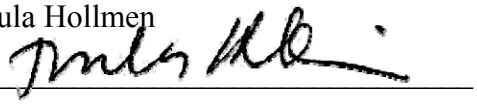
PROPOSAL SIGNATURE FORM

THIS FORM MUST BE SIGNED BY THE PROPOSED PRINCIPAL INVESTIGATOR AND SUBMITTED ALONG WITH THE PROPOSAL. If the proposal has more than one investigator, this form must be signed by at least one of the investigators, and that investigator will ensure that Trustee Council requirements are followed. Proposals will not be reviewed until this signed form is received by the Trustee Council Office.

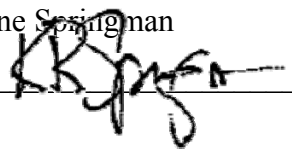
By submission of this proposal, I agree to abide by the Trustee Council's data policy (*Trustee Council Data Policy**, adopted July 9, 2002) and reporting requirements (*Procedures for the Preparation and Distribution of Reports***, adopted July 9, 2002).

PROJECT TITLE: Evaluating injury to harlequin ducks (*Histrionicus histrionicus*) caused by sublethal hydrocarbon exposure in Prince William Sound using species-specific cell lines

Printed Name of PI: Tuula Hollmen

Signature of PI:  Date: Sep 3, 2008

Printed Name of co-PI: Kathrine Springman

Signature of co-PI:  Date: Sep 3, 2008

* www.evostc.state.ak.us/Policies/data.htm

** www.evostc.state.ak.us/Policies/Downloadables/reportguidelines.pdf

Trustee Council Use Only Project No._____ Date Received: _____

FY07 INVITATION
PROPOSAL SUMMARY PAGE

(to be filled in by proposer)

Project Title: Evaluating injury to harlequin ducks (Histrionicus histrionicus) caused by sublethal hydrocarbon exposure in Prince William Sound using species-specific cell lines

Project Period: October 1, 2008 – September 30, 2009 (FY09)

Proposer(s):

Tuula Hollmen, DVM, PhD, Research Associate Professor, Alaska SeaLife Center and University of Alaska Fairbanks, 301 Railway Ave, PO Box 1329, Seward AK 99664-1329, tuula_hollmen@alaskasealife.org, (907) 224-6323.

Kathrine Springman, PhD, P.O. Box 315, Littleriver, CA 95456; krspringman@gmail.com; (707) 937-6212

Study Location: Prince William Sound, Alaska SeaLife Center (Seward)

Abstract:

Evaluation of harlequin duck (*Histrionicus histrionicus*) population trends, survival measures, and biomarker indicators of exposure suggests that the species is recovering, but has not fully recovered from the effects of the 1989 *Exxon Valdez* oil spill (EVOS) in the Prince William Sound (PWS). In areas oiled by the EVOS, elevated cytochrome P4501A biomarker induction has been observed in harlequin ducks as recently as March 2007, providing evidence of continued exposure. The magnitude of injury and its implications for populations of harlequin ducks caused by chronic oil exposure and long-term induction of central enzymatic processes is unknown. This study applies a panel of *in vitro* harlequin duck and surrogate cell line bioassays for a species-specific toxicological assessment of site-specific hydrocarbons from PWS. A combination of bioassays that measure direct effects on cell viability and DNA damage provide a new method to assess and quantify injury. Also, a battery of laboratory bioassays provides a method to link P4501A biomarker induction with other measures of cellular injury, and a comprehensive assessment of potential short- and long-term toxicity.

Funding:

EVOS Funding Requested: FY09 $ 229.6

TOTAL: $ 229.6 (includes 9%GA)

Non-EVOS Funds to be used: $ 0

TOTAL: $ 229.6

Date: September 3, 2008

PROJECT PLAN

NEED FOR PROJECT

Statement of Problem

Evaluation of harlequin duck (*Histrionicus histrionicus*) population trends, survival measures, and biomarker indicators of exposure through 2005 suggests that the species is recovering, but has not fully recovered from the effects of the 1989 *Exxon Valdez* oil spill (EVOS) in the Prince William Sound (PWS) (Esler et al. 2002; *Exxon Valdez* Oil Spill Trustee Council 2006). Evidence that this species has not fully recovered includes findings of proportionately lower numbers of females than males in oiled areas and higher levels of cytochrome P450 biomarker in tissues of ducks captured at oiled areas compared to unoiled areas (Trust et al. 2000; Rosenberg et al. 2005; *Exxon Valdez* Oil Spill Trustee Council 2006). In oiled areas, elevated cytochrome P450 biomarker induction has been observed in harlequin ducks as recently as November 2006 and March 2007, providing evidence of continued exposure (D. Esler, pers. comm.).

