



Abstract—The results of this study provide additional evidence of ovarian masculinization in the Atlantic croaker (*Micropogonias undulatus*) in and around the hypoxic zone in the Gulf of Mexico and the first evidence of ovarian masculinization in the spot (*Leiostomus xanthurus*) and bigeye searobin (*Prionotus longispinosus*). In some specimens of Atlantic croaker and spot, we observed this phenomenon to a degree that has not been reported in either species. We also found evidence of smaller gonads in spot from hypoxic sites compared with the gonads in spot from sites with normal oxygen concentrations. These findings indicate that ovarian masculinization and reproductive impairment associated with hypoxia exposure affect a wider range of species than was previously known, and they highlight the importance of continued research on this topic.

Ovarian masculinization and reproductive impairment in 3 species of groundfish in and around the hypoxic zone in the Gulf of Mexico

Michael V. Cyra (contact author)^{1,2}

Alexander J. Ledet^{2,3}

Henry L. Bart Jr.²

Email address for contact author: michael.cyra@jhuapl.edu

¹ Applied Physics Laboratory
Johns Hopkins University
11100 Johns Hopkins Road
Laurel, Maryland 20723-6099

³ Albert Einstein College of Medicine
1300 Morris Park Avenue
Bronx, New York 10461

² Ecology and Evolutionary Biology Department
Tulane University
201 Lindy Claiborne Boggs Center
6823 St. Charles Avenue
New Orleans, Louisiana 70118-5698

Manuscript submitted 20 May 2022.
Manuscript accepted 25 January 2023.
Fish. Bull. 121:36–49 (2023).
Online publication date: 16 February 2023.
doi: [10.7755/FB.121.1-2.4](https://doi.org/10.7755/FB.121.1-2.4)

The views and opinions expressed or implied in this article are those of the author (or authors) and do not necessarily reflect the position of the National Marine Fisheries Service, NOAA.

In the last century, hypoxia has emerged as a significant threat to coastal ecosystems. *Hypoxia*, or hypoxic conditions, refers to low levels of dissolved oxygen in bodies of water. Coastal hypoxia, which was first observed in the 1930s (Diaz and Rosenberg, 2008), has increased 3-fold during the last 30 years (Diaz and Rosenberg, 2008; Vaquer-Sunyer and Duarte, 2008). The range of dissolved oxygen values used to define an area as hypoxic is variable, and the term *hypoxia* conventionally indicates dissolved oxygen values below 2.0 mg/L (Rabalais et al., 2002; Vaquer-Sunyer and Duarte, 2008). Although hypoxic events are known to occur naturally throughout the world (Helly and Levin, 2004), the global increase in coastal hypoxia can mostly be attributed to the increased use of synthetic nitrogen fertilizers since their creation in the 1940s (Diaz and Rosenberg, 2008; Galloway et al., 2008). Increased nitrogen runoff from fertilizers can result in large algal blooms in coastal waters. When algal cells die, their decomposition consumes dissolved oxygen in the water column.

This process can result in hypoxia if the water column is stratified, given that subsurface waters are unable to mix with oxygen-rich surface waters because of differences in water density.

Hypoxia has wide-ranging ecological and physiological effects on marine life. Exposure to hypoxic conditions alters the behavior (Eriksson and Baden, 1997) and interspecific interactions of marine organisms (Sandberg, 1997). Hypoxic conditions have also been associated with changes in community composition (Dauer, 1993; Pihl, 1994) and biomass (Rosenberg and Loo, 1988). Community recovery following episodes of hypoxia is highly variable and dependent upon a variety of environmental and biotic factors (Wu, 2002), including community complexity and the severity and duration of hypoxic conditions (Boesch and Rosenberg, 1981). At the organismal level, endocrine disruption (Wu et al., 2003) and reproductive impairment (Zhou, 2001) are 2 of the most well-documented physiological effects of hypoxia exposure in fish.

Fish exposed to hypoxic conditions tend to have lower values for the gonadosomatic index (GSI), an indicator of reproductive condition, than fish exposed to normal oxygen concentrations, as has been observed in the Gulf killifish (*Fundulus grandis*) (Cheek et al., 2009), common carp (*Cyprinus carpio*) (Zhou, 2001), and Atlantic croaker (*Microponias undulatus*) (Wu et al., 2003; Thomas et al., 2007; Thomas and Rahman, 2012). Both episodic (diel) and chronic hypoxia have been shown to reduce the reproductive capacity of estuarine fish species (Landry et al., 2007; Cheek et al., 2009). Chronic exposure to hypoxic conditions has been further linked to masculinization in female fish (Landry et al., 2007; Cheek et al., 2009; Thomas and Rahman, 2012) and to reduced sperm production and viability in male Atlantic croaker (Thomas et al., 2015). Masculinization of female Atlantic croaker resulting from chronic exposure to hypoxia has been documented in both field and laboratory studies (Thomas et al., 2007; Thomas and Rahman, 2012).

In this study, we assessed whether the well-established reproductive effects of seasonal hypoxia on Atlantic croaker in the Gulf of Mexico (GOM) (Thomas and Rahman, 2012) extend to other cohabiting species of fish, specifically the spot (*Leiostomus xanthurus*) and bigeye searobin (*Prionotus longispinosus*). Both the Atlantic croaker and spot are members of the family Sciaenidae (order Perciformes). The bigeye searobin belongs to the family Triglidae (order Scorpaeniformes). Finding evidence of reproductive abnormalities at the family and order levels would demonstrate that the effects of seasonal hypoxia in the GOM are more widespread taxonomically, with broader implications for fisheries management, than previously thought.

Materials and methods

Sample collection and study area

Fish were collected aboard the NOAA Ship *Oregon II* during legs I and II (June–August) of the Southeast Area Monitoring and Assessment Program (SEAMAP) summer groundfish surveys in 2016, 2017, and 2019 (Fig. 1). Sampling sites were scattered throughout the area in which hypoxia seasonally develops in the GOM, ranging from the coast of Texas to waters off Louisiana. Samples were collected as described in the SEAMAP sampling protocol (GSMFC¹) at survey sites with depths ≤ 30 m because hypoxia in the GOM occurs less frequently at depths below 30 m. Whole specimens caught during trawl surveys were fixed in a 10% formalin solution and transferred to ethanol for storage. Dissolved oxygen values were recorded immediately prior to trawling, according to SEAMAP protocol for collecting data on water conditions, by using a shipboard array equipped with dissolved oxygen, conductivity, temperature, and depth sensors and water bottles for measuring other water properties (Table 1). A site was

defined as hypoxic if dissolved oxygen was 2.0 mg/L or lower, a level that is consistent with the recent literature on hypoxia (Vaquer-Sunyer and Duarte, 2008).

Female specimens of Atlantic croaker, spot, and bigeye searobin were reproductively immature at the time of sampling, as they did not contain any oocytes past the previtellogenic stage (Anderson et al., 2018). Male specimens of Atlantic croaker and spot were also reproductively immature, as no spermatids or spermatozoa were present. Some male bigeye searobin were reproductively mature. The natural history of Atlantic croaker and spot and histological examination of gonads of bigeye searobin strongly indicate that specimens would have reproductively matured within the year, making any evidence of reproductive abnormalities indicative of reproductive impairment. It is important to note that, although the majority of specimens were reproductively immature, the sampled fish were not juveniles and the term *immature* is meant to describe the state of the gametes within the gonads.

We obtained reference samples of Atlantic croaker and spot from the Royal D. Suttkus Fish Collection at the Tulane University Biodiversity Research Institute (TUBRI). Reference samples consisted of whole, formalin-fixed specimens from which gonads were dissected. We selected reference samples collected during the summer (June–September) at either the Chandeleur Islands or the Rigolets, both located in Louisiana. We selected samples collected between 1955 and 1969 because we wanted specimens from a time before the 1980s, when eutrophication dramatically increased in the western GOM, as evidenced by increased carbon deposition (Rabalais et al., 2007). The Chandeleur Islands and the Rigolets are not known to be hypoxic during the summer; therefore, both the location and time of the collection of our reference samples makes it unlikely that reference fish were exposed to hypoxic conditions prior to sampling.

Sample processing

Subsamples were selected from male and female Atlantic croaker, spot, and bigeye searobin collected from both hypoxic sites and sites with normal oxygen levels. These selected specimens were wet weighed (to the nearest 0.001 g) and measured for total length (to the nearest millimeter), and then they had their gonads removed and wet weighed. The gonads were mounted, sectioned, and stained with hematoxylin and eosin. Only a subsample of these gonads were histologically processed and examined.

