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Effects of an Environmental Estrogen on Male Gulf Pipefish, *Syngnathus scovelli* (Evermann and Kendall), a Male Brooding Teleost

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Environmental estrogens have been implicated in hormonal disruption in wildlife populations (McLachlan and Korach 1995). Reported effects include increased plasma levels of estrogen responsive proteins, testes atrophy, reproductive dysfunction and elevated intersex occurrence. Aquatic species are often most heavily impacted because of both the source of exposure and the exposure mechanisms unique to the aquatic environment. Xenoestrogenic sources in the aquatic environment have included pharmaceuticals in wastewater effluents (Hemming et al. 2001), phytoestrogens (Degen 1990; Makela et al. 1995), pesticides, surfactants and dispersants (Sumpter and Jobling 1995), and PCBs (Garcia et al. 1997). The egg yolk protein precursor, vitellogenin (VTG), has been used as a biological indicator for the presence of xenoestrogens in fish models (Heppell et al. 1995; Sumpter and Jobling 1995; Garcia et al. 1997). To date, there have been no reports of xenoestrogenic effects on reproduction of animals exhibiting sex-role reversal and male brooding. In the current study, the effects of xenoestrogenic compounds on male reproductive fitness of gulf pipefish, *Syngnathus scovelli* were assessed using a combination of histological and biochemical approaches.

The gulf pipefish is an euryhaline species found along the Atlantic coast of the United States from Georgia to Florida, throughout the Gulf of Mexico to the Yucatan Peninsula, the Caribbean coasts of Central and South America, and south to the northern coast of Brazil (Shipp 1986) in salinities ranging from 0 to 30 practical salinity units (psu). A member of the family Syngnathidae, these animals are oviparous, with a reversal of sex roles during the mating and brooding stages (Vincent et al. 1995). Females produce telolecithal eggs, which are inserted into the brood pouch of the male. The eggs are immediately fertilized and the male incubates the embryos throughout development, approximately 12-21 days (Vincent et al. 1995).

MATERIALS AND METHODS

Gulf pipefish, *Syngnathus scovelli*, were collected with seines at Meaher Park, Baldwin County, Alabama (N 30°42'26.2" W 87°53'38.8") . The effects of 17α-ethinylestradiol (EE2), a synthetic estrogen analog, on non-brooding male

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pipefish were examined in a laboratory based experiment. EE2 was chosen as a model for environmental estrogens because of its relative stability. Fish were held in the laboratory for more than two weeks prior to exposure, housed in 40 L aquaria at temperatures of 25 - 28°C. Reverse osmosis water supplemented with 0.54 mM $MgSO_4$, 0.058 mM KCl, 0.44 mM $CaSO_4$ and 1.32 mM $NaCO_3$ in order to obtain an alkalinity of 65 mg/L and a hardness of 100 mg/L was used. The salinity was brought to 5 psu by the addition of 51.3 mM NaCl to decrease parasitism and fungal infection. Fish were fed a diet of 24 h old *Artemia* nauplii twice daily. Fish were then separated into a control group and an EE2 exposure group with three replicate tanks for each group and five fish per tank. This resulted in a biological load of 0.12±0.01 g/L. Fish in the EE2 group were exposed to EE2 at a concentration of ~$3.37x10^{-9}$M (1 ⌈g/L) in 95% ethanol (final concentration of ethanol 0.16 M). A single dose of EE2 was chosen for the current study and reflects maximal levels of estrogenic chemical analytes fish would be expected to be exposed to in an effluent receiving estuary (Hemming et al. 2004). Control fish were exposed to the solvent alone. The exposure was ten days. Ten percent water changes occurred daily and tanks were supplemented with EE2 to maintain a concentration of ~$3.37x10^{-9}$M throughout the experiment. Preliminary studies examining degradation of EE2 over time indicated that daily supplementation was necessary to maintain EE2 levels at the desired level (unpublished data), however, absolute concentrations were not measured in the current study.