Lingering oil from the 1989 *Exxon Valdez* oil spill has proven resistant to degradation, is widely accessible to wildlife throughout western PWS, and has been found in areas used by harlequin ducks (*Exxon Valdez* Oil Spill Trustee Council 2006). Lingering oil may lead to population-level impacts particularly in species with near shore feeding habits and a high degree of site fidelity, such as harlequin ducks. Furthermore, oil stranded on PWS beaches has the potential to affect harlequin ducks for many years. In 2004, a small patch of semi-liquid fuel oil released from fuel tanks in the 1964 earthquake was encountered in the course of field studies in PWS; this fuel oil has retained the ability to trigger a strong biomarker response in exposed organisms even after four decades, providing evidence for potential of long-term effects of lingering oil on biota.

The magnitude of injury and its implications for populations of harlequin ducks caused by chronic, sublethal oil exposure and long-term induction of central enzymatic processes is unknown. Assessment of continuing exposure should link site-specific bioavailable hydrocarbons to the physiological or morphological injury they can cause. Cell lines developed from species of concern (e.g., harlequin ducks) offer a novel method to test for species-specific effects under laboratory conditions. These methods provide a tool to assess injury and physiological effects at the cellular level, and can be linked to previously measured biomarkers of P450 enzyme induction. At a later date, laboratory models may be expanded to assess oil injury at different life stages of harlequin ducks by development of *in ovo* assays, or immunological effects by the development of immune function bioassays. These methods provide tools to quantify injury, project future risks, and develop long-term remediation strategies.

Research that addresses these needs in cellular bioassays is possible due to recent advances in methods and technologies. Primary sea duck cell lines have been developed by Dr. Tuula Hollmen, and surrogate mallard (*Anas platyrhynchos*) cell lines have been used for laboratory bioassays. Existing cell lines and further development of harlequin duck and surrogate cell lines allow species-specific and high sample-volume testing of cellular damage following sublethal oil exposure at different hydrocarbon doses. This injury includes cell death, cytopathology, enzyme

induction, and DNA anomalies. Dr. Kathrine Springman has expanded the use of semipermeable membrane devices (SPMD) to include *in vivo* toxicity testing as well as analytical assessment of bioavailable nonpolar organics. The extracts of these samplers can be used for *in vitro* testing of injury from site-specific hydrocarbon exposure. Linking these methods correlates multiple endpoint assays to quantify injury resulting from exposure to the same field sample, and enables high sample volume, multiple dose testing for greater statistical strength and more reliable results.

Relevance to 1994 Restoration Plan Goals and Scientific Priorities

1. Development of cellular bioassays provides new and expanded strategies to evaluate and quantify injury due to hydrocarbon exposure. The method provides a novel tool to evaluate injury at the cellular level, and link injury at cellular level to P450 biomarker data.
2. Injury observed at the cellular level will be linked to site-specific hydrocarbon exposure of harlequin ducks. The method provides a tool to evaluate species-specific effects of site-specific contaminants.
3. This study will determine physiological effects that could have population-level implication. Validation of injury caused by hydrocarbon exposure in harlequin duck cell lines provides methods to link cellular injury to cytochrome P4501A (CYP1A) enzyme activity with the ethoxyresorufin-α-deethylase (EROD) bioassay. Data gathered from cellular-level bioassays facilitate linkage of previous studies with new approaches to injury assessment, i.e., integration of biological, chemical, and biochemical data for harlequin ducks
4. Dose-response relationships established in this study will provide a baseline for projections of anticipated injury, and establish foundations for further research on the effects of hydrocarbon remaining in PWS on reproductive success and population dynamics in harlequin ducks. Linking life stage specific effects on demographic parameters allows incorporation of laboratory results to population models.

PROJECT DESIGN

Objectives

Objective 1: *Develop harlequin duck and surrogate (mallard) cell lines to evaluate injury from site-specific hydrocarbons in harlequin ducks*

Objective 2: *Develop bioassays using harlequin duck and surrogate (mallard) cell lines to assess and quantify injury due to lingering oil in PWS*

Objective 3: *Evaluate injury due to site-specific lingering oil in PWS in harlequin ducks at the cellular level*

Objective 4: *Link analytical chemistry results from known oil-contaminated sites to injury assessments in harlequin ducks at the cellular level*

Objective 5: *Develop methods to link injury due to site-specific lingering oil in PWS in harlequin duck cell lines to harlequin duck population parameters and population level impact*

Procedural and Scientific Methods

Objective 1: Develop harlequin duck and surrogate (mallard) cell lines to evaluate injury from site-specific hydrocarbons in harlequin ducks

Primary cell lines will be developed from harlequin ducks and mallards, or domesticated breeds of mallards, and a reference duck embryo cell line will also be obtained from American Type Culture Collection (Rockville, MD, USA). Harlequin duck cell lines will provide a species-specific laboratory model, and mallard cell lines will provide a surrogate cell system. Surrogate cell lines will provide reference material for assay development, validation, and high volume testing. Preparation of cell lines involves isolation of primary cells, refinement of maintenance techniques, and characterization of isolated primary cell lines (growth rates, life span, morphology).