Gonadosomatic index

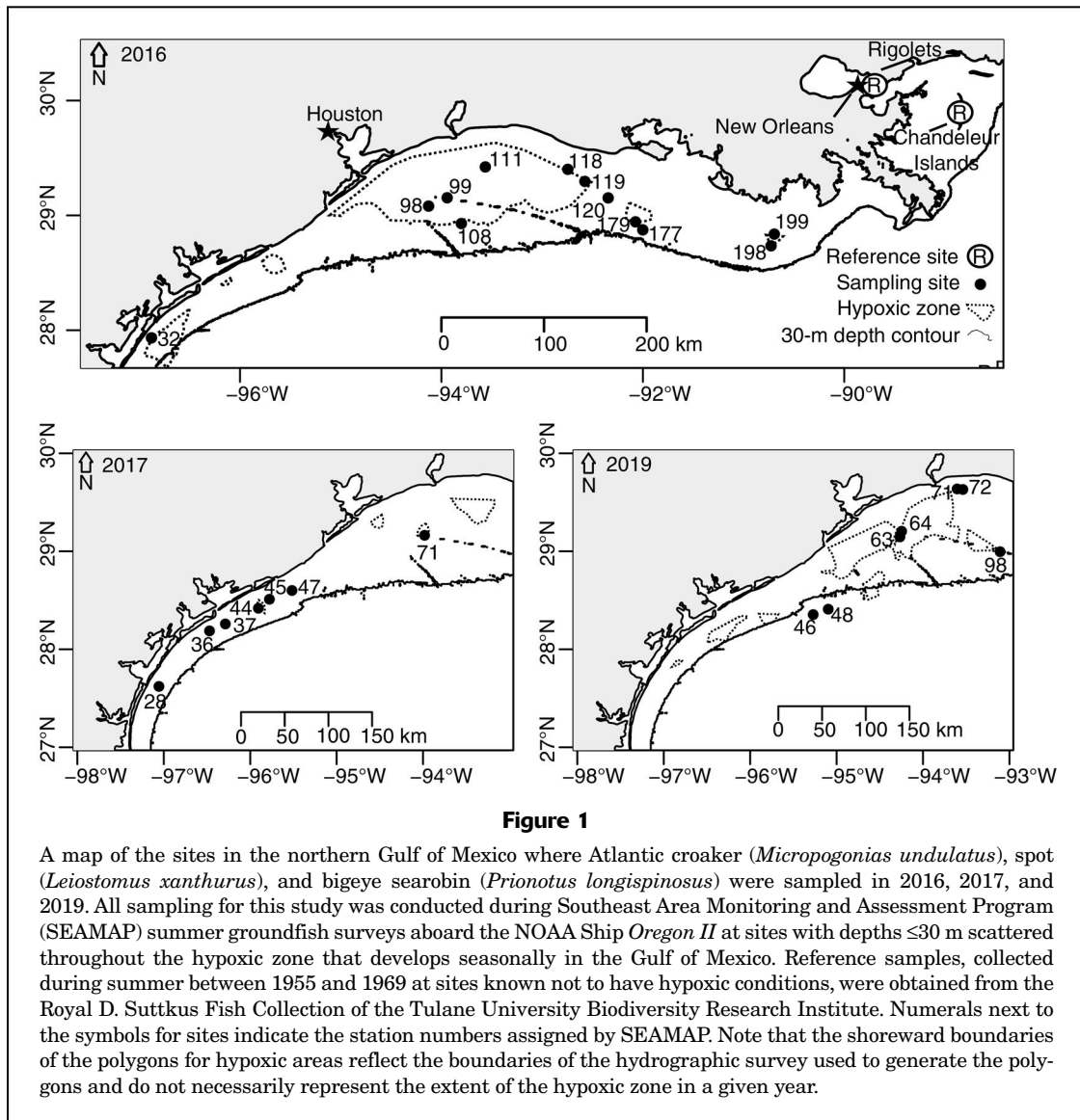
The GSI was calculated by using this formula:

$$\frac{Weight_{\text{gonads}}}{(Weight_{\text{fish}} - Weight_{\text{gonads}})} \times 100,$$

where $Weight_{\text{gonads}}$ = the wet weight of both gonads post fixation; and

$Weight_{\text{fish}}$ = the wet weight of the whole fish post fixation.

¹ GSMFC (Gulf States Marine Fisheries Commission). 2019. SEAMAP operations manual for trawl and plankton surveys, 63 p. Gulf States Mar. Fish. Comm., Ocean Springs, MS. [Available from [website](#).]



In order to compare the GSI of fish of varying sizes, a residual index of the GSI values was computed. The residual index has been shown to be an effective way to reduce the influence of body size in analyses of organismal condition (Jakob et al., 1996) and has been proven to be effective in the analysis of fish morphometric data (Reist, 1985; Jakob et al., 1996). The residual index is the difference between the observed GSI value and the predicted GSI value, which is determined by using a linear trendline generated with pooled data (for specimens from both hypoxic and normal oxygen concentrations). Positive values for the residual index indicate that the observed GSI is greater than the predicted GSI, whereas negative values indicate that the observed GSI is less than the predicted GSI.

Overall body condition

Overall body condition was calculated as wet weight divided by total length. These condition index values for

specimens were compared between hypoxic and normal sites by using the residual index (polynomial trendline) of the length–weight relationship based on data pooled for males and females, under the assumption that there is no sexual dimorphism in any of the species included in this study. In order to compare both sexes simultaneously, the weight used for the overall condition analysis was the wet weight of each fish minus the weight of its gonads.

Statistical treatment

Mean residual GSI and condition index values were compared between fish from hypoxic sites and those from sites with normal oxygen levels by using sites as replicates. The residual indices of both overall body condition and GSI for both male (hypoxic: $n=31$; normal: $n=28$) and female (hypoxic: $n=36$; normal: $n=31$) spot and female Atlantic croaker (hypoxic: $n=37$; normal: $n=44$) were normally distributed (Shapiro–Wilk test: $P>0.05$) (Shapiro

Table 1

Recorded dissolved oxygen (DO) values and depths at the sites sampled for this study in the northern Gulf of Mexico during 2016, 2017, and 2019. The site numbers correspond to the numbers assigned to “Pascagoula” stations by the Southeast Area Monitoring and Assessment Program.

Site no.	Year	DO (mg/L)	Depth (m)
15	2016	5.7	22.4
32	2016	1.1	15.4
98	2016	1.4	19.6
99	2016	1.1	18.7
108	2016	2.3	22.8
111	2016	0.0	15.0
118	2016	0.9	14.4
119	2016	1.0	15.8
120	2016	5.1	13.9
177	2016	4.1	26.4
179	2016	2.2	23.8
198	2016	2.0	18.1
199	2016	1.8	18.8
28	2017	5.8	23.6
29	2017	6.3	19.4
36	2017	5.4	18.0
37	2017	2.5	21.6
44	2017	1.4	20.1
45	2017	3.4	18.8
47	2017	3.2	19.3
48	2017	5.9	15.6
71	2017	1.4	17.8
46	2019	3.0	33.0
63	2019	0.5	14.0
64	2019	0.5	13.0
72	2019	1.5	10.0
98	2019	1.5	26.0

and Wilk, 1965) and had statistically indistinguishable variances (Bartlett’s test: $P > 0.05$) (Bartlett, 1937). Therefore, we used a 2-way analysis of variance (ANOVA), and a significance level (α) of 0.05, to compare the residual indices of these samples. The residual GSI for male Atlantic croaker was not normally distributed (Shapiro–Wilk test: $P < 0.05$); therefore, we used a Kruskal–Wallis test, with a α of 0.05, for these data (hypoxic: $n=48$; normal: $n=26$) (Kruskal and Wallis, 1952).

Results

Ovarian masculinization

Evidence of masculinization was found in 6 of the 17 ovaries from Atlantic croaker that were examined in this study, with masculinized ovaries observed only for specimens collected at hypoxic sites. In the masculinized ovaries from Atlantic croaker, testicular tissue was found primarily in connective tissue along the ovary periphery (Fig. 2). The testicular tissue consisted of clusters

of spermatids within connective tissue, similar to the tissue conditions used in determinations of masculinization made in previous studies (Thomas and Rahman, 2012) (Fig. 2). The spermatogenic tissue observed within ovarian tissue resembled spermatogenic cysts of lobular type testes. One specimen, the gonads of which externally resembled testes, had gonadal tissue that is best described as transitional (i.e., an ovary that was transitioning to a testis). The gonads of this specimen organizationally resembled testes; however, degenerated oocytes were present and surrounded duct-like structures. Spermatogenic tissue was not present. The ovaries of reference specimens contained no evidence of spermatogenic tissue or ovarian anomalies.

The masculinized ovaries from spot (Fig. 3) had a masculinization profile similar to that of the masculinized ovaries from Atlantic croaker. Masculinization in peripheral connective tissue was found in 3 of the 7 ovaries from spot that were examined in this study, with one of the ovaries coming from a specimen collected at a site with normal oxygen concentrations.

