Exxon Valdez Oil Spill
Restoration Project Final Report

Development of Culture Technology to Support Restoration of Herring in Prince William Sound: Use of *in vitro* Studies to Validate and Optimize Restoration Actions

Project 080821
Final Report

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Study History: In 2007 the *Exxon Valdez* Oil Spill Trustee Council awarded project 070821 as the first phase of the multi-year project "Development of Culture Technology to Support Restoration of Herring in Prince William Sound: Use of *in vitro* studies to validate and optimize restoration actions." The project's first year focused on herring culture techniques. Developments were reported in quarterly, annual and final reports. Project 080821, funded in 2008 as the second year investigation, subsequently addressed herring stress, disease and yolk proteins as stated under 080821 Objective 2: Conduct an assessment of yolk proteins and products in female herring that affect gamete quality and potential larval recruitment during the spawning cycle in PWS during 2008. This document reports the findings of that investigation.

Abstract: Spawning female Pacific herring (*Clupea pallasi*) caught in Prince William Sound (PWS) were examined to determine if pathogens such as viral hemorrhagic septicemia virus (VHSV) and Icthyophonus (ICTH) were associated with physiological stress and altered reproductive function. Serum levels of cortisol, vitellogenin, total calcium were measured along with free amino acids in ovulated eggs. A total of n=19 (59.1%) of 32 female herring caught in early April tested positive for ICTH. Cortisol levels in infected fish were significantly higher than in non-infected fish, but there were no significant differences in either vitellogenin (Vg) or total calcium. However, there was a significant, positive correlation between cortisol and vitellogenin levels within ICTH infected fish. Among free amino acids measured in ovulated eggs, valine was significantly higher in infected fish, whereas glycine was higher in non-infected fish. Higher levels of cortisol found in infected fish support suggestions that PWS herring are subject to environmental stress, but the effect on female reproduction is unclear. Proposed stock supplementation projects for PWS herring may benefit from improved understanding of factors that affect reproductive function.

Key Words: Pacific herring (*Clupea pallasi*), disease, viral hemorrhagic septicemia virus, Icthyophonus, vitellogenin, cortisol, reproductive function.

Project Data: *Description of data* – Length, weight of spawning female herring, concentrations of serum cortisol and vitellogenin, concentrations of free amino acids in ovulated eggs. *Format* – MS Excel and Word documents, .pdf and .jpg formats. *Custodian* – All herring data are available upon request via the Alaska SeaLife Center website: www.alaskasealife.org. The custodian of these data is Howard Ferren, Alaska SeaLife Center, 301 Railway Avenue, P.O. Box 1329, Seward, AK 99664, howard_ferren@alaskasealife.org. *Limitations* – None.

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EXECUTIVE SUMMARY

Previous studies indicate that the *Exxon Valdez* oil spill impacted recruitment of the 1989 year class, but its effects on the longer term population is unclear (Carls et al. 1998, 2001). Other stressors including a large population size, poor over-winter rearing conditions and disease within the herring population from viral hemorrhagic septicemia virus (VHSV) and Icthyophonus (ICTH) also appear to have been important. The prevalence of pathogens such as VHSV and ICTH can have a direct effect on population abundance through increased mortality in pre- and post-spawning adult herring. However, the diseases that result from these pathogens are also reflective of stress within the population, which can potentially impair reproductive function and further impact recruitment. When stressed, levels of glucocorticoids such as cortisol increase to mediate various physiological mechanisms that regulate growth, immune system function and reproduction.

Vitellogenin (Vg) is a large, calcium binding protein that is synthesized in the liver and transported in the blood stream to the developing oocytes in the ovary. Research has shown that stress interferes with the production of Vg in brook trout (Roy et al. 1990), rainbow trout (Lethomonier et al. 2000), Atlantic salmon (King et al. 2003) and Arctic char (Berg et al. 2004). Such effects may be manifest through reduced fecundity or egg size, changes in the chemical composition of eggs, delayed ovulation and lower survival rates of progeny (Campbell et al. 1992, Contreras-Sanchez et al. 1998).

We hypothesized that cortisol levels in infected fish would be higher than in non-infected fish, which would reduce serum Vg and potentially lead to changes in (a) blood calcium, through the action of Vg as calcium binding protein, (b) the amino acid composition of the eggs, or (c) both.

To test this hypothesis spawning female Pacific herring (*Clupea pallasi*) caught in Prince William Sound (PWS) were examined to determine if pathogens such as viral hemorrhagic septicemia virus (VHSV) and Icthyophonus (ICTH) were associated with physiological stress and altered reproductive function. Serum levels of cortisol, vitellogenin, total calcium were measured along with free amino acids in ovulated eggs. A total of n=19 (59.1%) of 32 female herring caught in early April tested positive for ICTH.