The source tissues for cell line development will be embryonic connective tissues (fibroblast cell lines) and liver (hepatocyte cell lines). In conjunction, additional organ tissues may be cryopreserved for potential future cell line development. The cells for primary cultures will be digested into suspensions using enzymatic treatment, and separated using centrifugation protocols (Docherty and Slota 1988, Brendler-Schwaab et al. 1994). For each stock processed, primary cultures will be initiated from fresh suspensions to determine cell morphology and viability. All remaining material will be cryopreserved as reserves for future testing. A control of each lot will be thawed after 48 hr and cultured to determine viability and recovery rate of the lot.

Cell culture media, reagents and subculturing techniques will be optimized for each cell line. Cell lines will be incubated at 37C in 5% CO_2 atmosphere, and monitored for status, health, and morphology using microscopy (described in Docherty and Slota 1988, Hollmen et al 2002). The primary cell lines will be subcultured at weekly intervals to determine the number of viable passages. For subculturing, cell layers will be dissociated into suspensions using proteolytic enzymes (such as trypsin) and chelating agents as necessary (such as EDTA). Cell morphology, yields, and growth rates will be evaluated to determine any passage-dependent effects and suitable passage number with consistent cell performance will be selected for further bioassay development. Cell counts will be performed using the hemocytometer method, and growth curves established for lag phase, log phase, and plateau phase. The passages selected for assay development will be further tested to control for any non-specific toxic effects from reagents and solvents. Cell lines will be subcultured into appropriate vessel platforms for bioassay development and high throughput testing.

Quality control protocols will follow standard laboratory operating procedures appropriate for cell culture methodology (Freshney 2000). Cell culture stocks and reagents will be assigned individual lot and tracking numbers, and aseptic techniques will be used for all cell culture protocols. Regular sterility checks will be performed to monitor for microbial contamination.

Objective 2: Develop bioassays using harlequin duck and surrogate (mallard) cell lines to assess and quantify injury due to lingering oil in PWS

Surrogate (mallard) cell lines will be used to validate appropriate molecular toxicology assays. The mixtures used for dosing will begin with those of known composition and progress to more relevant material of unknown toxicity. In order of dosing, these mixtures are:

A. Standard mixture of 16 priority pollutant polycyclic aromatic hydrocarbons (PP-PAH)
B. Standard mixture of chrysene and substituted chrysenes (CHRY)
C. Alaska North Slope Crude-intact (ANS-I)
D. Alaska North Slope Crude-laboratory weathered (ANS-W)

Semipermeable membrane devices (5) will be spiked with PP-PAH and processed as per Huckins et al. (2000). The same volume of the spiking material will be diluted in the solvent of choice as its QA/QC partner. This will be repeated with CHRY. For petroleum, 5 SPMDs will be constructed using 1 ml ANS-I for each in lieu of triolein. These will be processed as previously mentioned. This will be repeated for ANS-W. An aliquot of each extract will be sent to Auke Bay Laboratory for analysis, and the remainder processed as per Springman et al. (submitted).

Range finding studies will be performed to determine the median lethal concentration (LC_{50}) of each mixture and its partner for mallard and harlequin cell lines. Five different concentrations of each mixture and its partner below the LC_{50} will be used for each assay. These assays will include both positive/negative controls and solvent controls.

Assays will include:

A. Measures of cytotoxicity include evaluation of cytopathic effects, cell viability, and apoptosis. A test panel of bioassays will be optimized from a battery of assays (microscopy, dye exclusion and uptake, LDH enzyme release, caspase activity, and special stains to evaluate apoptosis) (Freshney 2000).
B. CYP1A activity as measured by the EROD assay in hepatocyte cultures, as per Kennedy et al. (1995).
C. A battery of assays to evaluate chromosome damage and genotoxicity will be conducted, from which the top or top two most promising assays will be selected (Poirier 1993; Fairbairn et al. 1995; Fenech 2000; Freshney 2000). This candidate list includes:
    1. Comet assay
    2. Micronuclei formation
    3. Sister chromatid exchange (SCE)
    4. PAH/DNA adduct formation

In fibroblast cell cultures some important enzymatic processes such as CYP1A will not be active, requiring the addition of a standardized external liver homogenate, or S9 fraction. Each assay using these cells will be performed for 5 doses with and without S9.