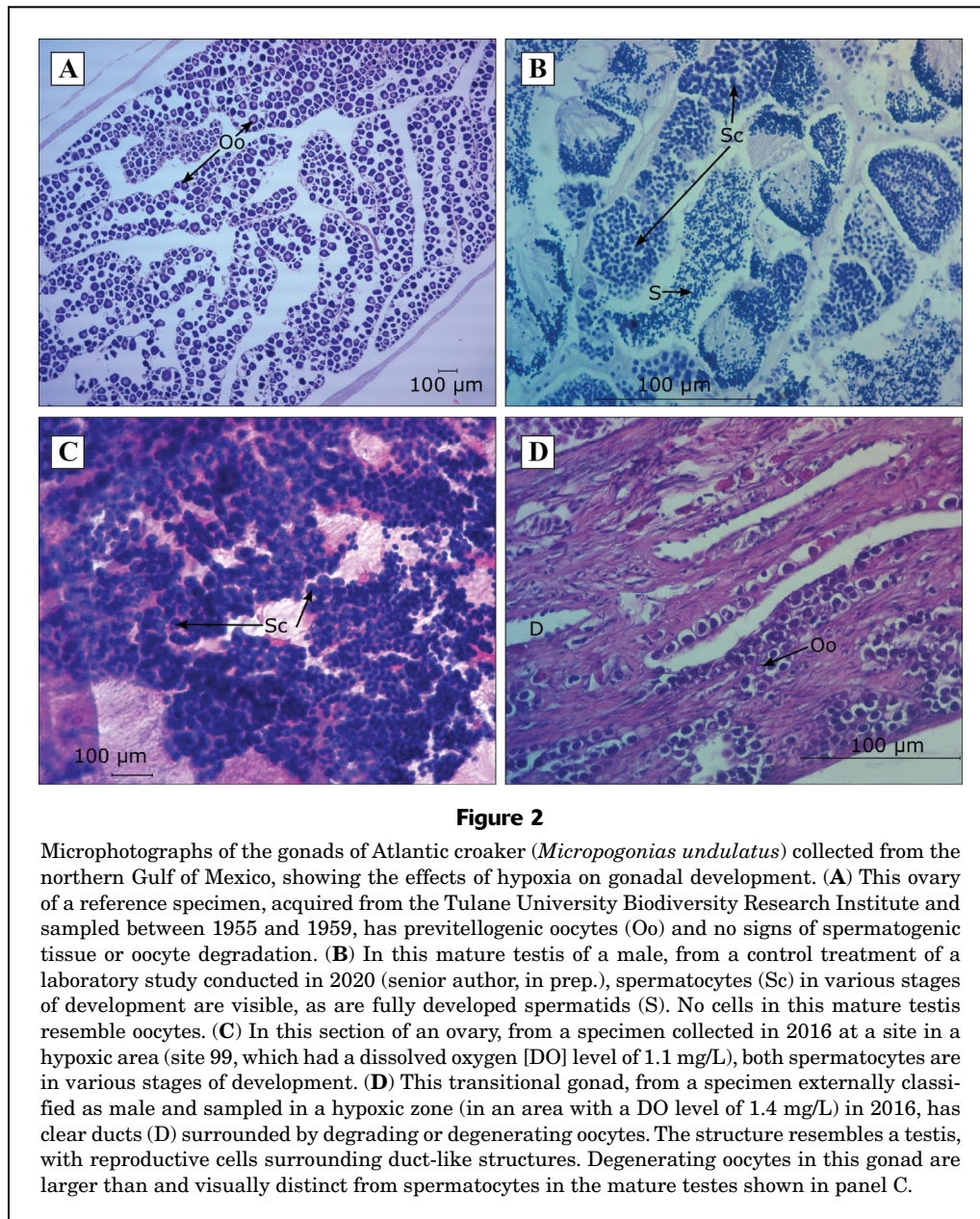
Two of the spot we examined contained ovaries that are best described as in a transition from female to male tissue (Fig. 3). These transitional gonads had structures more like a typical testis, with ducts surrounded by connective tissue that was interspersed with spermatogenic tissue. One of these transitional gonads contained numerous atretic follicles and a large lumen filled with what looked like ovum remnants and large melano-macrophage centers (Agius and Roberts, 2003). The other transitional gonad contained atretic follicles and a testicular-like structure but lacked ovum remnants and melano-macrophage centers. The ovaries of the reference specimens used in this study appeared more organized compared with the gonads of specimens collected in 2016, and no evidence of spermatogenic tissue was found within them.

Masculinization in bigeye searobin manifested as spermatogenic cells in various stages of development interspersed among oocytes and peripheral tissue (Fig. 4). Evidence of this phenomenon was found in 1 of the 7 ovaries from bigeye searobin that were examined in this study.

Gonadosomatic index and body condition

The mean residual index of overall body condition based on pooled data for female and male Atlantic croaker was significantly higher for fish from hypoxic sites than for fish from sites with normal oxygen concentrations (Kruskal–Wallis test: $P < 0.05$) (Figs. 5 and 6.). The residual GSI of both male and female Atlantic croaker from hypoxic sites did not significantly differ from that of males and females from sites with normal levels of oxygen (males: $P=0.65$, $df=7$, $f=0.21$; females: $P=0.77$, $df=11$, $f=0.08$) (Figs. 5 and 6).

The mean residual index of overall body condition for female and male spot combined did not significantly differ between specimens from hypoxic sites and specimens from normal sites ($P=0.12$, $df=14$, $f=0.60$) (Figs. 7 and 8). The mean residual GSI of female spot from hypoxic sites



was significantly lower (ANOVA: $P < 0.05$) than that of females from sites with normal oxygen concentrations (Figs. 7 and 8), and male spot from hypoxic sites also had significantly lower GSI values than males from normal sites (ANOVA: $P < 0.05$).

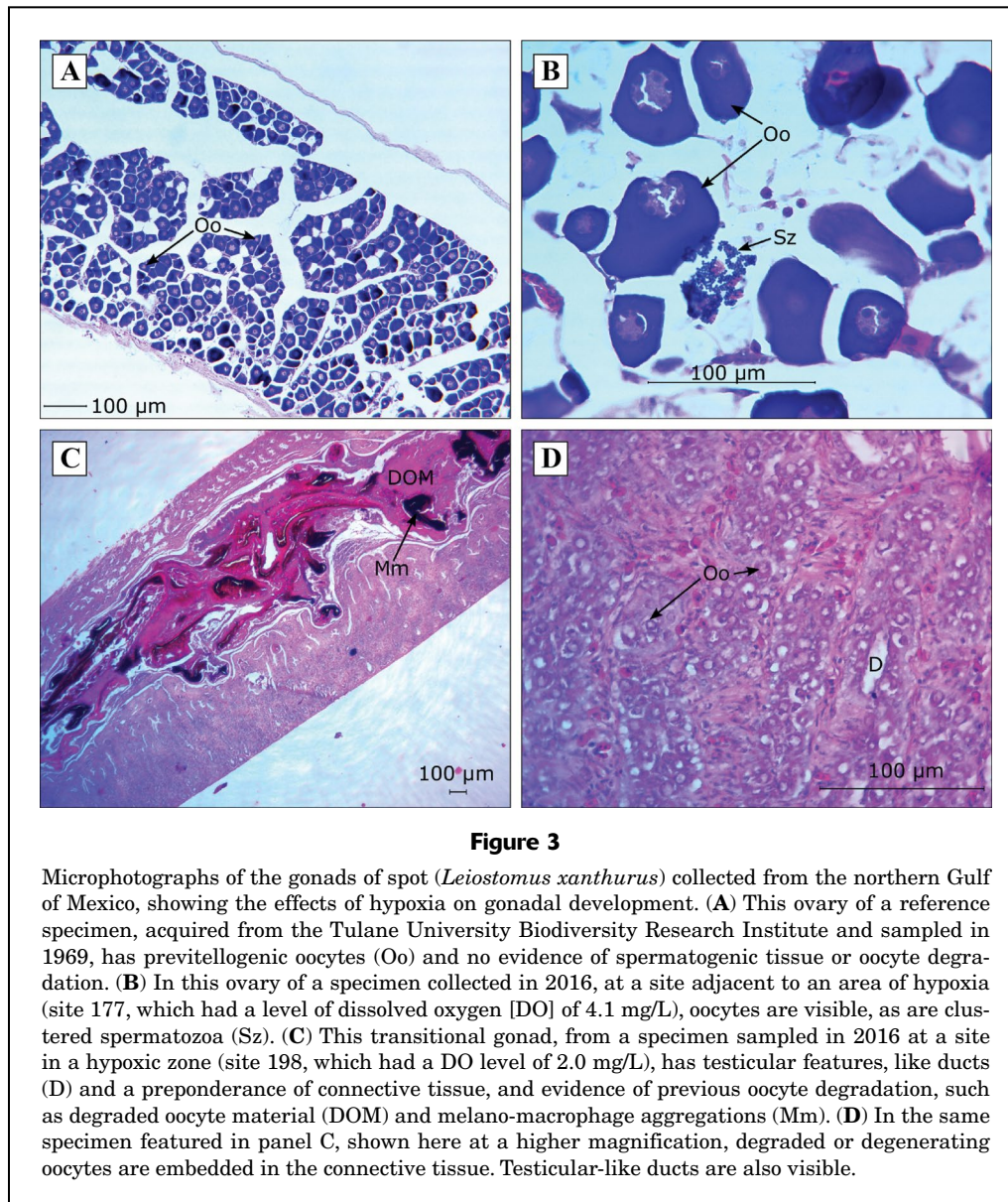
Discussion

Scope and possible causes of ovarian masculinization

Prior to this study, the only species from the GOM for which evidence of ovarian masculinization had been reported was the Atlantic croaker (Thomas and Rahman, 2012). This study is the first to report this phenomenon in

2 additional GOM species, the spot and bigeye searobin, with 2 of 7 subsampled ovaries from spot (Fig. 3) and 1 of 7 subsampled ovaries from bigeye searobin (Fig. 4) containing male reproductive cells. Additionally, such evidence was found in 6 of 17 ovaries of Atlantic croaker examined in this study (Fig. 2).