Cortisol levels in infected fish were significantly higher than in non-infected fish, but there were no significant differences in either vitellogenin (Vg) or total calcium. However, there was a significant, positive correlation between cortisol and vitellogenin levels within ICTH infected fish. Among free amino acids measured in ovulated eggs, valine was significantly higher in infected fish, whereas glycine was higher in non-infected fish.

Higher levels of cortisol found in infected fish support suggestions that PWS herring are subject to environmental stress, but the effect on female reproduction is unclear. Proposed stock supplementation projects for PWS herring may benefit from improved understanding of factors that affect reproductive function.

INTRODUCTION

Pacific herring abundance in Prince William Sound increased in synchrony with other Alaskan herring stocks beginning in the late 1970s before peaking at more than 100,000 tons annually between 1989 and 1993 (Gray et al. 2002). Although biomass estimates differ, the herring population declined significantly thereafter and is presently at levels similar to those observed in the 1970s. Previous studies indicate that the *Exxon Valdez* oil spill impacted recruitment of the 1989 year class, but its effects on the longer term population is unclear (Carls et al. 1998, 2001). Rather, other stressors including a large population size, poor over-winter rearing conditions and disease within the herring population from viral hemorrhagic septicemia virus (VHSV) and Icthyophonus (ICTH) also appear to have been important. These pathogens have reportedly caused massive and recurring mortality in herring (Marty et al. 2004) and, combined with marked variation in local food availability (Foy and Norcross 1999, Norcross et al. 2001) and predation (Stokesbury et al. 2002), may be acting as major pressures contributing to persistent low population abundance.

The prevalence of pathogens such as VHSV and ICTH can have a direct effect on population abundance through increased mortality in pre- and post-spawning adult herring. However, the diseases that result from these pathogens are also reflective of stress within the population, which can potentially impair reproductive function and further impact recruitment. When stressed, levels of glucocorticoids such as cortisol increase to mediate various physiological mechanisms that regulate growth, immune system function and reproduction. Reproduction can be adversely affected by both acute and chronic stress because increased demand for cortisol synthesis diverts energy to actions that are needed to cope with the stressor(s) and away from reproductive functions such as gamete formation.

Gamete formation in female teleost fish occurs through the accumulation of vitellogenin (Vg) and its structural proteins, lipovitellin and phosvitin (Hiramatsu et al. 2002, Patino and Sullivan 2002). Vitellogenin is a large, calcium binding protein that is synthesized in the liver and transported in the blood stream to the developing oocytes in the ovary. The derived yolk proteins supply amino acids and lipids to the developing embryo (lipovitellin – Lv) and provide calcium binding sites for skeletal development (phosvitin – Pv). Synthesis of Vg is initiated by rising17-β-estrodiol levels in the blood in response to environmental and endogenous cues that act on the hypothalamus-pituitary-gondal axis (Patino and Sullivan 2002). In Pacific herring, vitellogenesis begins from late summer to early fall, rises to a peak in March prior to spawning, then declines rapidly through April and May (Koya et al. 2003). The rise in serum Vg coincides with increasing concentrations of estrodiol-17β (E2), although levels of this hormone peak 2 months in advance of Vg.

Research has shown that stress interferes with the production of Vg in brook trout (Roy et al. 1990), rainbow trout (Lethomonier et al. 2000), Atlantic salmon (King et al. 2003) and Arctic char (Berg et al. 2004), and that these effects can occur at both transcriptional and post-transcriptional levels. For example, Lethomonier et al. (2000) reported that cortisol implants caused marked decrease in both estrogen (ER) receptor and Vg mRNA levels in rainbow trout liver, whereas *in vitro* studies showed that glucocorticoid receptors (GR) inhibit E2 stimulated transcription of the ER promoter, suggesting that the adverse affects of stress or cortisol on

vitellogenesis may arise from transcriptional interference of GR on ER mRNA activity. In contrast, Berg et al. (2004) found that injections of Arctic char with high levels of cortisol increased plasma Vg, but that in combination with E2 reduced circulating Vg while having no effect on Vg mRNA level. These results suggested that cortisol acts on Vg on the post-transcriptional level in Arctic char. Ultimately such effects may be manifest through reduced fecundity or egg size, changes in the chemical composition of eggs, delayed ovulation and lower survival rates of progeny (Campbell et al. 1992, Contreras-Sanchez et al. 1998).

OBJECTIVE

We suggested that Pacific herring in PWS could be similarly affected by environmental stress, and that such stress would be reflected by the expression of VHSV or ICTH at the time of spawning. More specifically, our objective was to:

> Determine if circulating levels of cortisol and Vg in adult female herring that tested positive for VHSV or ICTH differed from fish that did not express these pathogens.

We hypothesized that cortisol levels in infected fish would be higher than in non-infected fish, which would reduce serum Vg and potentially lead to changes in (a) blood calcium, through the action of Vg as calcium binding protein, (b) the amino acid composition of the eggs, or (c) both.

METHODS

Animals: Female Pacific herring (n=32) were caught individually by hook and line at the time of spawning on April 8, 2008 near Port Gravina in eastern PWS. After capture, each fish was checked for ripeness by applying pressure to the abdomen. Fish that expressed eggs freely were considered mature, killed immediately by blunt trauma and measured for total length and weight. We did not attempt to stage maturation by any other observational method (Hay 1985). Approximately 0.5-1.0 ml of blood was then drawn from a caudal vessel using a non-heparinized syringe. The ovaries were dissected, weighed and bagged, and with the blood and fish transported in a cooler to the Alaska SeaLife Center (ASLC). Serum was separated by centrifuging at 4000 rpm for 10 min at room temperature. Serum was stored at -80°C and ovaries at -20°C until analyzed. Samples of the kidney, liver, spleen, heart and blood smears were collected from each fish and mailed to the Alaska Department of Fish & Game Pathology Lab (Anchorage, AK) to determine the incidence of VHSV, VENV and ICTH.

Measurement of cortisol and vitellogenin: Cortisol and Vg levels were determined by enzyme-linked immunosorbent assay (ELISA). The procedure for the cortisol assay was provided by the manufacturer (American Research Products, Belmont MA). Briefly, 20 µL from each of seven standards ranging from 0-2208 nmol/L and samples was loaded in duplicate onto a 96 well plate coated with anti-cortisol monoclonal antibody. The standards and samples were then mixed with 200 µL of anti-cortisol antiserum conjugated to horseradish peroxidase and incubated at room temperature for 60 min. After incubation the wells were rinsed three times with the manufacturers wash solution and the plate patted on absorbent paper to remove residual droplets. Each well was filled with TMB (tetramethylbenzidine) substrate solution and incubated for 15 min. The reaction was stopped using 0.5 M H_2SO_4 and the optical density (OD) read after 5 min

with a microplate reader (Molecular Devices) at 450 nm. The serum concentration of cortisol was determined by fitting the mean values for each sample to the standard curve of the known cortisol dilutions.

Serum levels of herring Vg were determined using antibodies, purified standards and protocol generously provided by T. Matsubara (Japan Fisheries Agency, Hokkaido National Fisheries Research Institute - HNFRI). Ninety-six well plates (Nalge Nunc Int., Rochester NY) were prepared by coating with primary antibody (purified rabbit immunoglobulin anti- Pacific herring Vg) diluted 1:400 in sodium bicarbonate buffer (0.01 M Na_2CO_3, 0.04 M $NaHCO_3$, pH 9.6) and incubating over night at 4.0°C. The wells were then washed with phosphate buffered saline solution (PBS, 0.05% Tween 20) and blocked for 1 hr by adding 250 µL per well of 1.0% bovine serum albumin (BSA) and 5.0% skimmed milk in sodium bicarbonate buffer (SBB). After washing, 100 µL of the standards (purified Pacific herring Vg diluted 1:50-25,600) and samples (diluted 1:1000) in 0.5% BSA-PBS were added in triplicate to the wells and incubated at room temperature for 90 min. The wells were washed again with PBS then loaded and incubated 90 min with 100 µL of the second antibody (purified rabbit immunoglobulin $F(ab')_2$ biotinylated anti- Pacific herring Vg) diluted at 1:600 in 0.5% BSA-PBS. Following incubation the wells were washed with PBS solution then loaded with 100 µL of Streptavidin / horseradish peroxidase (Dako NA, Carpinteria CA) in 1.0% BSA-PBS wash for 60 min. After final washing the reactions were developed for 10 min with 100 µL per well of TMB, stopped with 1.0 M H_2SO_4 and read at 450 nm. The mean OD values for each sample were fit to the standard curve of purified Vg to calculate the serum concentrations of Vg in the samples.

Total blood Ca^{2+} was determined by colorimetry following the manufacturer's procedures (Stanio Laboratory, Boerne TX).