Fish were anesthetized in 0.008% 3-aminobenzoic acid ethyl ester (methanesulfonate salt/ MS222, Sigma, St. Louis, MO) for approximately 15 minutes (Allen et al. 1999). Fish weights were recorded and blood plasma was collected for vitellogenin (VTG) analyses (see below). Liver and gonads were removed and weighed for hepatosomatic index [HSI = (liver weight / body weight) X 100] and gonadosomatic index [GSI = gonad weight / body weight) X 100] determinations (Hemming et al. 2001). Liver and gonads were then prepared for light and transmission electron microscopy. Samples were removed as above and fixed in 3% glutaraldehyde and 1.1% cacodylate buffer. Samples were then washed in cacodylate buffer and post fixed in 1% osmium tetroxide for one hour. Samples were again washed with cacodylate buffer, dehydrated in a graded alcohol series, embedded in Poly/Bed 812, and cured at 63° C. Thick sections (1 μm) were taken and stained with toluene blue for light microscopy. Ultrathin sections (60 – 80 nm) were cut and stained with uranyl acetate and Reynold's lead citrate and viewed under a FEI/Phillips CM 100 transmission electron microscope (Hillsboro, OR) at the University of South Alabama Medical Center. Pigmentation patterns of fish were noted and qualitatively recorded. Differences in HSI and GSI values between control and experimental fish were compared using a Student's *t*-test ($\leq$=0.05) (Zar 1984). For comparative purposes, HSI, GSI, and VTG concentrations were also determined for field-collected females (n=37). These fish were processed as described above within 24 h of collection.

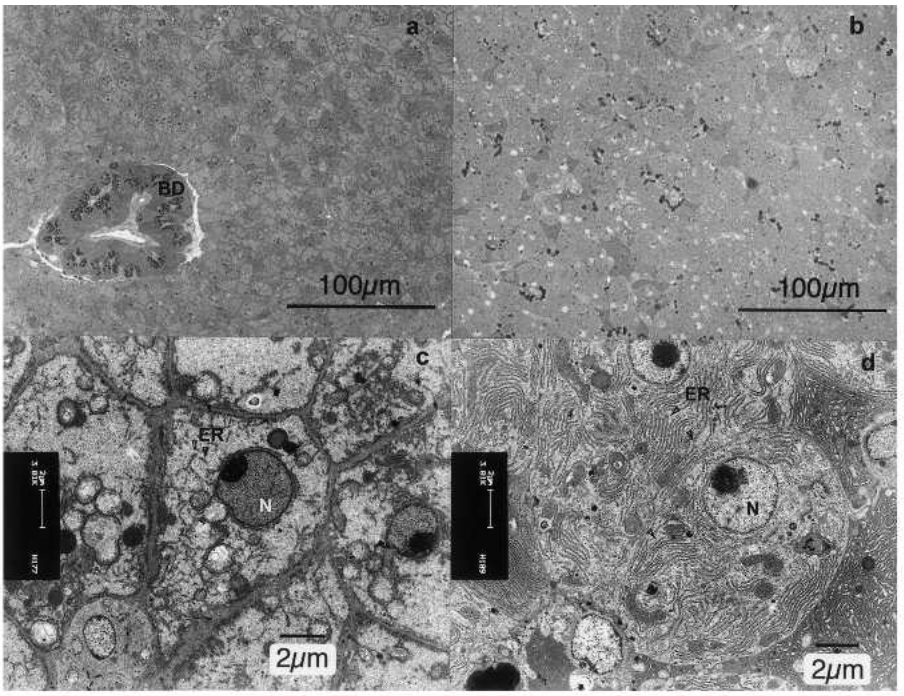
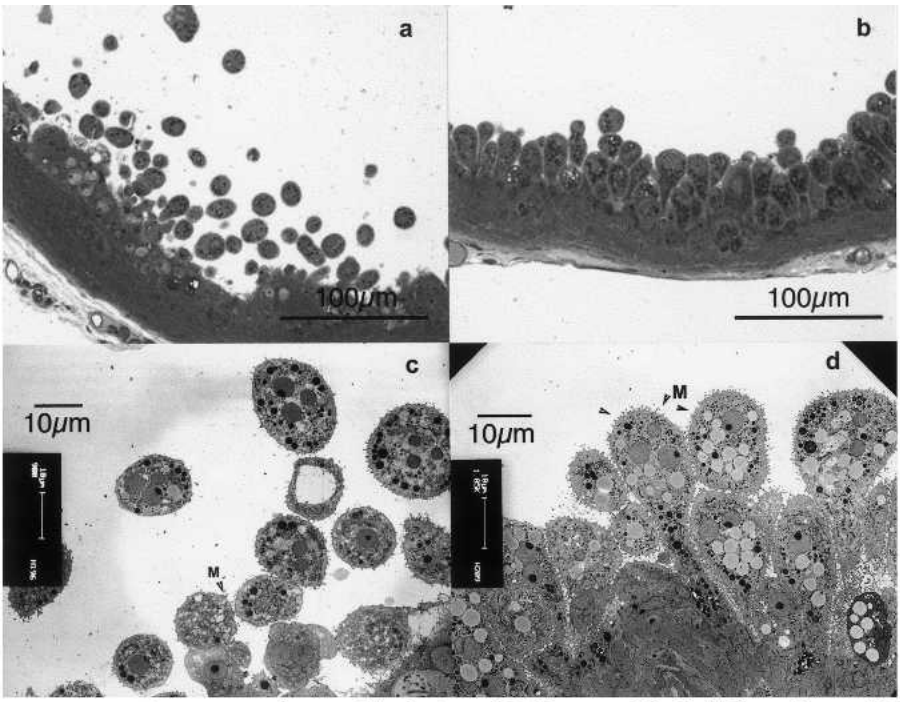


Figure 1. Microscopy of control (a & c) and EE2-treated (b & d) liver tissue (BD = bile duct, N = nucleus, ER = endoplasmic reticulum, arrows) from non-brooding male *Syngnathus scovelli*. Note: Panels a & b = light microscopy, c & d = transmission electron microscopy

Plasma VTG concentrations were determined by dot blot analyses (GIBCO-BRL, Life Technology, Inc.) with sheepheads minnow (*Cyprinodon variegatus*) VTG (University of Florida, ICBR – Biomarker and Protein Core, Gainesville, FL) used as a standard. Plasma samples from 4-5 fish per replicate were pooled for VTG analyses using mouse anti-killifish VTG (1:500 dilution, Biosense Laboratories, Bergen Norway). A chemiluminescent system (Cell Signaling Technology, Beverly, MA) was used for detection (detection limit 2.54×10^{-5} g VTG/ml). Relative intensity of dots for vitellogenin analysis was determined using Image J (NIH, USA). A VTG standard curve was produced between relative intensities of dots and logarithmically transformed VTG standard concentrations.

RESULTS AND DISCUSSION

Exposure to EE2 had significant effects on non-brooding male gulf pipefish. HSI values for EE2-exposed males were significantly higher than those for control fish (control=1.52±0.46%, EE2=2.25±0.63%, P=0.001). A similar pattern was seen for GSI (control=0.80±0.22%, EE2=1.04±0.20%, P=0.01). Field collected non-gravid females had HSI (1.28±0.44) and GSI (0.74±1.04) values similar to those



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of control males. There were histological alterations in both liver and gonadal tissue of EE2 exposed males. The control livers had uniformly shaped, tightly packed cells (Figure 1a). The EE2 exposed liver cells were not as tightly packed and showed an increase in dark-staining vesicles (Figure 1b). Exposed liver cells showed a dramatic increase in endoplasmic reticulum abundance (Figures 1d vs. 1c). Both the increase in number of vesicles and endoplasmic reticulum suggest an increase in synthesis and secretory processes and are probably linked to the increase in VTG production (see below). The control gonads had spermatogonia detached from the testicular wall that had a few projections resembling microvilli (Figure 2a & 2c). This is typical of what is seen in non-brooding males of other syngnathids (Kornienko and Drozdov 1999; Wantanabe et al. 2000). In the EE2 exposed gonads, the spermatogonia were tightly packed along the testicular wall and had a notable increase in number of microvilli-like projections (Figure 2b & 2d). VTG levels in control fish were below detection. However, EE2-exposed male fish had VTG levels of 39.17±4.04 ⌠g/ml. These values were higher than field-collected female fish (3.82±2.71 ⌠g/ml). It was also noted that the pigmentation pattern of EE2-treated males shifted from that of normal males to that of normal females. Females have an olive brown color with V-shaped metallic bands that run dorso- ventrally on the anterior portion of the trunk, while

males are lighter in color and lack the metallic banding (Jones and Avise 1997). Feminization of pigmentation pattern, including overall darkening and development of the banding pattern occurred in EE2 exposed males fish. This suggests that, at least for the non-brooding stage of *S. scovelli*, exposure to environmental estrogens can lead to disruption of the endocrine system and feminization of males.

These results parallel what has been seen for non sex-role reversed fish exposed to xenoestrogens or other contaminants (Allen et al. 1999; Hemming et al. 2001; Verslycke et al. 2002). The previously reported results also implicate external, as well as physiologic feminization of male fish exposed to xenoestrogens. The extent to which physiologic changes impede species fitness is uncertain, however, the loss of masculine phenotypic sexual cues used in mating rituals would be expected to rapidly lead to decreases in reproductive success. If females recognize pigmented and banded males as "females" then a greater time will be spent in female-female competition as opposed to mating behavior. To clarify potential effects on species fitness, future studies will focus on the effects of environmental estrogens on brooding males and their developing embryos, and on mating rituals. In addition, dose-response relationship between concentration environmental estrogens and the biological and behavioral effects on this species will be developed.

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