in salinity (0.6 to 2.9 in the present study) are unlikely to alter reproduction.

Differences in body size of sampled fish did not drive between-site variation in reproductive capacity. Regardless of whether body sizes were similar or different between sites, reproductive capacity was lower at hypoxic sites. Size-related increases in male 11KT, female T and female GSI were significantly dampened at hypoxic sites. Superficially, this suggests that large fish are more severely impacted by hypoxia. In fact, all sizes of fish are equally likely to become regressed at sites with severe or prolonged diel hypoxia, but small fish are more likely to be regressed at sites with moderate diel hypoxia, suggesting that small fish are more sensitive.

Differences in the timing of sampling between sites did not explain differences in reproductive capacity. Reproductive parameters did not vary with time of day, probably because mean time of sample collection was either similar between sites or occurred late in the afternoon, which is a period when GSI and hormones change little in either sex (Emata et al. 1991). Over the 2 to 3 d collection period, GSI and steroid hormones varied with lunar phase. However, even when samples from the hypoxic site were collected earlier in the lunar phase, the site effect explained more of the variation than did lunar day. When samples were collected later at the hypoxic site, lunar effects might be expected to override the inhibitory effect of low DO, but reproductive capacity was still dramatically reduced at the hypoxic sites. Thus, site differences in DO, rather than timing differences in sample collection are more likely to explain differences in reproductive capacity.

Environmental hypoxia clearly reduces the reproductive capacity of *Fundulus grandis*. Changes in *F. grandis* populations have the potential to alter trophic structure in marshes and bays. *Fundulus* spp. are important vectors of energy flow within the marsh community. Kneib's (1997) trophic-relay hypothesis suggests that marsh resident species such as *F. grandis*

move carbon from the upper marsh surface to the lower marsh, both directly in the form of increased biomass and indirectly as prey for juveniles of larger fish species—e.g. drums (sciaenids) and flounders which depend upon the marsh edge as a nursery habitat and are of commercial and recreational importance. To fully understand the impact of hypoxia on marsh resident populations and support ecological modeling, measures of fitness (e.g. fecundity or number of fertilized eggs) under varying intensities and durations of hypoxia will be necessary.

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