Quantification of the free amino acid concentrations in the eggs was performed using high performance liquid chromatography (HPLC) modified from Vasanits et al. (2000). The HPLC system consisted of a dual solvent module (Beckman Coulter System Gold 125) and fluorescence detector (Jasco FP 2020 Plus). A Beckman Coulter Ultrasphere C18 column (5 µ, 4.5 x 150 mm) was used for separations. Approximately 1.0-1.5 gm of eggs from each female was homogenized in 0.5 ml of 0.1 M HCl, digested for 3 hr than centrifuged at 4,000 rpm for 10 min. The supernatant was stored at 4.0°C until analyzed. Samples and standards were derivatized with *o*-phthaldialdehyde-*N*-acetyl-L-cysteine (OPA-NAC) prepared at least 24 hours in advance and allowed to react for at least 30 min prior to analysis. Nor-valine was used as the internal standard for all samples. Standards (10 µmol) were made for 16 amino acids (all L-isomers): aspartic and glutamic acid, histidine, serine, glycine, threonine, arginine, alanine, tyrosine, methionine, valine, cysteine, tryptophan, phenylalanine, leucine and lysine to identify sample peaks. The mobile phase consisted of two eluents: (A) 0.05 M sodium acetate, 1.0% tetrahydofuran, pH 6.0 and (B) 46% 0.1 M sodium acetate, 44% acetonitrile, 10% methanol, pH 7.2. The eluent program was 100% A (5 min), a linear increase to 75% B (5-20 min), then 100% A (20-25 min). The flow rate was 2.0 ml min^{-1} and all analyses were conducted at room temperature.

Statistical analyses: The effects due to the presence of pathogens (infected, non-infected) on serum vitellogenin, cortisol and calcium levels, as well as the amino acid composition of the

eggs was tested by analysis of variance (ANOVA). Correlations between these factors were tested by linear regression. Significance levels for all tests were 5% (Sokal and Rohlf 1981).

RESULTS

The physical and biochemical characteristics for both infected and non-infected fish are shown in Table 1. All values are reported as means ± S.E.M. Of the 32 female herring that were caught and sampled, a total of 19 (59.1%) tested positive for ICTH (Table 1). None of the fish we sampled tested positive for either VHSV or VENV. There were no significant differences in the length, weight, ovary weight or GSI between infected and non-infected fish ($P \geq 0.09$).

Serum cortisol levels were significantly higher ($P = 0.04$) in infected fish than in non-infected fish. In contrast, there was no significant difference in either vitellogenin or total calcium between the two groups ($P \geq 0.13$). However, there was a significant positive association between the circulating cortisol and vitellogenin levels ($r = 0.63$, $P = 0.004$) in the infected fish combined, but not among the non-infected fish ($r = 0.08$, $P = 0.79$). These relationships are shown in Figure 1.

Among the 16 free amino acids that we could identify by HPLC, only glycine ($P = 0.03$) and valine ($P = 0.04$) differed significantly between infected and non-infected fish. Glycine was higher in non-infected (570.1 ± 36.9 nmol • gm^{-1}) than in infected fish (374.2 ± 24.3 nmol • gm^{-1}), whereas valine was higher (522.0 ± 26.1 nmol • gm^{-1}) among infected than non-infected females (443.9 ± 25.7 nmol • gm^{-1}).

		ICTH +	ICTH -	P
Animals				
	N	19	13	
	Length (mm)	229.9 (5.7)	241.4 (6.6)	0.19
	Weight (gm)	153.5 (11.2)	187.2 (16.1)	0.09
	Ovary weight (gm)	42.9 (4.4)	33.4 (3.4)	0.10
	GSI (%)	14.1 (1.2)	17.3 (1.5)	0.11
Serum				
	Cortisol (ng • ml-1)	231.7 (27.9)	159.1 (20.5)	0.04
	Vitellogenin (μgm • ml-1)	66.3 (14.6)	37.9 (8.3)	0.13
	Calcium (mMol)	10.2 (0.3)	10.4 (0.5)	0.80
Egg Amino Acids*				
	Alanine	1146.2 (68.7)	1174.5 (57.2)	0.77
	Arginine	464.7 (26.6)	435.9 (47.9)	0.57
	Aspartic acid	65.1 (14.7)	61.2 (9.9)	0.83
	Cysteine	93.7 (11.5)	108.2 (14.7)	0.79
	Glutamic acid	141.8 (18.5)	138.0 (15.3)	0.87
	Glycine	374.2 (24.3)	570.1 (36.9)	0.03
	Histidine	385.4 (13.7)	408.8 (16.6)	0.29
	Leucine	864.6 (56.7)	759.5 (57.7)	0.21
	Lysine	477.4 (30.8)	503.9 (46.2)	0.62
	Methionine	894.2 (53.0)	873.8 (42.9)	0.77
	Phenylalanine	315.8 (16.3)	298.5 (20.7)	0.52
	Serine	625.1 (49.7)	752.5 (67.3)	0.14
	Threonine	412.2 (22.9)	362.7 (21.9)	0.13
	Tryptophan	273.7 (25.0)	300.3 (44.9)	0.58
	Tyrosine	465.7 (17.2)	448.0 (30.4)	0.59
	Valine	522.0 (26.1)	443.9 (25.7)	0.04

