

Final Report Executive Summary
ACES Project Summary: Part 2 Final Technical Report

Period Covered by the Report: 6/01/01 – 12/31/03
Date of Report: 12/31/03
EPA Agreement Number: R-827072-01-1
Title: Effects of Estrogen Pollution on the Reproductive Fitness of the Gulf Pipefish, *Syngnathus scovelli*
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Research Category: Small Grant for Exploratory Research
Project Period: 06/01/01-6/30/03

Background and Objectives:

Developmental and sex related changes in fish resulting from environmental estrogen exposure have been implicated in causing adverse population level effects. Recent research has focused on the influence of environmental estrogens on adult fish, although it has long been known that juvenile fish will experience sex reversal with hormone treatment at critical stages of development. The influence of estrogens on oviparous fish fecundity and offspring sexual development has been assessed using sentinel species including the fathead minnow, *Pimephales promelas*, the sheepshead minnow, *Cyprinidon variegatus*, and the rainbow trout, *Oncorhynchus mykiss*. The current research assesses the effects of xenoestrogenic exposure on the reproductive fitness of the male-brooding Gulf pipefish, *Syngnathus scovelli*. Such species possess adaptations that may limit the effect environmental estrogens have on their offspring. Baseline studies on untreated fish were used to establish benchmark conditions of various physiologic parameters and for comparison with subsequent exposure derived data. The specific objectives were to:

1. Determine baseline blood and brood pouch fluid osmolality, total glucose, total protein, and plasma vitellogenin, and examine normal brood pouch development, gonad morphology, gonado-somatic and hepato-somatic Indices for reference fish.
2. Determine the sensitivity of *S. scovelli* to estrogenic exposure utilizing vitellogenin induction levels.
3. Examine the effect of estrogen exposure on the gonad and liver morphology, and gonado-somatic and hepato-somatic Indices.

Material and Methods:

Collection and Maintenance of Fish

S. scovelli were collected from Meaher Park, Baldwin County, AL using dip nets and seines. At the time of collection, fish were visually sexed and checked for embryos and the developmental stage of the embryos recorded. Fish were held in 10-gallon aquariums. The fish were allowed to acclimate for at least two weeks prior to baseline studies and approximately six months prior to estrogenic treatment. It has been noted that estrogenic effects can be detected up to 5 months after exposure ceases (Hemming, *et al.*, 2001). Tanks were filled with distilled water reconstituted with MgSO₄, CaSO₄, KCl, and NaHCO₃. Tanks were kept at a constant temperature of 25° C and on a 12:12 light:dark schedule. All fish were fed a diet of 24-48 hour old *Artemia* twice a day.

Baseline Studies

Benchmark conditions for various physiological and histological parameters were established using field collected fish held for at least two weeks under control conditions. This data was used to understand better the role of males in embryonic development and was compared with subsequent exposure derived data. Changes in the inorganic and organic makeup of brood pouch fluid and the blood of brooding males were examined (see biochemical analyses below). In addition, baseline GSI and HSI measurements were taken, and gonad and brood pouch morphology was assessed. Pipefish were sampled each week during their brood cycle and post brooding.

Estrogen Treatment

Male pipefish were divided between a treatment group and a control group. Three replicate tanks for each group with five fish per replicate were used. An estrogen (ethynylestradiol) concentration of 1.0 µg/L was used with exposure renewed daily for 10 days. This estrogen concentration is at the upper end of concentrations found in effluent receiving systems (Hemming, *et al.*, 2001). An initial experiment was run with male fish at the no brood stage. Trials are currently being set-up with fish that have accepted a brood within the past 24 h. Estrogenic responses of the adult organisms were evaluated via changes in GSI and HSI, plasma vitellogenin concentration, and gonad morphology. Each of these endpoints has been shown to be influenced by estrogenic stimuli (Folmar, *et al.*, 1996; Hemming, *et al.*, 2001).

Gonadal-Somatic and Hepatic-Somatic Indices

At the termination of baseline acclimations and post exposure for experimental studies, length (cm) and weight (g) measurements were taken and fluids extracted (see below). Fish were then sacrificed (modification of methods by Allen, *et al.*, 1999), testes and liver weight (g) measured and GSI and HSI calculated. Gonad and liver samples were placed in 2.5% phosphate (0.1M at pH 7.0) buffered glutaraldehyde (Allen, *et al.*, 1999) for morphological examinations (see below).