The cause of the ovarian masculinization found in this study cannot be determined. However, hypoxia is a likely possibility. It is well known that estrogen, the synthesis of which is regulated by the enzyme aromatase, is a primary factor in the sex determination of fish. Guiguen et al. (2010) discussed results of a number of experiments that indicate that suppression of estrogen synthesis (aromatase inhibition) in fish can result in the formation of testicular tissue. Aromatase inhibitors have been shown to induce

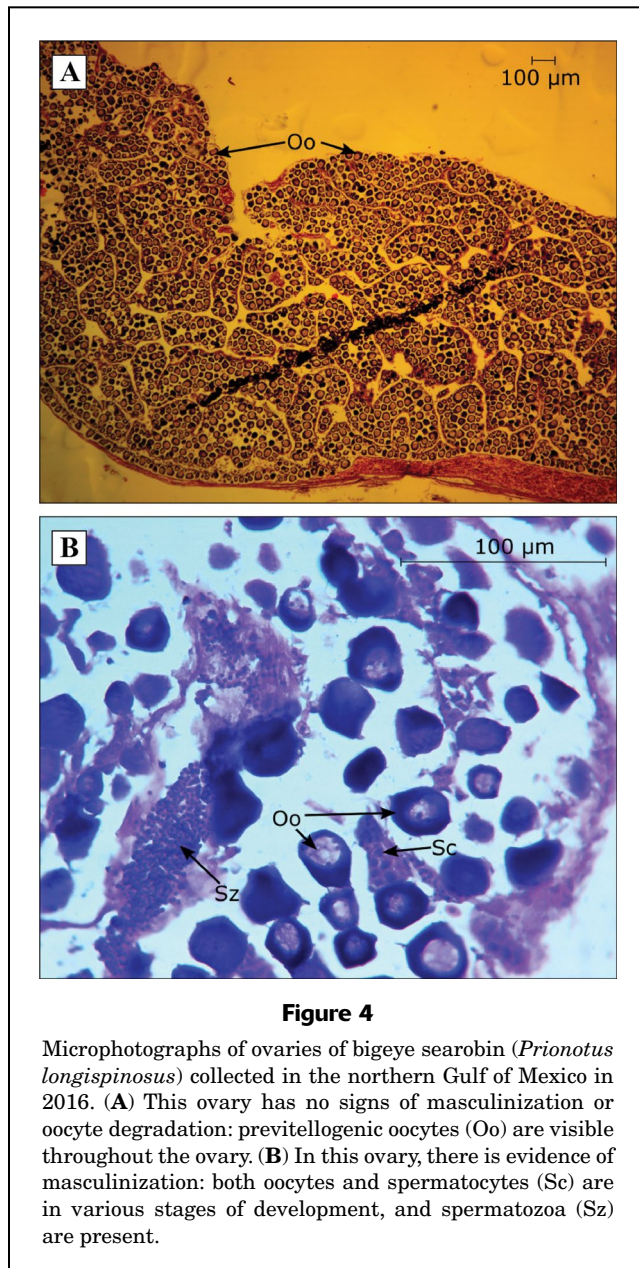


masculinization in several species of fish, including the naturally hermaphroditic honeycomb grouper (*Epinephelus merra*) (Bhandari et al., 2004), red grouper (*E. morio*) (Guiguen et al., 2010), and European seabass (*Dicentrarchus labrax*) (Navarro-Martín et al., 2009) and in the naturally gonochoristic common carp (Ogawa et al., 2008). Long-term aromatase suppression has even been reported to convert functional ovaries into functional testes in tilapia (Sun et al., 2014). Hypoxic conditions have been shown to function as an aromatase inhibitor in Atlantic croaker, decreasing mRNA expression and aromatase activity in fish exposed to hypoxia both in the wild and in the lab (Thomas and Rahman, 2012).

It is likely that the ovarian masculinization observed in fish from hypoxic sites in our study could be a consequence of hypoxia-induced inhibition of aromatase and the

concomitant suppression of estrogen synthesis. The well-documented connection between aromatase suppression and the development of intersex gonads in fish, coupled with the broad geographic extent of hypoxia encountered during the sampling for this study, strongly indicates that the ovarian masculinization observed in this study was hypoxia related.

The presence of intersex specimens from sites with normal oxygen levels is a novel discovery; however, the relatively low sample size prevents us from offering a definitive interpretation. Specifically, the normal sites that yielded intersex specimens were immediately adjacent to areas of hypoxia (Fig. 1) and differed by only 0.2 mg/L in the level of dissolved oxygen, with the concentration at these normal sites falling right on the line between our definition of hypoxic and normal conditions. Although this closeness in



location and dissolved oxygen could indicate either that the intersex specimens had passed through the hypoxic area and were subsequently masculinized or that the dissolved oxygen concentration at the normal site was low enough to induce the development of intersex gonads, there are not enough data to identify a causal relationship. Sampling numbers were not sufficient to explain the source of ovarian masculinization in fish from normal sites; therefore, further sampling efforts and laboratory exposure experiments are needed to fully explain this phenomenon. Similarly, it is unclear what effect the masculinization observed in this study would have had on fish if they had reached reproductive maturity. Although the molecular pathway involved in ovarian masculinization in fish is well understood, the parameters that trigger this

pathway and how they affect fish at different stages of reproductive development is not well understood and should be further studied.

Our findings highlight the importance of continued work in this area, particularly in the development of intersex gonads in additional fish species and the exposure parameters (e.g., duration of exposure, dissolved oxygen level, and reproductive stage at time of exposure). A better understanding of the exposure parameters that trigger the development of intersexuality in fish would help not only elucidate the findings of this study but also better inform management decisions regarding resources in the GOM and in other areas affected by hypoxia.

Our observation of ovarian masculinization in Atlantic croaker and 2 other groundfish species in the GOM extends the taxonomic breadth of this phenomenon, which was first reported by Thomas and Rahman (Thomas et al., 2007). If our finding of testicular tissue in the ovaries of wild-caught specimens is not related to hypoxia, the phenomenon either is due to some other environmental factor or it represents naturally occurring background intersexuality. Bahamonde et al. (2013) reviewed this phenomenon in great detail, with his work including studies of gonochoristic species in which intersex individuals were found to occur naturally (Bahamonde et al., 2013). In one such study involving the freshwater species smallmouth bass (*Micropterus dolomieu*), significantly more occurrences of intersex fish during the prespawning period were observed than during the reproductive period (Blazer et al., 2007). The spawning period of Atlantic croaker in the GOM begins as early as October and ends as late as June (Parker, 1971), a protracted spawning period. Results from research on bigeye searobin in the GOM indicate that this species also has a protracted spawning period, from January through June (Hoff, 1992). The spot has been reported to be a winter spawner in the GOM, with the exact timing of spawning contingent on longitude (Parker, 1971). Even though our ovarian samples were taken from fish collected primarily in the prespawning period for their species, it is unlikely that the evidence of masculinization we observed was the result of the inherent intersex characteristics described by Bahamonde et al. (2013).

The vast majority of examples of naturally occurring intersex individuals in gonochoristic fishes outlined by Bahamonde et al. (2013) are male fish with testicular tissue that contained oocytes, given that the study was focused primarily on the process of male feminization. The feminization of testicular tissue indicates aromatization of androgens into estrogens—the opposite hormonal mechanism from that presumed to occur in ovarian masculinization, making it incompatible with our interpretation. Additionally, our observation of heavily masculinized specimens, including a specimen of spot best characterized as in the process of undergoing a sex change, with degenerating oocytes, indicates that specimens were transitioning from female to male, similar to the transition of ovaries to functional testes in tilapia as a result of aromatase suppression (Sun et al., 2014).