* (nmol • gm-1)

Table 1. Physical characteristics, blood chemistry and egg amino acid composition for ICTH infected and non-infected PWS herring.

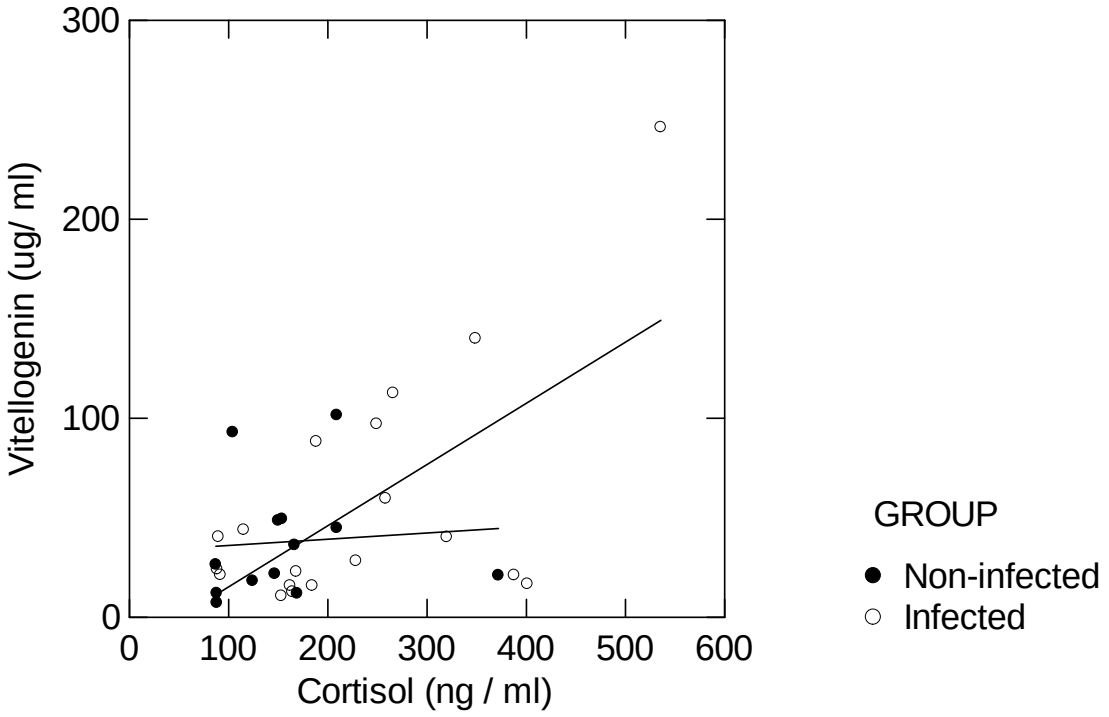

Figure 1. The relation between serum cortisol and vitellogenin for ICTH infected (P = 0.004) and non-infected PWS herring (P = 0.79).

DISCUSSION

Stress can exert profound effects on physiological processes in fish that are mediated through behavioral, nervous system and endocrine responses. Endocrine responses in particular have widespread actions that influence a variety of functions including metabolism, growth, disease resistance and reproduction. In the wild, fish routinely encounter and cope with stress in a manner that effectively minimizes adverse effects to their health and survival. However, under conditions of highly elevated or chronic stress, the energetic cost of the response can result in reduced growth, disease, impaired reproduction and mortality. For PWS Pacific herring, the presence of pathologies associated with VHSV, VENV and ICTH suggests that environmental stressors may be acting to limit population recovery.

We proposed that in addition to the direct effects that disease may have herring mortality, stress may also limit recruitment early in the life cycle through its affects on reproduction. More specifically, stress hormones such as cortisol can inhibit the synthesis of the egg yolk precursor protein vitellogenin (Vg) by acting on either or both the hepatic estrogen receptor (ER) and its ligand 17-β-estrodiol (E2) that are up-regulated to initiate Vg synthesis (Berg et al. 2004, Lethimonier et al. 2000). The potential effects on reproductive performance can include delayed ovulation, reduced fecundity, smaller egg size and lower hatching success (Contreras-Sanchez et al. 1998, Campbell et al. 1992). Since the presence of disease causing pathogens in PWS herring can be indicative of environmental stress (Carls et al. 1998), we wanted to determine if reproductive function in these fish might also be impaired or altered compared to non-infected fish.

Our data indicate that infected fish had significantly higher levels of serum cortisol than non-infected fish, but there was no significant difference in Vg levels between infected and non-infected fish. Elevated cortisol in the infected fish is consistent with stress induced immune system impairment (Pickering and Pottinger 1989). Induced stress (i.e., capture, transport, confinement) in captive herring can produce rapid and pronounced disease related mortality in Pacific herring (Hershberger et al. 2006), so significantly increased cortisol levels among infected herring in the wild might also be expected.

In contrast, experimental evidence suggests that elevated cortisol can interfere with the synthesis of Vg (Berg et al. 2004, Lethimonier et al. 2000), which we did not observe. Rather, within infected fish there was a significant positive relationship between circulating cortisol and Vg. Synthesis of Vg in Pacific herring increases gradually over 6-7 months in response to E2-ER mediated stimulation and before peaking approximately one month prior to spawning, but then falls rapidly to pre-stimulation levels. Since our opportunity to collect samples was limited to a single day, we could not characterize temporal changes that occurred before and after the time of spawning. Hence, our samples likely reflect a collection of fish in which Vg had either peaked or declined from pre-spawning levels. High levels of cortisol (> 350 ng • ml^{-1}) associated with very low levels of Vg (< 50) support this suggestion (Figure 1). Moreover, high levels of cortisol (~2500 ng • ml^{-1}) stimulate Vg production in Arctic char in the absence of E2 (Berg et al. 2004). In herring, E2 levels decline rapidly at about the same time that Vg synthesis peaks (Koya et al. 2003), which suggests that spawning herring in PWS probably have very low levels of E2 as well (although we did not measure this hormone), such that added stress among infected fish may act to increase or maintain Vg above seasonally determined levels.

We should also note that the highest levels of Vg that we detected in PWS herring (~ 200 μg • ml^{-1}) approximate the baseline levels for pre- and post-spawning fish reported by Koya et al. (2003) for captive herring. The timing of our sampling (spawning) likely contributed to the low levels we observed since Vg synthesis declines rapidly during the spawning season. The lack of any correlation between plasma Vg and calcium also suggests that most of the fish we sampled were either late or post-vitellogenic fish since these two factors have been shown to be highly correlated in pre- and vitellogenic fish (Linares-Casenave et al. 2003). Secondly, the reactivity of Vg anti-sera was reportedly low (personal communication, T. Matsubara), which was confirmed by comparison of purified Vg standards between our laboratory and the HNFRI (~50% lower). Hence, the absolute levels of Vg in spawning PWS herring may be closer to double those reported here.

Quantitatively, the most abundant amino acids in the eggs of PWS herring were alanine, methionine, leucine, serine, which represented ~ 51% of the total free amino acid pool in both groups of fish. The concentrations of these amino acids as well as their proportion of the total pool are in general agreement with those reported for Baltic herring *Clupea harengus* (Kristoffersen and Fin 2008). Free amino acids in eggs of marine fish provide substrate for metabolism and protein synthesis in the developing embryo, and are believed to have an important role as osmolytes during oocyte maturation and hydration (Finn et al. 2002, Hiramatsu et al. 2002, Thorsen and Fyhn 1996, Fyhn and Serigstad 1987, Craik and Harvey 1987). As eggs mature, proteolysis of Vg components (Lv and Pv) gives rise to increasing amounts of free

amino acids, which are then metabolized during embryonic development. In herring, the contribution of the free amino acid pool to oocyte hydration is minor compared to inorganic ions (Kristoffersen and Finn, 2008), which is typical of marine species that spawn benthic eggs compared to those that spawn pelagic eggs (Finn et al. 2002).

With the exceptions of glycine ($P = 0.03$) and valine ($P = 0.04$), the free amino acid levels did not differ significantly between infected and non-infected fish. Moreover, the differences we did observe were not directional. Glycine was higher in infected (570.1 ± 36.9 nmol • gm^{-1}) than in non-infected ($374. \pm 24.3$ nmol • gm^{-1}) fish, whereas valine was lower in infected (522.0 ± 26.1 nmol • gm^{-1}) compared to non-infected fish (443.9 ± 25.7 nmol • gm^{-1}). Whether these specific amino acid differences have any functional effect on oocyte hydration or subsequent embryonic development is not clear. Both are neutral amino acids and therefore would not affect the electroneutrality of the developing oocyte that is evidently maintained during hydration (Kristoffersen and Finn 2008). They are also both minor components with respect to the total free amino acid pool (<15% combined in both groups of fish) and thus the observed differences would not likely have a material effect on the free amino acid contribution to oocyte osmolality.

The absence of any major differences in free amino acid content between the infected and non-infected fish is not surprising in light of similar levels of Vg in the two groups. Although a more detailed analysis of yolk proteins derived from Vg (e.g. Lv(A), Pv or β'-component) and temporal changes in these proteolytic products may have revealed differences related to the stage of maturation (i.e., degradation of these specific proteins has been shown to be or suggested as specific sources of free amino acids during oocyte maturation (Finn et al. 2002, Hiramatsu et al. 2002, Matsubara et al. 1999)), such an analysis was beyond the scope of this project.

CONCLUSION

Disease has been identified as important pressures limiting recovery of PWS herring. We suggested that if disease or the prevalence of pathogens was indicative environmental stress(ors), than such stress could also impair reproductive function and further impact population recruitment. Our results indicate that PWS herring infected with ICTH exhibit elevated serum cortisol levels consistent with increased stress. However, rather than having an attenuating effect on circulating Vg, Vg increased in direct relation to cortisol among infected fish. This may reflect, in part, sampling timing since circulating Vg levels in herring has seasonal periodicity that peaks shortly before spawning and declines rapidly afterward. There were also few significant differences in the pool of free amino acids between infected and non-infected fish.

Expanding the sampling period (early fall – late spring) and frequency (monthly) to better characterize seasonal changes in cortisol, Vg products and ovarian development would improve understanding of the role of stress on reproductive function in PWS herring. Such information may also aid in refining techniques for artificial propagation of early life history stages (e.g. develop brood stock screening criteria to characterize gametes that lead to high fertilization and hatching success).

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ADDENDUM

The scope of services funded as Project 080821 included the objective to:

Plan and coordinate travel for Dr. Takahiro Matsubara and an associate or designee. Travel was to include:

- Traveling to Alaska and attending the Marine Science Symposium in January 2008.
- Participation in an *EVOS* Trustee Council sponsored workshop on herring stock restoration.
- Visiting the fish culture facilities at ASLC, the Seward Shellfish Hatchery and U.S. Geological Survey (USGS) Marrowstone Field Laboratory (Nordland, Washington).
- Touring potential stock rearing and release sites in Prince William Sound (PWS) (e.g., Tatitlek).
- Meeting with scientists and interested parties involved in the Prince William Sound herring restoration effort.

Background

Dr. Matsubara is an internationally recognized expert in the field of fish endocrinology with a specific focus in the area of reproduction. He has authored over 50 peer reviewed papers and has collaborated on multiple occasions with fish physiology researchers in the U.S. As the Section Chief for the Resources Enhancement Section of the Hokkaido National Fisheries Research Institute, he directs all aspects of fish culture investigations at the Akkeshi Field Station, including those for Pacific herring.

In partial fulfillment of Project 070721, Dr. Tim Linley traveled to Japan to meet with Dr. Matsubara, inspect the Akkeshi Field Station herring culture facility, and to learn about herring culture techniques practiced in Japan. Contact, travel and collaboration with Japanese herring culture researchers were facilitated by Dr. Shinji Harakawa of Hakuju Institute for Health Science Co., Ltd. (scientific collaborator of MariCal, Inc.) and Mr. Naoki Tojo of Hokkaido University. Dr. Harakawa and Mr. Tojo provided initial contact information with the principal investigators involved in herring culture research and coordinated obtaining approval to meet the researchers and view their operations. New regulations adopted by the Fishery Research Agency of Japan (FRA), which recently took control of the previously independent Fishery Research Institutes, required that all information exchange on the part of institute scientists be cleared through FRA. After nearly two months of effort, written approval was provided by Dr. Koji Nakamura, International Coordinator for the Incorporated Administrative Agency at the FRA. Dr. Linley was then able to travel to Japan and meet personally with herring researchers and observe stock supplementation efforts as part of this official visit. Despite the complications associated with obtaining approval for the visit, and the timing of the visit coinciding with several national holidays, a total of four days of meetings, facility tours and presentations occurred in late April 2007. This included time at both the Hokkaido National Fishery Research Institute (HNFRI) in Kushiro, as well as the Akkeshi Field Station. The herring investigators with whom Dr. Linley interacted included Dr. Takahiro Matusbara, Section Chief Resources Enhancement Section, Coastal Fisheries and Aquaculture Division, HNFRI; Dr. Masaaki Fukuda, Director of the Coastal Fisheries and Aquaculture Division, HNFRI; Dr. Nobuyuki

Ohkubo of the HNFRI; Mr. Murakami Naoto and Mr. Fukunaga Kyohei of the Akkeshi Research Station and Dr. Ryuzo Yanagimachi of the University of Hawaii.

For Objective 1 of Project 080821, we proposed that Dr. Matsubara and an associate journey to the U.S. to present at and EVOSTC-sponsored herring restoration workshop to be held in conjunction with the Marine Science Symposium in January 2008. The goal of the proposed workshop was to provide a forum for the exchange of information related to past, present and future efforts to restore herring stocks in Japan and PWS. Dr. Matsubara and his colleague(s) were to describe the research, development and implementation of herring culture techniques for stock supplementation that has taken place in Japan during the last 20 years. This was to include presentation on the technical problems encountered during the early phases of the program, the economic bottlenecks in scaling up production from the laboratory to large-scale releases for stock supplementation, and post-release evaluation to determine thee contribution to recruitment.

In conjunction with this travel, we planned for Dr. Matsubara and his colleague(s) to travel to Seward to tour the ASLC and Seward Shellfish Hatchery where they could view and assess those facilities as to their potential use for conducting culture related research for herring. The visit would include a review of the ASLC – MariCal herring culture program progress to date and discussions to guide future research efforts and topics. Travel for Dr. Matsubara and his colleague(s) would further include visiting a potential field site for a pilot-scale herring rearing and release project at Tatitlik. The purpose of this visit would be to broaden the interaction between scientists involved in herring stock restoration and PWS community stakeholders. Such interaction would help provide a foundation for community participation in the recovery efforts in PWS, much as they have in Japan where community based enhancement projects play a major role in supplementing wild herring stock production.

Finally, we proposed to coordinate with Dr. Paul Hershberger of the USGS for Dr. Matusbara and his colleague(s) to visit the Marrowstone Field Station in Nordland, WA before or after travel to Alaska. The endocrine studies proposed as part of Objective 2 (Project 080821) are directly linked to environmental stress and disease expression in PWS herring (e.g., VHS) and this meeting would be used to develop hypotheses regarding the role of disease on reproductive function in herring, and the potential implications for stock restoration in PWS. It would also give the Japanese the opportunity to view and assess the culture techniques employed at a major herring research facility in the U.S.

With the approval of funding to support this travel, Dr. Takahiro Matsubara and selected colleague Mr. Hiroyuki Okouchi of the Fisheries Research Agency of Japan agreed to travel to the U.S. in May 2008 to provide details about herring culture operations in Japan. The travel itinerary prioritized the first stop in Seattle where Dr. Linley, flying in from Maine, met with Dr. Matsubara. Joining the Japanese scientists and Dr. Linley were *EVOS* Trustee Council staff including Executive Director Michael Baffrey, Science Director Catherine Boerner and Dr. Doug Hay. Tours and meetings were held at the Marrowstone Field Research Station with Dr. Paul Hershberger, Jake Gregg and other Station personnel. A second tour and meeting was held at the USGS Western Fisheries Center where Dr. Matsubara and Mr. Okouchi presented on their work related to herring stock enhancement and reproductive endocrinology in marine fish. Additionally, the participants traveled to Alaska where Dr. Matsubara and Mr. Okouchi gave

seminars on herring culture at the Alaska SeaLife Center. *EVOS* Trustee Council Science Director, Drs. Linley, Matsubara and Mr. Okouchi also flew to Tatitlek to tour and assess the Tatitlek Shellfish Hatchery as a possible facility to conduct herring culture in Prince William Sound.

Following the Seattle-Alaska tour and presentations, Dr. Matsubara and Mr. Okouchi returned to Japan. Communication and collaboration continued over the following months with Dr. Matsubara who provided assay materials for Dr. Linley to conduct the herring yolk assessments described under Project 080821 Objective 1, to which this Final Report is largely dedicated. In addition, Dr. Matsubara was willing to provide Dr. Linley the documented 20 year history of herring culture investigations in Japan. The document, *Herring Fry Production Techniques*, was translated from Japanese to English and submitted to the EVOS Trustee Council as an objective of the Supplemental Grant to Project 080821.