Blood and Brood Pouch Fluid Extraction

Fish were anesthetized using MS 222 and the tail was severed at the base of the caudal peduncle. The blood was collected in 10 µl heparinized capillary tubes and centrifuged so that plasma could be collected. This plasma was used for all tests conducted. Brood pouch fluid was extracted from *S. scovelli* by inserting a 10 µl syringe between the flaps, which compose the brood pouch (Quast and Howe, 1980). Fluid samples from multiple fish were pooled for analyses.

Biochemical Analyses

Fluids for baseline studies were sub-sampled for determination of total protein concentration, glucose, and osmolality. Additional fish were sampled for assay of vitellogenin concentrations. Fish from the estradiol experiment were also assayed for vitellogenin levels. The Bradford-based micro-Biorad assay (Hercules, CA) was used for protein determinations. Bovine serum albumin was used as a standard and all samples were run in triplicate. Fluid glucose concentrations were quantified using the Sigma Diagnostic #315 colorimetric assay with glucose standards. All samples were run in triplicate. Fluid osmolality was analyzed using a Wescor vapor pressure osmometer (Model #5500; Logan, Utah). Wescor Opti-Mole osmolality standards were used for calibration. All samples were run in triplicate. Plasma vitellogenin was quantified via dot-blot immunoassay using an anti-vitellogenin antibody from the University of Florida in the Protein Chemistry Molecular Biomarkers Laboratory (Rangel, 2000). Vitellogenin served as the standard.

Histology of Gonads and Livers

Following fixation in glutaraldehyde, the male livers and gonads from the estradiol treatment experiments are being held for EM analyses. These will be carried out during the spring of 2004.

Histology of the Brood Pouch

Brood pouches were excised from baseline pipefish each week during their brood cycle and post brooding. Surface morphology of the lining of the brood pouch was examined with a scanning electron microscope (SEM). These samples were fixed in 2.5% phosphate (0.1M at pH 7.0) buffered glutaraldehyde and viewed with Phillips XL20 SEM. Cellular specializations of the surface epithelial cells of the brood pouch and the surface of the egg membrane were examined with transmission electron microscopy (TEM). These samples were also be fixed in glutaraldehyde, embedded in plastic, sectioned with an ultramicrotome, and stained with uranyl acetate and lead citrate solutions.

Statistical Analyses

For the baseline studies variations in plasma and brood pouch fluid protein concentrations, glucose concentrations, osmolality and in the GSI and HSI among brood stages were compared using separate Model 1 one-way ANOVAs and Tukey's multiple comparison tests (Zar, 1984). For the estrogenic exposure experiments, variations in plasma vitellogenin levels and in the GSI and HSI between treatments were analyzed using separate Student's *t*-tests (Zar, 1984). The alpha value for all experiments was set at 0.05.

Results and Discussion:

The brood cycle of the Gulf pipefish lasts approximately 15 days. Four brood stages have been defined using the development of embryos as markers. These stages are termed: A) no brood stage - the male has not received eggs from the female (0 day); B) pharyngula stage - only embryonic eye pigmentation visible through the brood pouch (~5 day); C) protruding snout stage - all embryonic fins developed, snout only slightly protruding, large amount of yolk still remaining (~10 day); and D) embryonic/juvenile stage - embryos fully developed, little or no yolk remaining, ready for release (~15 day) (Figure 1). Baseline structural and physiological data for each stage was collected during the first year of the study. Histological data shows that at the no brood stage, the surface of the brood pouch is flat, smooth, and lined with pavement epithelial cells. During incubation, the pouch inner surface forms shallow depressions with low walls, arranged in longitudinal rows (pharyngula stage). From the pharyngula through the embryonic/juvenile stage the walls increase in height, particularly the medial walls (Figure 2). The flap shows similar changes and by the protruding snout stage or shortly thereafter, the pouch and flap walls meet, completely separating the embryos. Epithelial cells lining the floor of the depressions differ from those of the wall during incubation, and the floor pouch epithelium appears to be the site of attachment for the egg chorion through the protruding snout stage. These structural changes are paralleled by changes in specific reproductive markers and in the organic and inorganic make up of the blood and brood pouch fluid of male pipefish. GSI values decrease significantly through the protruding snout stage, increasing thereafter (Figure 3). HSI values did not vary with brood stage. Blood and brood pouch fluid protein and glucose values do not vary with the brood cycle. Blood osmolality values stayed constant throughout the brood cycle and were hyperosmotic relative to the environment. Brood pouch fluid osmolality was initially higher than blood osmolality, then decreased just prior to juvenile release. Based on differences seen in baseline studies, the focus of xenoestrogen exposure studies focused on comparisons between the no brood and protruding snout stages.

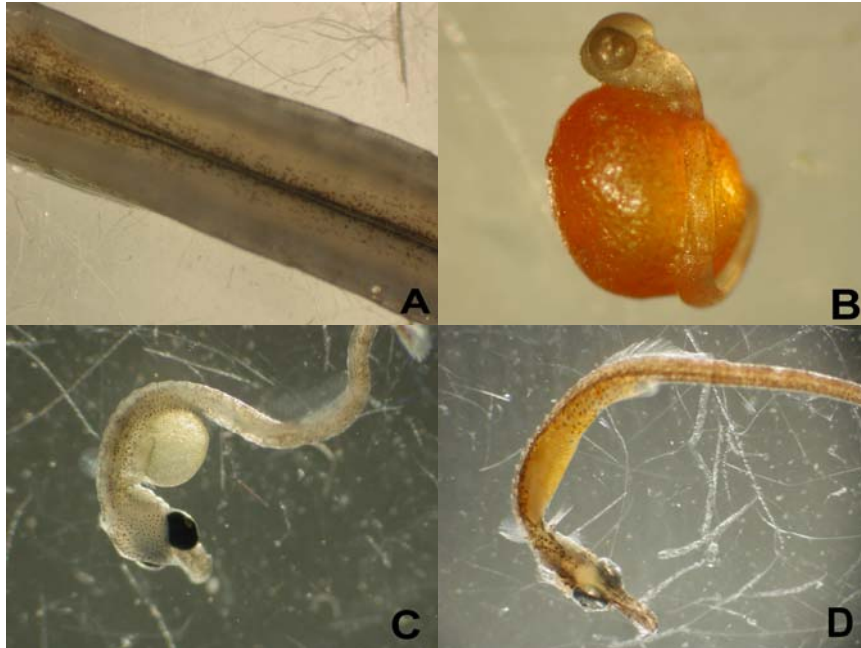


Figure 1. Embryonic stages for gulf pipefish, *S. scovelli*: (A) non-brooding – empty brood pouch shown, (B) pharyngula equivalent stage, (C) protruding snout equivalent stage, (D) juvenile equivalent stage. (photos by Charlyn Partridge)

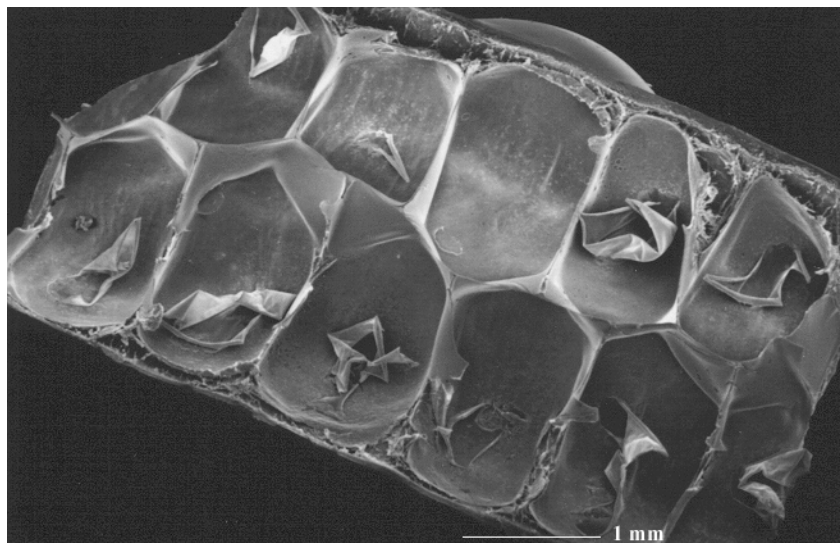


Figure 2. Egg compartment formation during the 6-10 day incubation of a *S. scovelli* male. Fragments of tissue in most compartments are pieces of chorion that remained when embryos were removed.

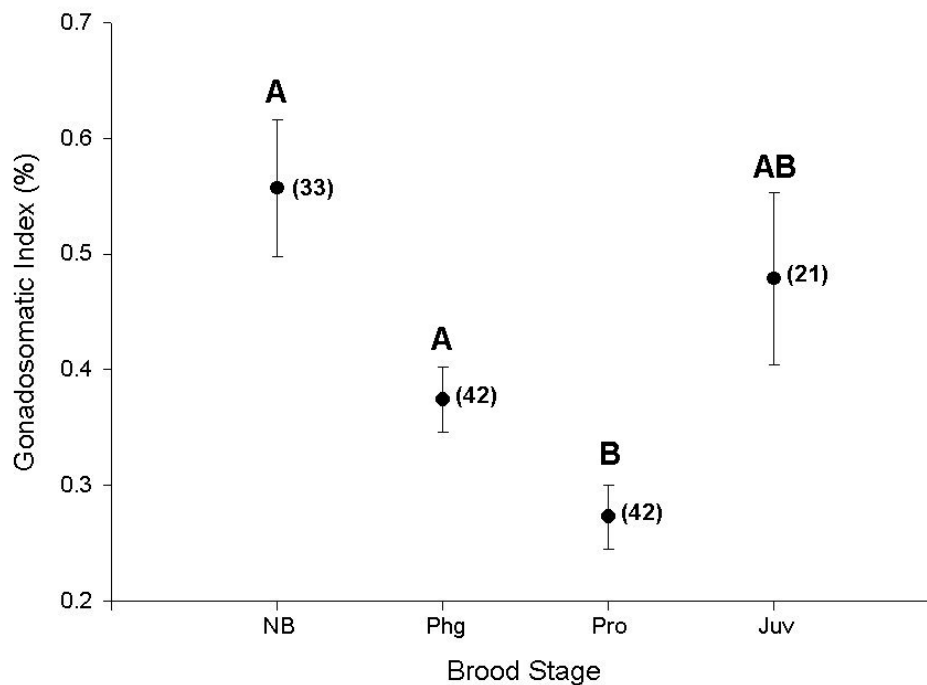


Figure 3. Mean gonadosomatic indices (%) of laboratory-bred male gulf pipefish, *S. scovelli*, at the non-brooding stage (NB), pharyngula stage (Phg), protruding snout stage (Pro) and juvenile stage (Juv). Vertical error bars on means indicate standard error. Samples sizes indicated in parentheses. Treatment results with the same letter above error bar are not significantly different at $P < 0.05$.

The synthetic estrogen, ethynyl estradiol (EE2) was used for exposure experiments. Preliminary EE2 stability experiments showed that exposure experiments can be run under the normal light:dark regime for culture of pipefish, but require daily EE2 renewals. In the original proposal, fish blood samples were to be sent out for vitellogenin analyses. However, Dr. Tim Sherman (University of South Alabama, Biology) offered his assistance in the development of an ELISA based assay for the pipefish samples using antibodies available from Cayman Chemical Company. The results obtained are comparable to those available through the University of Florida, Protein Chemistry and Molecular Biomarkers Laboratory. Vitellogenin levels were below detection limits for all brood stages of male field-collected fish. Levels for female fish (1-7 $\mu\text{g/ml}$) were comparable to levels found in other species of fish from low exposure environments. Exposure of laboratory-cultured male fish to EE2 leads to high vitellogenin levels (10-175 $\mu\text{g/ml}$). Exposure to EE2 also lead to increases in HSI values and to a change from normal male pigmentation patterns to female pigmentation patterns. GSI was not affected.

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- Zar, J.H. 1984. *Biostatistical Analysis*, 2nd ed. Prentice-Hall, Inc. Englewood Cliffs, NJ.

Changes in Scope or Objectives:

Based on the results of the first year of study, which indicated that the greatest differences among brood stages were between the no brood stage (0 day) and the protruding snout stage (10 day), our focus for the estradiol exposure experiments was on these two stages. In the original proposal, fish blood samples were to be sent out for vitellogenin analyses. However, Dr. Tim Sherman, Department of Biology, USA, offered his assistance in the development of an ELISA based assays for the pipefish samples using antibodies available from Cayman Chemical Company. The results obtained were comparable to those available through the University of Florida, Protein Chemistry and Molecular Biomarkers Laboratory.

Publications/Presentations:

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- Bolland, J. and A. Boettcher. In prep. Breeding cycles and population survey of euryhaline *Syngnathus scovelli* in a freshwater portion of Mobile Bay.
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- Partridge, C. and J. Shardo. 2002 Morphological changes in the brood pouch of the Gulf pipefish, *Syngnathus scovelli*, during egg incubation. Poster presentation, 2002 Benthic Ecology Meeting, Orlando, FL. March 2002.
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- Rozelle, J., C. Partridge, A. Boettcher, and D. Forbes. 2002. Use of gas chromatography in the elucidation of carbohydrates in the body fluids of the Gulf pipefish, *Syngnathus scovelli*. Poster presentation. University of South Alabama, University Committee for Undergraduate Research 4th Undergraduate Research Week. October 2002.

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