Objective 3: Evaluate injury due to site-specific lingering oil in PWS in harlequin ducks at the cellular level

Methods used in previous EVOS Trustee Council funded research (Restoration Project 060740) will be repeated to collect samples of bioavailable site-specific lingering EVOS oil in PWS. Selection of up to 10 SPMD deployment sites will be coordinated with previous studies using criteria based on harlequin duck distribution, evidence of induction of cytochrome P450 in tissues of harlequin ducks, and occurrence of oil in substrate. Site selection will be coordinated with D. Rosenberg (presence and density of harlequin ducks), D. Esler (evidence of P450 induction in harlequin ducks), and J. Rice and J. Short (presence of oil). Sites shared by chemical, biomarker, and ecological studies of PWS are central to this work, and will be prioritized in selection of field sites. In addition to sites selected based on these criteria, SPMDs will be deployed at one reference site and in McClure Bay at a site of semi-liquid 44 year old oil.

The SPMDs will be deployed as described in previous studies (Rice et al. 2006; Springman et al. (submitted)) and retrieved following a standard deployment period of 28 d. The samples will be processed at Environmental Sampling Technologies Laboratory, St. Joseph, MO, as per Huckins et al. (2000). The resulting extracts will be sent to Auke Bay Laboratory for polycyclic aromatic hydrocarbon (PAH) analysis as per Short et al. (1996). Next, the samples will be processed for *in vitro* toxicity testing as described in Springman et al. (submitted). These samples will arrive at ASLC for testing, and will be archived until ready for use.

Phase I (validation) of this study provides the procedural details necessary for the appropriate assays, and protocols for exposure times, solvent choice, and dosing details. The optimized battery of bioassays will be repeated with the SPMD extracts from the selected sites in PWS to evaluate injury due to site-specific contaminants at the cellular level. The results will be merged with the PAH analysis results.

The emphasis of this objective is to use the validated cellular endpoint bioassays to evaluate and quantify injury to site-specific hydrocarbons. Because multiple endpoint assays will be performed and correlated to quantify injury resulting from exposure to the same field sample, injury can be evaluated through a range of responses (cellular pathology, cell death, enzyme activity, and DNA damage). Furthermore, the battery of bioassays allows evaluation of relationships among response variables, particularly that of enzyme biomarker induction with cellular pathology. The laboratory protocols also enable high sample volume and multiple dose testing for greater statistical strength and more reliable results.

Objective 4: Link analytical chemistry results from known oil-contaminated sites to injury assessments in harlequin ducks at the cellular level

With SPMDs, the bioavailable fraction of nonpolar hydrocarbons can be used for toxicology testing, and these results integrated with the chemical composition of the sample used in these bioassays. This strategy has proven effective when used for *in vivo* testing (Short et al. (submitted) and with the *in vitro* EROD assay using fish hepatoma cells (PLHC-1; Parrott et al.

1999). We propose to test SPMD extracts from sites with remaining EVOS oil in a battery of bioassays in harlequin and mallard (surrogate) duck cell lines, and compare the damage caused by lingering oil at the cellular level with the analytical chemistry results from those sites.

Alaska North Slope crude oil released from *T/V Exxon Valdez* on March 29, 1989 was of uniform composition. Since then, the oil has interacted with the environmental characteristics of the sites where it is found today. Features relating to beach geomorphology and hydrology, sediment composition, and others factored in determining its bioavailability, toxicity, and to some extent the chemical nature of what remains at these sites, as described in Springman et al. (submitted) and Short et al. (submitted). These studies clarify that oiled sites differ as a result of the characteristics of each location. Here, we evaluate the consequences of those interactions by examining the chemical profiles from the SPMDs deployed at these sites as per Short et al. (1996). The sample extracts will be analyzed chemically for suites of 2- to 5-ring PAHs (including alkyl-substituted homologues), listed in Appendix 1. Briefly, the hexane aliquot for PAH analysis will be purified by column chromatography (1 g silanol) following addition of a suite of perdeuterated PAHs used as internal standards. Purified PAHs will be measured by gas chromatography/mass spectrometry (GCMS) operated in the selected ion monitoring mode, and will be quantified on the basis of a 5-point calibration curve of the most similar PAH standard available. To evaluate these results, concentrations of PAH in the SPMD will be normalized to total PAH (TPAH) concentration of that sample. Profiles of PAH composition, useful in distinguishing PAH sources, will be assembled.

The oil in the patches at the ANS sites has previously been confirmed as remnants from the 1989 *Exxon Valdez* oil spill (Short et al. 2004). In a study by Short et al. (submitted), the Monterey Formation source of the oil at one of the proposed sites (McClure Bay) was confirmed on the basis of tricyclic and tetracyclic terpane analysis as described by Kvenvolden et al. (1995).

Curve fitting will be used to mathematically describe the relationship between the contents of the SPMD and the results observed in the battery of cellular assays (an example from Short et al. (submitted) is included in Appendix 2). For curve fitting, results of SPMD chemical analysis and the results from one bioassay with the doses used will be exported to TableCurve 2D v5.01 (SYSTAT Software Inc.), software that facilitates the fitting of data to both linear and nonlinear equations. The chemical concentrations will be entered as the independent variable (x), and mean assay results from each assay as the dependent variable (y). Model selection criteria including the number of parameters and the goodness-of-fit (as determined by adjusted R^2 and F-statistic) will be used to rank the candidate equations for each data set. As there are multiple assays from the same sample, these analyses will be nested where appropriate. Multidimensional analysis (TableCurve 3D) may be used if shows promise for these applications.

The emphasis of this objective is to uncover the interactions that different components of these site-specific complex mixtures have with the results obtained from a battery of assays. As 44 PAH will be analyzed and 5 doses used in each of the bioassays, many different associations are possible. The results will be analyzed to determine salient relationships between a site-specific contaminant and an elicited response at the cellular level.

Objective 5: Develop methods to link injury due to site-specific lingering oil in PWS in harlequin duck cell lines to harlequin duck population parameters and population level impact

This project will provide a new method to evaluate injury and will generate a large body of data. Further, the toxicological, cytological, biochemical, and chemical data are all linked to each other and to other studies performed at the same sites. These data can be used to clarify evaluations of other data and can provide information on the drivers behind toxicological effects that remain in PWS.

Results from our laboratory study will be incorporated into modeling efforts in collaboration with Dr D. Esler (Simon Fraser University). Quantifying injury at the cellular and developmental levels, and linking cellular injury to CYP1A activity provides new methods to assess effects on the host organism. Incorporating results into demographic parameters provide tools to link contaminant effects to specific life stages, and to population models under development. Model structures can be used to examine and evaluate population level effects, and the potential of individual and life stage specific injury to impact population projections.

Data Analysis and Statistical Methods

Methods used for analyzing data will depend on the nature of the data and its characteristics. For PAH analysis, methods used with the same type of data from PWS will be employed (Short et al. (submitted); Short et al., 1996). The distribution of all cellular data will be examined for normality. If distribution is normal or is normal with log transformation, parametric statistics will be employed. If distributions are not normal, robust nonparametric methods can be used to evaluate the significance of differences between two means of assay values from any two sites. First, differences between responses from different sites will be evaluated using a two-sample permutation test (Ephron and Tibshirani 1993; for further details see Appendix 3). Second, we will use the parametric Student's *t*-test following logarithmic transformation of the assay data values. We will evaluate the underlying assumption of normality by applying the Kolmogorov-Smirnov (K-S) test to the group identified by the permutation test above after log-transformation. Our application of Student's t-test to comparisons of treatment groups with the control group assumes approximate normality of the treatment groups. Other studies (Parrott et al. 1999; Kennedy et al. 1995; Kennedy et al. 1996) provide guidance for other measures of data analysis and statistical methods in studies using cell bioassay derived data. Briefly, the dose-dependent effects of PAH on EROD activity (Short et al., submitted) can be examined in cell lines, and fitted to Gaussian curves. The parameters from these curves can then be used to obtain the median effective concentration (EC_{50}) to complement the LC_{50} values obtained in the validation phase of this work.

Description of Study Area

Laboratory studies will be conducted at the Alaska SeaLife Center in Seward, Alaska. SPMDs will be deployed in Prince William Sound to obtain site-specific environmental samples for laboratory bioassays.

Coordination and Collaboration with Other Efforts

This project will complement other studies focusing on harlequin duck population status and dynamics, as well as lingering oil in PWS. We will coordinate field site selection with other investigators focusing on harlequin duck population measures and trends, biomarker indicators of exposure to oil, and occurrence of oil in PWS (D. Rosenberg, D. Esler, J. Rice, and J. Short). Results from our study will also be incorporated into modeling efforts (D. Esler). Results at cellular and developmental level incorporated into demographic parameters provide tools to link contaminant effects to specific life stages, and can be evaluated for population level implications using model structures under development.

SCHEDULE

Project Milestones

Objective 1. Develop harlequin duck and surrogate (mallard) cell lines to evaluate injury from site-specific hydrocarbons in harlequin ducks.
To be met in part by December 2008 (Year 1 cell isolation) an din part by December 2009 (Year 2 cell line isolation).
To be met in full by October 2010.

Objective 2. Develop assays using harlequin duck and surrogate (mallard) cell lines to assess and quantify injury due to lingering oil in PWS.
To be met by September 2009.

Objective 3: Evaluate injury due to site-specific lingering oil in PWS in harlequin ducks at cellular level
To be met by March 2010.

Objective 4: Link analytical chemistry results from known oil-contaminated sites to injury assessments in harlequin ducks at cellular level.
To be met by December 2010.

Objective 5: Link injury due to site-specific lingering oil in PWS in harlequin duck cell lines to harlequin duck population level impact.
To be met by December 2010.

Measurable Project Tasks

FY09, 1st quarter (October 1, 2008 – December 31, 2008)

Cell line characterization
Start endpoint bioassay development

FY09, 2nd quarter (January 1, 2009 – March 31, 2009)

Annual Marine Science Symposium
Endpoint bioassay development

FY09, 3rd quarter (April 1, 2009 – June 30, 2009)

Endpoint bioassay development
Year 2 cell line isolation

FY09, 4th quarter (July 1, 2009 – September 30, 2009)

Year 2 cell line characterization
Start testing PWS samples in cell lines
Submit annual report

RESPONSIVENESS TO KEY TRUSTEE COUNCIL STRATEGIES

Community Involvement and Traditional Knowledge (TEK):

A community involvement plan will be developed if this proposal is funded. The plan will specify how relevant communities and other stakeholders will be informed and engaged with the project, and how ASLC public displays and exhibits can be involved in communicating more about this project and its results. Findings from this study will be presented to the scientific community and resource managers through presentations, publications, and at EVOS meetings.

Resource Management Applications

The project will develop and validate bioassays that allow dosing of cellular test models of species of concern in a controlled laboratory setting using the contents of an SPMD. The laboratory dosing will provide means to relate the toxicity of the sample to cellular injury and mechanism of toxicity in the species of concern. The injury assessment at cellular level will directly apply to the species of concern, and provides a linkage between bioavailable contaminants and host response. The bioassays also provide methodology options for monitoring of environmental conditions in response to clean up protocols, restoration and remediation via biomonitoring of field sites for site-specific and species-specific effects of bioavailable contaminants.

PUBLICATIONS AND REPORTS

Proposed Peer Reviewed Publications

Manuscripts will be prepared for publication in peer reviewed journals. Candidate target journals include Analytical Biochemistry, Environmental Toxicology and Chemistry, Environmental Health Perspectives, Journal of Aquatic Animal Health, Marine Ecology Progress Series, Aquatic Toxicology, Journal of Toxicology and Environmental Health, Part A, Marine Pollution Bulletin, Cell Biology and Toxicology, Comparative Biochemistry and Physiology.

REFERENCES

Brendler-Schwaab, S.Y., Schmezer P., Liegiebel U., Weber S., Michalek K., Tompa A., and Pool-Zobel B. L.. 1994. Cells of different tissues for *in vitro* and *in vivo* studies in toxicology: compilation of isolation methods. Toxicology In Vitro 8: 1285-1302.

Docherty, D. E., and Slota P.G.. 1988. Use of muscovy duck embryo fibroblasts for the isolation of viruses from wild birds. Journal of Tissue Culture Methods 11:165-170.

Ephron B., and Tibshirani R. 1993. *An Introduction to the Bootstrap*. Chapman & Hall, London.

Esler, D., Bowman T. D., Trust K. A., Ballachey B. E., Dean T. A., Jewett S. C., and O'Clair C. E.. 2002. Harlequin duck population recovery following the 'Exxon Valdez' oil spill: progress, process, and constraints. Marine Ecology Progress Series 241:271-286.

Exxon Valdez Oil Spill Trustee Council. 2006. Update on Injured Resources and Services. Exxon Valdez Oil Spill Trustee Council, Anchorage, AK, 41 pp.

Fairbairn D.W., Olive P.L., O'Neill K.L. 1995. The comet assay: a comprehensive review. Mutation Research 339: 37-59.

Fenech M. 2000. The in vitro micronucleus technique. Mutation Research 455:81-95.

Freshney, R. I. 2000. Culture of Animal Cells: A Manual of Basic Technique, 4th ed. Wiley-Liss, Inc., 577 pp.

Hollmén, T., Franson J.C., Kilpi M., Docherty D.E., Hansen W.R., and Hario M. 2002. Isolation and characterization of a reovirus from common eiders (*Somateria mollissima*) from Finland. Avian Diseases 46:478-484.

Huckins J.N., Petty J.D., Prest H.F., Clark R.C., Alvarez D.A., Orazio C.E., Lebo J.A., Cranor Walter L., Johnson B.T. 2000. *A Guide for the Use of Semipermeable Membrane Devices (SPMDs) as Samplers of Waterborne Hydrophobic Contaminants*. Columbia, MO, Columbia Environmental Research Center, U.S. Geological Survey.

Kennedy S.W., Jones S.P., Bastien L.J. 1995. Efficient analysis of cytochrome P4501A catalytic activity, porphyrins, and total proteins in chicken embryo hepatocyte cultures with a fluorescence plate reader. Analytical Biochemistry 226:362-370.

Kennedy S.W., Lorenzen A., Jones S.P., Hahn M.E., Stegeman J.J., 1996. Cytochrome P4501A induction in avian hepatocyte cultures: a promising approach for predicting the sensitivity of avian species to toxic effects of halogenated aromatic hydrocarbons. Toxicity and Applied Pharmacology 141: 214-230.

Kvenvolden K.A., Hostettler F.D., Carlson P.R., Rapp J.B. 1995. Ubiquitous tar balls with a California-source signature on the shorelines of Prince William Sound, Alaska. Environmental Science and Technology 29: 2684-2694.

Parrott J.L., Backus S.M., Borgmann A.I., Swyripa M. 1999. The use of semipermeable membrane devices to concentrate chemicals in oil refinery effluent on the Mackenzie River, Arctic 52: 125-138.

Poirier, M.C. 1993. Antisera specific for carcinogen-DNA adducts and carcinogen-modified DNA: application for detection of xenobiotics in biological samples. Mutation Research 288:31-38.

Rosenberg D.H., Petrula M.J., Hill D.D., Christ A.M. 2005. Harlequin Duck Population Dynamics: Measuring Recovery from the Exxon Valdez Oil Spill. Restoration Project 040407 Final Report, 47 pp.

Short J.W., Jackson T.J., Larsen M.L., Wade T.L., 1996. Analytical methods used for the analysis of hydrocarbons in crude oil, tissues, sediments, and seawater collected for the natural resources damage assessment of the *Exxon Valdez* oil spill. In: S. D. Rice, R. B. Spies, D. A. Wolfe and B. A. Wright (Eds.). Proceedings of the Exxon Valdez Oil Spill Symposium, Symposium 18. American Fisheries Society, pp. 140-148.

Short J.W., Springman K.R., Lindeberg M.R., Holland L.G., Larsen M.L., Sloan C.A., Khan C., Hodson P.V., Rice S.D. 2008. Semipermeable membrane devices link site-specific contaminants to effects: Part II – a comparison of lingering *Exxon Valdez* oil with other potential sources of CYP1A inducers in Prince William Sound, Alaska. Marine Environmental Research (submitted).

Springman K.R., Short J.W., Lindeberg M.R., Maselko J.M., Kahn C., Hodson P.V., Rice S.D. 2008. Semipermeable membrane devices link site-specific contaminants to effects: Part I—induction of CYP1A in rainbow trout from contaminants In Prince William Sound, Alaska. Marine Environmental Research (submitted).

Trust, K.A., Esler D., Woodin B. R., and Stegeman J. J.. 2000. Cytochrome P450 1A induction in sea ducks inhabiting nearshore areas of Prince William Sound, Alaska. Marine Pollution Bulletin 40: 397-403.

Abbreviated Curriculum Vitae

Tuula Hollmén, D.V.M., Ph.D.

Research Associate Professor of Marine Science (University of Alaska Fairbanks)
Eider Program Manager (Alaska SeaLife Center)
Alaska SeaLife Center and University of Alaska Fairbanks
P.O. Box 1329, Seward, AK 99664
Phone: 907-224-6323; Fax: 907-224-6320; E-mail: tuula_hollmen@alaskasealife.org

EDUCATION

Ph.D. University of Helsinki, Helsinki, Finland (2002)
D.V.M. University of Helsinki, Helsinki, Finland (1992)

PROFESSIONAL EXPERIENCE

Administrative

Eider Program Manager, Alaska SeaLife Center (2002-present)

Research

Research Associate Professor of Marine Science (University of Alaska Fairbanks) (2005-present)
Research Assistant Professor of Marine Science (University of Alaska Fairbanks) (2002-2005)
Visiting Scientist, U.S. Geological Survey, National Wildlife Health Center, WI (1997-2002)
Assistant Professor, University of Helsinki, Department of Basic Veterinary Sciences, Finland (1992-1996)
Visiting Scientist, National Biological Survey, Pacific Islands Science Center, HI (1994)

5 PUBLICATIONS RELATED TO THE PROPOSED PROJECT

Miles, K., Flint, P., Trust, K., Ricca, M., Spring, S., Arrieta, D., Hollmén, T., and B. Wilson. 2007. Polycyclic aromatic hydrocarbon exposure in Steller's eiders and harlequin ducks in the eastern Aleutian Islands, Alaska. Environmental Toxicology and Chemistry 26:2690-2703.

Franson, J.C., Hoffman, D.J., Wells-Berlin, A.M., Perry, M.C., Shearn Bochsler, V., Finley, D.L., Flint, P.L., and T. Hollmén. 2007. Effects of dietary Selenium on tissue concentrations, pathology, oxidative stress, and immune function in common eiders (*Somateria mollissima*). Journal of Toxicology and Environmental Health 70:861-874.

Matson, C.W., J.C. Franson, T. Hollmén, M. Kilpi, M. Hario, P.L. Flint, and J.W. Bickham. 2004. Evidence of chromosomal damage in Common Eiders (*Somateria mollissima*) from the Baltic Sea. Marine Pollution Bulletin 49:1066-1071.

Franson, J.C., T. Hollmén, P.L. Flint, J.B. Grand, and R.B. Lanctot. 2004. Contaminants in molting long-tailed ducks and nesting common eiders in the Beaufort Sea. Marine Pollution Bulletin 48:504-513.

Hollmén, T., J.C. Franson, P.L. Flint, J.B. Grand, R.B. Lanctot, D.E. Docherty, and H.M. Wilson. 2003. An adenovirus linked to mortality and disease in long-tailed ducks in Alaska. Avian Diseases 47:173-179.

5 OTHER PUBLICATIONS

Nilsson, P. Hollmén, T., Atkinson, S., Mashburn, K., Tuomi, P., Esler, D., Mulcahy, D,. and D. Rizzolo. 2008. Effects of ACTH, capture, and short term confinement on glucocorticoid concentrations in harlequin ducks (*Histrionicus histrionicus*). Comparative Biochemistry and Physiology, in press.

Hollmén, T., and D.E. Docherty. 2007. Orthoreovirus. In: Infectious and Parasitic Diseases of Wild Birds, 2nd ed. (N.J. Thomas, D.J. Forrester, and D.B. Hunter, eds). Iowa State University Press, Ames, Iowa.

Skerratt, L.F., J.C. Franson, C.U. Meteyer, and T.E. Hollmén. 2005. Causes of mortality in sea ducks (Mergini) necropsied at the USGS-National Wildlife Health Center. Waterbirds 28(2): 193-207.

Hollmén, T., J.C. Franson, M. Kilpi, D.E. Docherty, W.R. Hansen, and M. Hario. 2002. Isolation and characterization of a reovirus from common eiders (*Somateria mollissima*) from Finland. Avian Diseases 46:478-484.

Hollmén, T., J.C. Franson, M. Hario, S. Sankari, M. Kilpi, and K. Lindström. 2001. Use of serum biochemistry to evaluate nutritional status and health of incubating common eiders (*Somateria mollissima*) in Finland. Physiological and Biochemical Zoology 74:333-342.

COLLABORATORS SINCE 2004

Russel Andrews, Alaska SeaLife Center and University of Alaska Fairbanks
Shannon Atkinson, University of Alaska Fairbanks
Daniel Esler, Simon Fraser University
Paul Flint, US Geological Survey
Kim Trust, US Fish and Wildlife Service
J. Christian Franson, US Geological Survey
James B. Grand, University of Alabama and US Geological Survey
Martti Hario, Finnish Game and Fisheries Research Institute
Sara Iverson, Dalhousie University
Mikael Kilpi, Aronia Research Institute
James Lovvorn, University of Wyoming
Keith Miles, US Geological Survey
John Pearce, US Geological Survey
Margaret Petersen, US Geological Survey
Abby Powell, University of Alaska Fairbanks and US Geological Survey
Nora Rojek, US Fish and Wildlife Service
Dan Rosenberg, Alaska Department of Fish and Game
Sandra Talbot, US Geological Survey
Pamela Tuomi, Alaska SeaLife Center
Markus Öst, