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Project Title: Ensuring biosecurity in the Atlantic salmon farming industry through a novel approach to inducing sterility: disrupting the early establishment of the GnRH system.
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Final Report

The main goal of this project was to develop a simple and efficient technology for inducing hypogonadism and sterility in Atlantic salmon that is based on disrupting the developmental establishment of the GnRH neuronal system. The proposed technical objectives sought to accomplish this goal by immersing Atlantic salmon embryos, during defined developmental time periods, in γ-aminobutyric acid (GABA), a naturally-occuring neurotransmitter.

Atlantic salmon eggs and milt were obtained from Heritage Salmon of Halifax, Nova Scotia, and transported to the aquaculture facilities of the Center of Marine Biotechnology where they were fertilized on December 11, 2003. Eleven groups of 700 embryos were placed at 11 ^{O}C in a Heath tray system that was extensively modified to meet the incubation requirements for these studies. Beginning at 21 days post-fertilization, groups of embryos at defined developmental timepoints were incubated in either 200 μM or 1 mM GABA for several days according to the following schedule:

Duration of treatment	200 μM GABA	1 mM GABA
21-26 dpf	Group 1	Group 6
27-32 dpf	Group 2	Group 7
33-38 dpf	Group 3	Group 8
39-44 dpf	Group 4	Group 9
45-50 dpf	Group 5	Group 10
51-60 dpf	Not treated	Group 11

Treatment incubations were done at 11 ^{O}C under static conditions, and treatment water was changed daily. After the treatment period, embryos are returned to flow-through conditions. Control embryos were kept under constant flow-through conditions. Twenty embryos are sampled regularly from each group, and either frozen for RNA extraction or processed for subsequent analysis of GnRH neuronal development via whole-mount *in situ* hybridization. Sac fry from all groups were transferred out of the Heath tray system into 90-gallon flow-through tanks at 70 days post fertilization. The embryos tolerated the treatments well, and mortality of treated groups was not different from controls.

In order to develop the molecular assays for analysis of treatment effects on the GnRH neuronal system, nucleotide sequences for the Atlantic salmon GnRHs were isolated. In salmonids, two isoforms of the mature GnRH peptide have been demonstrated: the

sGnRH and cGnRH-II decapeptides, which are also found in other teleost species. In tetraploid species, such as salmonids, each mature peptide is encoded by two distinct genes that differ in nucleotide sequence. Thus, two distinct mRNAs are found for each GnRH isoform. Using available sequence information for cloned GnRH cDNAs from other salmonid species, oligonucleotide primers were designed and used for PCR amplification of brain cDNA from adult Atlantic salmon. Four sequences of interest were cloned and characterized: two cDNAs encoding the sGnRH peptide [designated sGnRH(I) and sGnRH(II)] and two cDNAs encoding the cGnRH-II peptide [designated cGnRH-II(I) and cGnRH-II(II)]. Nucleotide sequence similarity between the Atlantic salmon sGnRH(I) and sGnRH(II) genes is 85% within the coding region. Sequence similarity between coding regions of the two Atlantic salmon cGnRH-II genes is 88%. In addition, nucleotide sequence similarity of the GnRH cDNAs from Atlantic salmon and other salmonid species ranges from 80-97%, with the closest seqeunce homologies between Atlantic salmon and rainbow trout. Because significant areas of high sequence homologies are found between each GnRH gene and among salmonid species, the molecular probes designed from these sequences are able to detect transcripts from both genes for a specific GnRH isoform. For the *in situ* hybridization assays used to localize the GnRH neurons, a digoxigenin-labelled RNA probe that recognizes transcripts from both the Atlantic salmon sGnRH(I) and sGnRH(II) genes was generated. Similarly, a probe was generated to measure transcripts from the Atlantic salmon cGnRH-II(I) and cGnRH-II(II) genes. Likewise with the real-time, fluoresence-based quantitative RT-PCR assays developed to analyse changes in sGnRH and cGnRH-II gene expression, both genes for a specific GnRH isoform can be measured with a single assay. In addition, the molecular assays developed for this project are readily useable in other salmonid species, in particular rainbow trout. This was done in order to provide deliverables that will make development of the proposed sterilization technology easily transferable to other sectors of the aquaculture industry.

Although whole-mount *in situ* hybridization assays revealed attenuated migration of the GnRH neurons from the nasal region, this affect was apparently transitory and GnRH neuronal migration had recovered by the parr stage of development. In addition, 50-100 pre-smolt fish from each experimental group were sacrificed at 15 months of age in April 2005, and immature gonadal tissues were seen in all sampled fish. Thus, it was decided to explore other pharmaceutical treatments that would potentially disrupt development of the hypothalamic GnRH neurons. Using zebrafish as a surrogate model, several other GABA-ergic compounds were tested: Baclofen (a $GABA_B$ receptor agonist), muscimol (a $GABA_A$ receptor agonist), bicuculine (a $GABA_A$ receptor antagonist) and dehydroepiandrosterone (an allosteric $GABA_A$ receptor antagonist). Of these compounds, treatment with dehydroepiandrosterone (DHEA) alone resulted in significant effects on the developing GnRH neurons (see below).

The use of zebrafish as a test model in this set of experiments enabled us to quickly determine whether any of these other compounds were worth persuing. Zebrafish reach sexual maturity fairly quickly (3 months) and have a rapid embryonic development (3 days to hatch), compared to salmonids. Moreover, our laboratory has studied the development of the GnRH system in zebrafish, and possesses the molecular tools

necessary for the relevant experimental analyses. In addition, zebrafish have two forms of GnRH, as is found in salmonids. In preliminary studies using DHEA treatment, zebrafish embryos were immersed in 100 μM DHEA for 24 hours at 1, 2, 3 or 4 days post fertilization (dpf). At 8 and 12 dpf, larvae were sampled and processed for localization of GnRH gene expression using whole-mount *in situ* hybridization. GnRH mRNA levels in the forebrain neurons were quantitated using image analysis software coupled with light microscopy. Relative levels of GnRH mRNA between groups were expressed as units of optical density. As illustrated in the graphs below, when assayed at 8 dpf, a significant decrease in GnRH gene expression was seen in embryos treated at 2 dpf with DHEA, when compared to untreated controls. This effect persisted until at least 12 dpf, when GnRH expression was seen to decrease even further to approximately 10% of normal levels. In addition, embryos treated with DHEA at 3 and 4 dpf eventually stopped expressing forebrain GnRH altogether by 12 dpf.

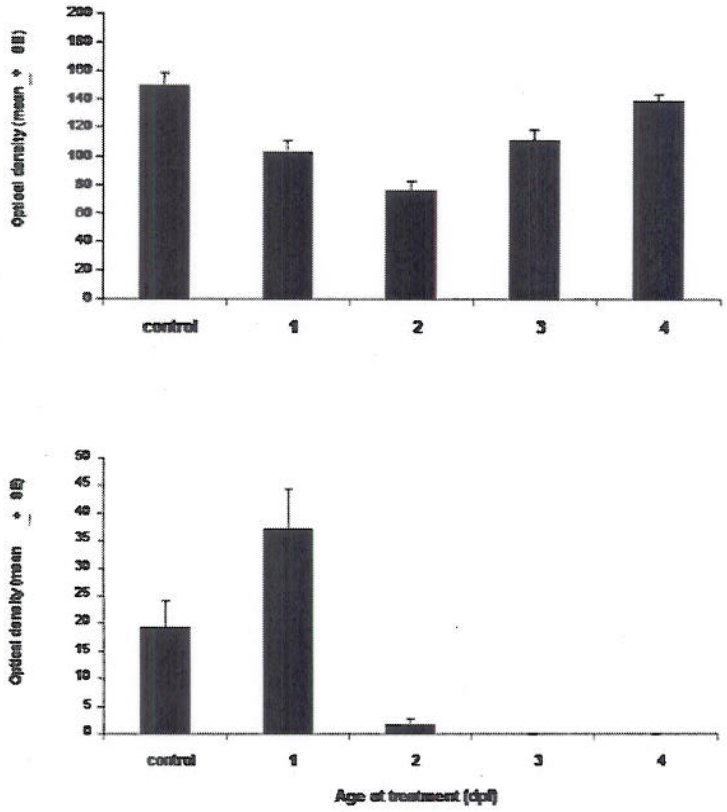

Figure 1: GnRH-I gene expression in zebrafish embryos, as determined by semi-quantitative whole-mount *in situ* hybridization. Top panel: relative amounts of GnRH mRNA in migrating neurons, assayed 8 dpf. Bottom panel: relative amounts of GnRH mRNA in migrating neurons, assayed 12 dpf. For both panels, groups were treated at 1, 2, 3 or 4 dpf. "Control" indicates untreated fish.

This effect was confirmed using *in situ* hybridization. Embryos were immersed at 2 days post-fertilization in 100 μM DHEA for 24 hours. DHEA-treated and untreated control fish were sampled at 8 days post-fertilization, and whole-mount in situ hybridization was used to localize GnRH expression in the nasal region. Figure 2 shows four representative fish each from the DHEA-treated and untreated control groups. DHEA-treated larvae (panels A-D) showed marked reduction in GnRH gene expression in the nasal region, indicated by dark purple staining, compared to untreated controls (panels E-H). The effects of DHEA on GnRH gene expression are not surprising in light of what is known about the effects of DHEA on the GnRH neurons in mammals. DHEA is an endogenous neurosteroid that has been shown to inhibit GnRH gene expression and GnRH peptide release (Cui et al., 2003; Zwain et al., 2002). These effects occur through modulation of $GABA_A$ receptors on the GnRH neurons (Sullivan and Moenter, 2003).

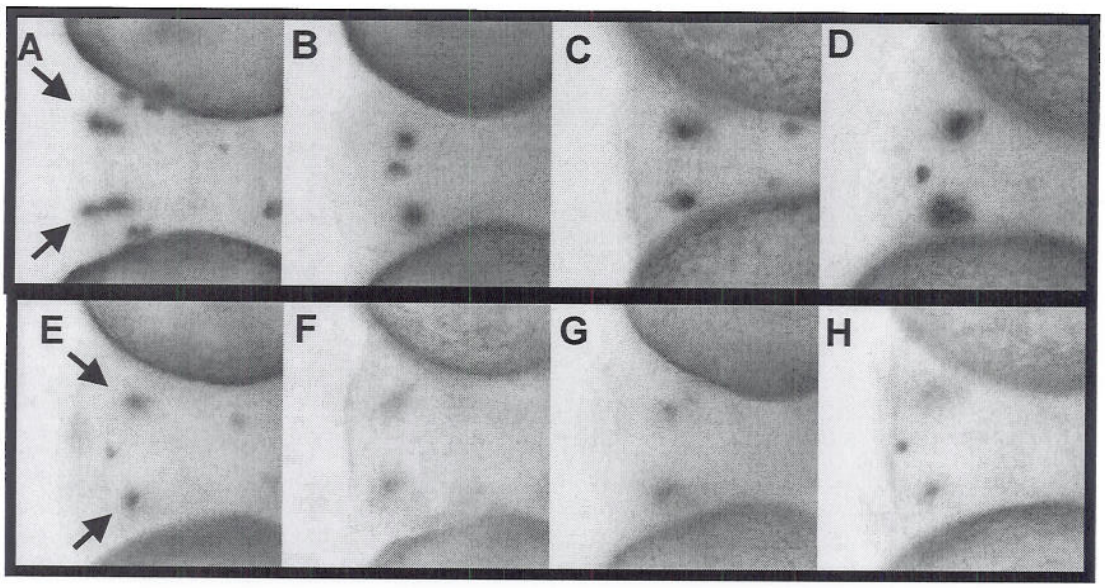

Figure 2: Localization of sGnRH gene expression in zebrafish larvae via whole-mount *in situ* hybridization. A-D: untreated, control larvae at 8 dpf. E-H: 8 dpf larvae treated with 100 μM DHEA during embryonic development. sGnRH gene expression is indicated by dark purple staining in the nasal region (arrows in left panels). All panels are dorsal views of the head, anterior to the left.

A direct assessment of the effects of DHEA treatment on GnRH neuronal migration in the developing forebrain was done using transgenic zebrafish that express a GFP reporter gene under regulatory control of the sGnRH promoter. This line of transgenic fish was developed in order to clearly and efficiently visualize the effects of various treatments on the development of the forebrain GnRH neuronal system. Visualization of GnRH neurons expressing GFP is done non-invasively in real-time, enabling a relatively quick assessment of treatment effects on development of the forebrain GnRH neurons, and

allowing us to follow these effects throughout development in individual fish. The fluorescent reporter technology is also extremely sensitive, and since the reporter is a translated protein we are able to discern the axonal processes as well as the cell bodies of the GnRH neurons. GnRH-GFP transgenic embryos were immersed in 200 μM DHEA for 24 hours at 2 days post-fertilization. GnRH neuronal development was followed in treated embryos by visualization with fluoresence micrscopy, and compared to untreated GnRH-GFP transgenic siblings. As seen in 12 days post-fertilization larvae and shown in Figure 3, posterior migration of the GnRH neurons out of the nasal region was severely attenuated in DHEA-treated fish. Proliferation of GnRH neurons, as indicated by fluorescent cell bodies, was not seen in DHEA-treated fish.

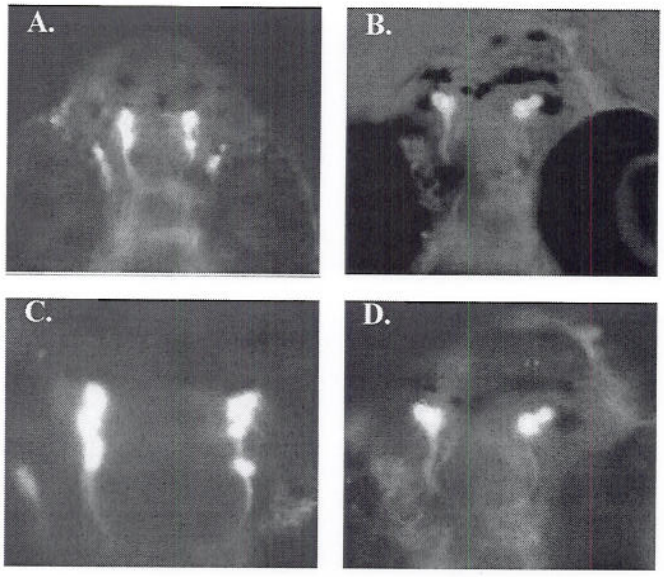

Figure 3: GnRH neuronal migration as seen in GnRH promoter/GFP reporter transgenic zebrafish larvae. A and C: Control, untreated larvae at 12 dpf showing posteriorly-proliferating GnRH cell bodies. B and D: DHEA-treated larvae at 12 dpf showing reduced proliferation of GnRH cell bodies, which remain in the nasal region. Panels C and D are 5X magnifications of views in A and B, respectively. All panels are dorsal views of the head, anterior at the top.

These effects were apparent in DHEA-treated larvae as long as GnRH-GFP fluorescent neurons could be visualized, up to 20 days post-fertilization. In addition, gross morphological examination of gonadal development in these DHEA-treated fish indicates altered gonad development.

Since DHEA treatment appears to be a potentially promising method of altering development of the forebrain GnRH system, we are coordinating with a commercial trout grower and a federal aquaculture research center in order to test this protocol in Rainbow trout, which will serve as an efficient model for application of this treatment in salmonid species.