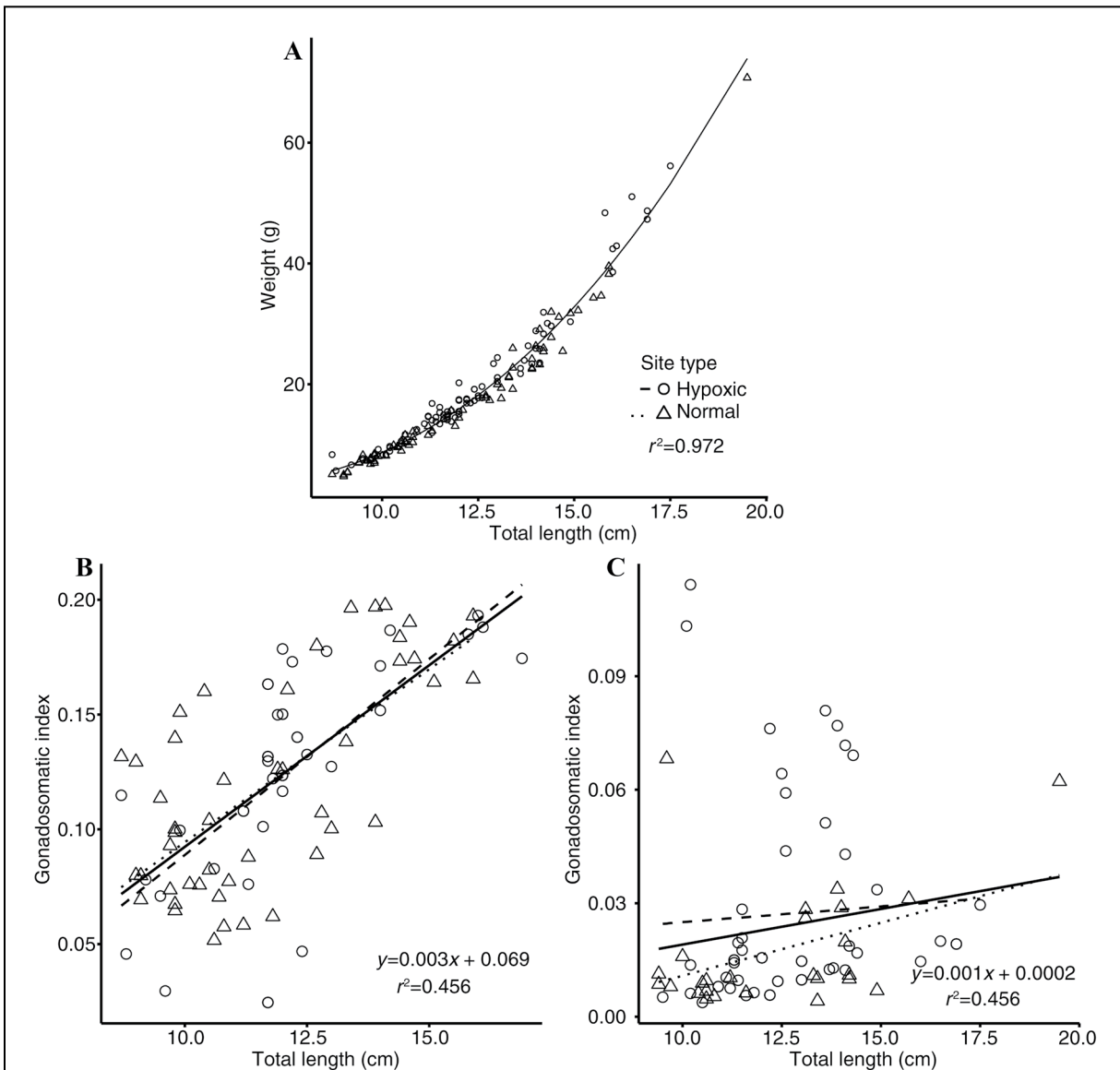


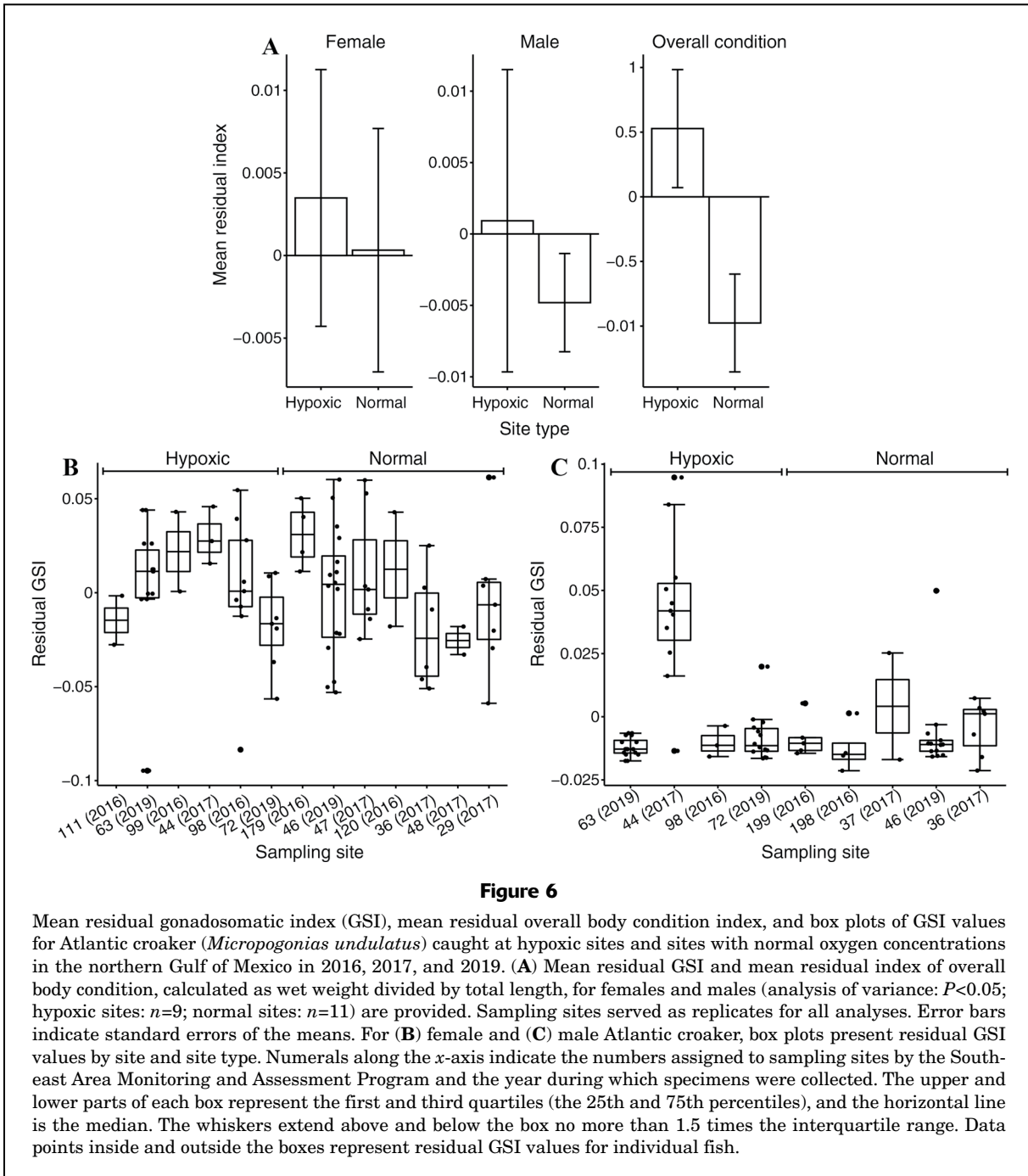
Figure 5

(A) Weight–length relationship for Atlantic croaker (*Micropogonias undulatus*), males and females combined, and the relationship between length and the gonadosomatic index for (B) female and (C) male Atlantic croaker collected in the northern Gulf of Mexico during 2016, 2017, and 2019. All specimens were caught off the coasts of Texas and Louisiana at sites in hypoxic areas and sites with normal levels of oxygen. The solid trend lines were calculated by using data pooled for fish from both hypoxic and normal sites, and the dashed and dotted trend lines represent data only for fish from hypoxic sites or only for fish from normal sites, respectively. Also provided are the coefficient of determination (r^2) in each graph and the equation for the solid trend line in each of the bottom graphs.

Therefore, although the occurrence of naturally intersex individuals in otherwise gonochoristic fishes should be seriously considered, the low number of examples of naturally masculinized individuals in the literature, compared to the high degree of masculinization in some of our specimens, as well as the lack of any observable intersexuality in our reference samples indicate that naturally occurring intersexuality is not a likely explanation for our observations.

Manifestation of masculinization

In previous reports of masculinization in Atlantic croaker, the process is described as “spermatogenic cyst-like structures” (Thomas and Rahman, 2012). This description fits the majority of masculinized ovaries from Atlantic croaker and the masculinized specimens of spot from a site in this study that had normal oxygen concentrations but was adjacent to the area with hypoxic conditions. In addition to



observing this more typical manifestation of masculinization in sampled fish, we also examined 3 specimens that were undergoing this phenomenon to a degree that has yet to be described in wild specimens from the GOM.

In 2 specimens of spot and 1 specimen of Atlantic croaker, we observed masculinization to the extent that their gonads were in transition from female to male tissue, as these specimens had degenerating or regressing female reproductive cells and a testes-like overall structure. The gonads of these specimens contained well-defined ducts that were surrounded by highly degraded oocytes.

The gonad of one of the spot specimens also contained a large duct or lumen space filled with material consistent with follicular atresia and melano-macrophages. The other specimen of spot had similarly degraded oocytes and well-defined male tissue; however, the gonad of this individual lacked a large lumen and melano-macrophage aggregations.

The evidence of oocyte degradation combined with the well-defined testicular structure and spermatogenic tissue in these specimens indicate masculinization beyond what has been observed previously in fish from the GOM.

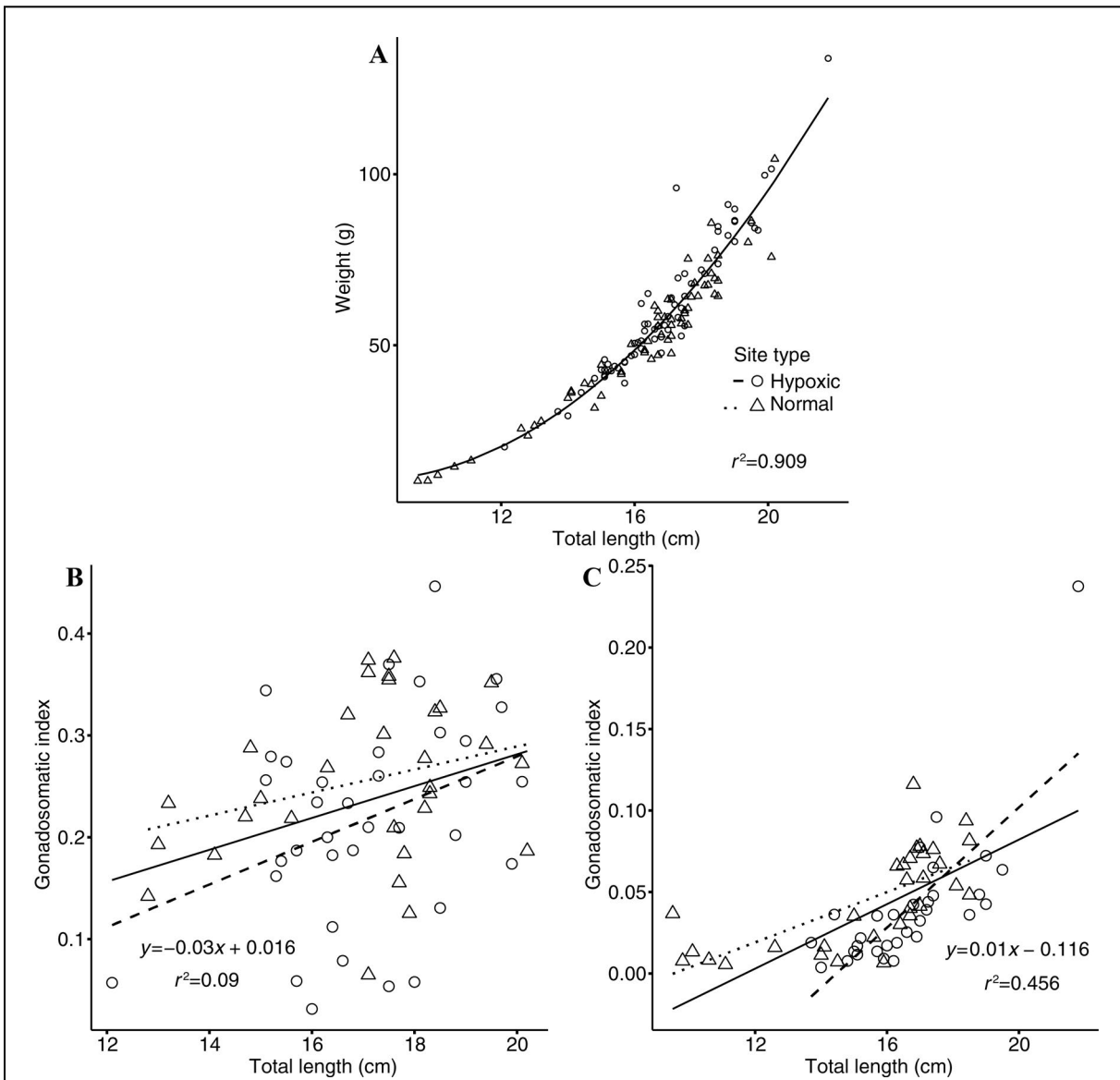


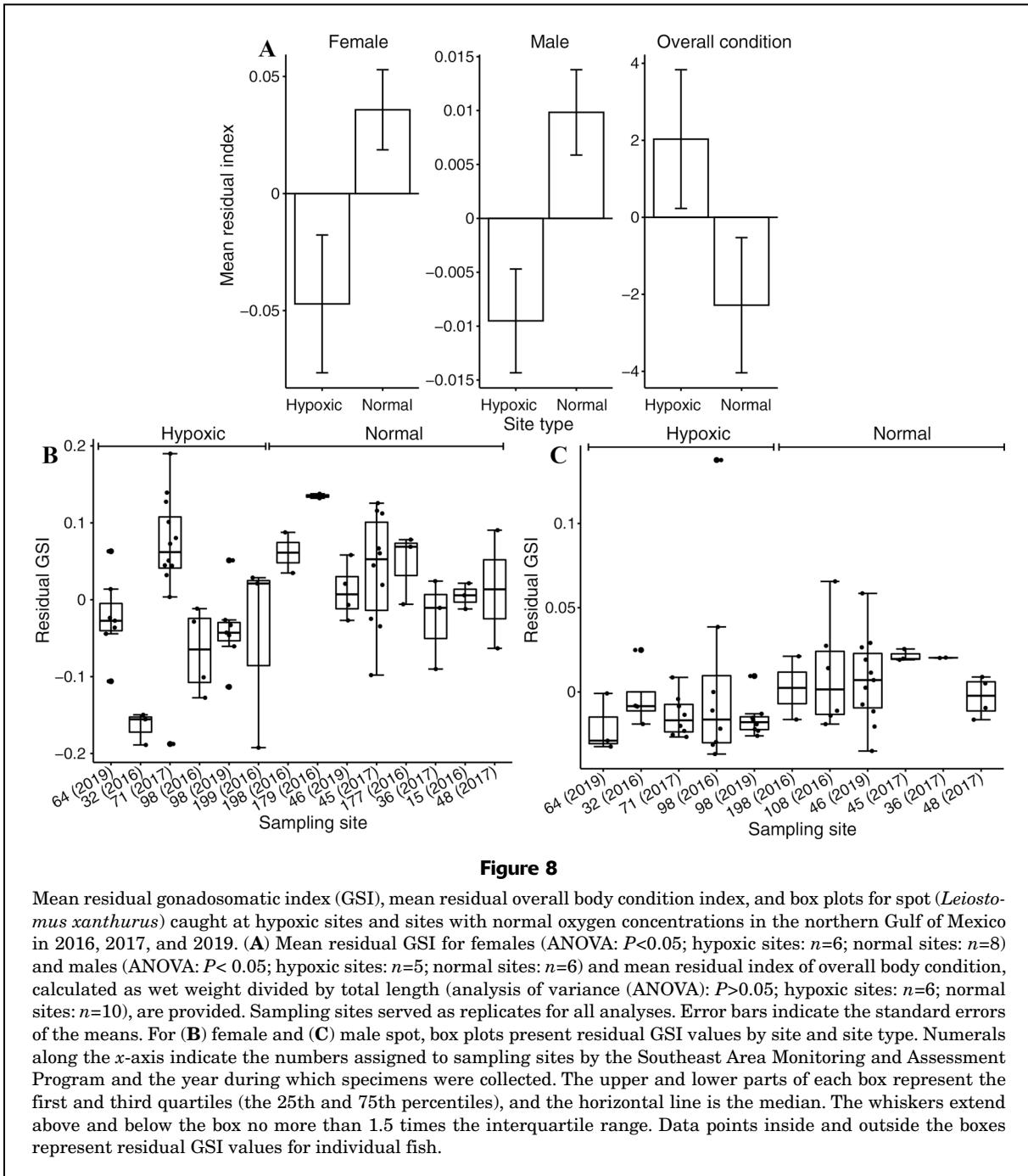
Figure 7

(A) Weight–length relationship for spot (*Leiostomus xanthurus*), males and females combined, and the relationship between length and the gonadosomatic index for (B) female and (C) male spot collected in the northern Gulf of Mexico during 2016, 2017, and 2019. All specimens were caught off the coasts of Texas and Louisiana at sites in hypoxic areas and sites with normal levels of oxygen. The solid trend lines were calculated by using data pooled for fish from both hypoxic and normal sites, and the dashed and dotted trend lines represent data only for fish from hypoxic sites or only for fish from normal sites, respectively. Also provided are the coefficient of determination (r^2) in each graph and the equation for the solid trend line in each of the bottom graphs.

The characterization of these individuals as females undergoing masculinization, as opposed to males undergoing feminization, is supported by work on tilapia by Sun et al. (2014) in which aromatase suppression led to a complete sex change in females, a change characterized by a degradation of oocytes and replacement of the oocytes by spermatogenic cells (Sun et al., 2014). Histological observations in naturally hermaphroditic fish transitioning from female to male provide similar evidence of oocyte

breakdown prior to the transition from female to male (Wang et al., 2017; Maxfield and Cole, 2019). Centers of melano-macrophages have been considered pathological signs of environmental degradation and hypoxia in fish (Agius and Roberts, 2003), including individuals of species such as the Atlantic croaker in the GOM (Fournie et al., 2001), albeit the aggregations were found in the spleen.

The more structurally complex nature of the examples of masculinization we observed in this study, coupled with



the discovery of transitional individuals, highlights the need for further research into this phenomenon. Fish specimens examined in previous work were sampled later in the season and were more reproductively developed than the fish examined in this study (Thomas and Rahman, 2012), indicating that hypoxia-induced reproductive impairment in fish in the GOM is more widespread and develops earlier than previously known. It is important to note that, although the results of this study indicate that ovarian masculinization in fish can occur earlier in gonadal development than previously thought, more research is

needed to understand how this early impairment translates to reproductive success later in the season.

Ovarian masculinization and reproductive impairment

For both female and male Atlantic croaker, no statistically significant differences were found in the residual GSI between hypoxic sites and sites with normal oxygen concentrations, although a trend of increased GSI in specimens from normal sites was observed. This finding contradicts those of previous studies in which

statistically significant decreases in GSI values were found for Atlantic croaker from hypoxic sites (Thomas and Rahman, 2012). Although unexpected, the results of our study could be explained by the difference in the severities of hypoxic conditions between the investigations, given that hypoxia in 2016 was less patchy and more widespread compared to that in 2007 (the year of Thomas and Rahman's field collections), or by differences in the sexual maturity of sampled specimens due to differences in how far into the seasons fish were sampled. These discrepancies highlight the importance of continued research into hypoxia and its effects, especially those on fish at different levels of reproductive maturity.

Both female and male spot from hypoxic sites had significantly lower ($P < 0.05$) residual GSI values than those for females and males from sites with normal oxygen levels. This finding supports observations from previous studies of Atlantic croaker and further highlights the potential effects of an environmental stressor, such as hypoxia, on fish reproduction at population and ecosystem levels.

Female and male Atlantic croaker and female spot sampled from hypoxic sites in 2017 had some of the highest GSI values observed for both species and both sexes (Figs. 6 and 8). The patchy areas of hypoxia from which these specimens were sampled in 2017 were a fraction of the size of the large, more continuous hypoxic zones sampled in 2016 and 2019 (the hypoxic zone was patchier in 2019 than in 2016, but the patches of hypoxia were much larger in 2019 than in 2017) (Fig. 1). The patchiness of hypoxia in 2017 could have led to hypoxia exposure that was less severe than that to which fish were exposed in 2016 and 2019. Although we have no diet data to support this interpretation, it is possible that the smaller areas of hypoxia in 2017 could have allowed benthic fish to feed on hypoxia-stressed infaunal invertebrates, similar to what has been observed in the more episodic zones of hypoxia that develop in Chesapeake Bay (Pihl et al., 1992). This interpretation could also explain why fish from hypoxic sites in 2017 had such high GSI values. Balancing the potential for increased foraging opportunities associated with hypoxic conditions with the physiological costs associated with hypoxia exposure is one explanation for the context-dependent nature of thresholds for hypoxia avoidance in groundfish in the GOM (Craig, 2012), highlighting the need for more research to place the reproductive costs of hypoxia in the broader ecological context of the GOM.

Conclusions

The results of our study indicate that reproductive impairment, specifically the presence of intersex individuals associated with hypoxic conditions, affects more species of fish than previously documented. We also found evidence of reproductive anomalies, such as the presence of ovum remnants in early stages of ovarian development, in fish in and around the hypoxic zone in the GOM in 2016. These findings, along with the lack of ovarian masculinization and reproductive anomalies in reference fish and

the significantly lower GSI values for spot from hypoxic sites than from sites with normal oxygen concentrations, strongly support the idea that hypoxic conditions can alter the reproductive health of fish.

The findings of this study expand upon previous reports of ovarian masculinization and reproductive impairment in fish in the GOM. They increase the number of species affected and provide novel examples and pathologies of this phenomenon. This study also resulted in evidence of ovarian masculinization occurring in fish sampled at sites bordering hypoxic areas and in fish at earlier developmental stages than have been reported previously. However, these findings need to be further explored to fully understand how they fit within the broader context of hypoxia-induced ovarian masculinization in fish in the GOM.

Resumen

Los resultados de este estudio aportan evidencia adicional de masculinización ovárica en la corvina (*Micropogonias undulatus*) dentro de la zona hipóxica del Golfo de México y sus alrededores, así como las primeras pruebas de masculinización ovárica en la croca (*Leiostomus xanthurus*) y el rubio ojón (*Prionotus longispinosus*). En algunos ejemplares de corvina y croca observamos este fenómeno en un grado que no se había reportado antes en ninguna de las dos especies. También, encontramos evidencia de gónadas más pequeñas en crocas de sitios hipóxicos en comparación con las gónadas crocas de sitios con concentraciones normales de oxígeno. Estos hallazgos indican que la masculinización ovárica y la deficiencia reproductiva asociados con la exposición a la hipoxia, afectan a una gama de especies más amplia de lo que se conocía hasta ahora y destacan la importancia de seguir investigando sobre este tema.

Acknowledgments

We would like to thank the scientists and staff at the National Marine Fisheries Service lab in Pascagoula, Mississippi, and the crew of the NOAA Ship *Oregon II* for all their time, expertise, kindness, and guidance during the sample collection process. We would also like to recognize J. Mann, the collections manager of the Royal D. Suttkus Fish Collection at the TUBRI, for helping to process and integrate the sizeable number of specimens generated by this work into the collection at the TUBRI.

Literature cited

- Agius, C., and R. J. Roberts.
2003. Melano-macrophage centres and their role in fish pathology. *J. Fish Dis.* 26:499–509. [Crossref](#)
- Anderson, J., D. McDonald, B. Bumguardner, Z. Olsen, and J. W. Ferguson.
2018. Patterns of maturity, seasonal migration, and spawning of Atlantic croaker in the western Gulf of Mexico. *Gulf Mex. Sci.* 34(1):19–31. [Crossref](#)

- Bahamonde, P. A., K. R. Munkittrick, and C. J. Martyniuk.
2013. Intersex in teleost fish: are we distinguishing endocrine disruption from natural phenomena? *Gen. Comp. Endocrinol.* 192:25–35. [Crossref](#)
- Bartlett, M. S.
1937. Properties of sufficiency and statistical tests. *Proc. R. Soc. Lond. A* 160:268–282. [Crossref](#)
- Bhandari, R. K., H. Komuro, M. Higa, and M. Nakamura.
2004. Sex inversion of sexually immature honeycomb grouper (*Epinephelus merra*) by aromatase inhibitor. *Zool. Sci.* 21:305–311. [Crossref](#)
- Blazer, V. S., L. R. Iwanowicz, D. D. Iwanowicz, D. R. Smith, J. A. Young, J. D. Hedrick, S. W. Foster, and S. J. Reeser.
2007. Intersex (testicular oocytes) in smallmouth bass from the Potomac River and selected nearby drainages. *J. Aquat. Anim. Health* 19:242–253. [Crossref](#)
- Boesch, D. F., and R. Rosenberg.
1981. Response to stress in marine benthic communities. *In* Stress effects on natural ecosystems (G. W. Barrett and R. Rosenberg, eds.), p. 179–200. John Wiley and Sons, New York.
- Cheek, A. O., C. A. Landry, S. L. Steele, and S. Manning.
2009. Diel hypoxia in marsh creeks impairs the reproductive capacity of estuarine fish populations. *Mar. Ecol. Prog. Ser.* 392:211–221. [Crossref](#)
- Craig, J. K.
2012. Aggregation on the edge: effects of hypoxia avoidance on the spatial distribution of brown shrimp and demersal fishes in the northern Gulf of Mexico. *Mar. Ecol. Prog. Ser.* 445:75–95. [Crossref](#)
- Dauer, D. M.
1993. Biological criteria, environmental health and estuarine macrobenthic community structure. *Mar. Pollut. Bull.* 26:249–257. [Crossref](#)
- Diaz, R. J., and R. Rosenberg.
2008. Spreading dead zones and consequences for marine ecosystems. *Science* 321:926–929. [Crossref](#)
- Eriksson, S. P., and S. P. Baden.
1997. Behaviour and tolerance to hypoxia in juvenile Norway lobster (*Nephrops norvegicus*) of different ages. *Mar. Biol.* 128:49–54. [Crossref](#)
- Fournie, J. W., J. K. Summers, L. A. Courtney, V. D. Engle, and V. S. Blazer.
2001. Utility of splenic macrophage aggregates as an indicator of fish exposure to degraded environments. *J. Aquat. Anim. Health* 13:105–116. [Crossref](#)
- Galloway, J. N., A. R. Townsend, J. W. Erisman, M. Bekunda, Z. Cai, J. R. Freney, L. A. Martinelli, S. P. Seitzinger, and M. A. Sutton.
2008. Transformation of the nitrogen cycle: recent trends, questions, and potential solutions. *Science* 320:889–892. [Crossref](#)
- Guiguen, Y., A. Fostier, F. Piferrer, and C.-F. Chang.
2010. Ovarian aromatase and estrogens: a pivotal role for gonadal sex differentiation and sex change in fish. *Gen. Comp. Endocrinol.* 165:352–366. [Crossref](#)
- Helly, J. J., and L. A. Levin.
2004. Global distribution of naturally occurring marine hypoxia on continental margins. *Deep Sea Res., I* 51:1159–1168. [Crossref](#)
- Hoff, J. G., Jr.
1992. Comparative biology and population dynamics of searobins (genus *Prionotus*) with emphasis on populations in the northwestern Gulf of Mexico. Ph.D. diss., 229 p. Va. Inst. Mar. Sci., Coll. William Mary, Gloucester Point, VA. [Available from [website](#).]
- Jakob, E. M., S. D. Marshall, and G. W. Uetz.
1996. Estimating fitness: a comparison of body condition indices. *Oikos* 77:61–67. [Crossref](#)
- Kruskal, W. H., and W. A. Wallis.
1952. Use of ranks in one-criterion variance analysis. *J. Am. Stat. Assoc.* 47:583–621. [Crossref](#)
- Landry, C. A., S. L. Steele, S. Manning, and A. O. Cheek.
2007. Long term hypoxia suppresses reproductive capacity in the estuarine fish, *Fundulus grandis*. *Comp. Biochem. Physiol., A* 148:317–323. [Crossref](#)
- Maxfield, J. M., and K. S. Cole.
2019. Patterns of structural change in gonads of the divine dwarfgoby *Eviota epiphanes* as they sexually transition. *J. Fish Biol.* 94:142–153. [Crossref](#)
- Navarro-Martín, L., M. Blázquez, and F. Piferrer.
2009. Masculinization of the European sea bass (*Dicentrarchus labrax*) by treatment with an androgen or aromatase inhibitor involves different gene expression and has distinct lasting effects on maturation. *Gen. Comp. Endocrinol.* 160:3–11. [Crossref](#)
- Ogawa, S., M. Akiyoshi, M. Higuchi, M. Nakamura, and T. Hirai.
2008. ‘Post-sex differentiatinal’ sex reversal in the female common carp (*Cyprinus carpio*). *Cybum* 32(2 suppl.): 102–103. [Crossref](#)
- Parker, J. C.
1971. The biology of the spot, *Leiostomus xanthurus* Lacepede, and Atlantic croaker, *Micropogon undulatus* Linnaeus, in two Gulf of Mexico nursery areas. Sea Grant Publ. TAMU-SG-71-210, 194 p. Tex. A&M Univ., College Station, TX. [Available from [website](#).]
- Pihl, L.
1994. Changes in the diet of demersal fish due to eutrophication-induced hypoxia in the Kattegat, Sweden. *Can. J. Fish. Aquat. Sci.* 51:321–336. [Crossref](#)
- Pihl, L., S. P. Baden, R. J. Diaz, and L. C. Schaffner.
1992. Hypoxia-induced structural changes in the diet of bottom-feeding fish and Crustacea. *Mar. Biol.* 112:349–361. [Crossref](#)
- Rabalais, N. N., R. E. Turner, and W. J. Wiseman Jr.
2002. Gulf of Mexico hypoxia, A.K.A. “the Dead Zone.” *Annu. Rev. Ecol. Syst.* 33:235–263. [Crossref](#)
- Rabalais, N. N., R. E. Turner, B. K. S. Gupta, D. F. Boesch, P. Chapman, and M. C. Murrell.
2007. Hypoxia in the northern Gulf of Mexico: does the science support the plan to reduce, mitigate, and control hypoxia? *Estuar. Coasts* 30:753–772. [Crossref](#)
- Reist, J. D.
1985. An empirical evaluation of several univariate methods that adjust for size variation in morphometric data. *Can. J. Zool.* 63:1429–1439. [Crossref](#)
- Rosenberg, R., and L.-O. Loo.
1988. Marine eutrophication induced oxygen deficiency: effects on soft bottom fauna, western Sweden. *Ophelia* 29:213–225. [Crossref](#)
- Sandberg, E.
1997. Does oxygen deficiency modify the functional response of *Saduria entomon* (Isopoda) to *Bathyporeia pilosa* (Amphipoda)? *Mar. Biol.* 129:499–504. [Crossref](#)
- Shapiro, S. S., and M. B. Wilk.
1965. An analysis of variance test for normality (complete samples). *Biometrika* 52:591–611. [Crossref](#)
- Sun, L.-N., X.-L. Jiang, Q.-P. Xie, J. Yuan, B.-F. Huang, W.-J. Tao, L.-Y. Zhou, Y. Nagahama, and D.-S. Wang.
2014. Transdifferentiation of differentiated ovary into functional testis by long-term treatment of aromatase inhibitor in Nile tilapia. *Endocrinology* 155:1476–1488. [Crossref](#)

- Thomas, P., and M. S. Rahman.
2009. Biomarkers of hypoxia exposure and reproductive function in Atlantic croaker: a review with some preliminary findings from the northern Gulf of Mexico hypoxic zone. *J. Exp. Mar. Biol. Ecol.* 381(Suppl.):S38–S50. [Crossref](#)
2012. Extensive reproductive disruption, ovarian masculinization and aromatase suppression in Atlantic croaker in the northern Gulf of Mexico hypoxic zone. *Proc. R. Soc. B* 279:28–38. [Crossref](#)
- Thomas, P., M. S. Rahman, I. A. Khan, and J. A. Kummer.
2007. Widespread endocrine disruption and reproductive impairment in an estuarine fish population exposed to seasonal hypoxia. *Proc. R. Soc. B* 274:2693–2702. [Crossref](#)
- Thomas, P., M. S. Rahman, M. E. Picha, and W. Tan.
2015. Impaired gamete production and viability in Atlantic croaker collected throughout the 20,000 km² hypoxic region in the northern Gulf of Mexico. *Mar. Pollut. Bull.* 101:182–192. [Crossref](#)
- Vaquer-Sunyer, R., and C. M. Duarte.
2008. Thresholds of hypoxia for marine biodiversity. *Proc. Natl. Acad. Sci. U.S.A.* 105:15452–15457. [Crossref](#)
- Wang, Q., Y. Liu, C. Peng, X. Wang, L. Xiao, D. Wang, J. Chen, H. Zhang, H. Zhao, S. Li, and H. Lin.
2017. Molecular regulation of sex change induced by methyltestosterone-feeding and methyltestosterone-feeding withdrawal in the protogynous orange-spotted grouper. *Biol. Reprod.* 97:324–333. [Crossref](#)
- Wu, R. S. S.
2002. Hypoxia: from molecular responses to ecosystem responses. *Mar. Pollut. Bull.* 45:35–45. [Crossref](#)
- Wu, R. S. S., B. S. Zhou, D. J. Randall, N. Y. S. Woo, and P. K. S. Lam.
2003. Aquatic hypoxia is an endocrine disruptor and impairs fish reproduction. *Environ. Sci. Technol.* 37:1137–1141. [Crossref](#)
- Zhou, B. S.
2001. Effects of hypoxia on metabolism, bioenergetics, growth, and reproduction of the common carp (*Cyprinus carpio* L.). Ph.D. thesis. City Univ. Hong Kong, Hong Kong, China.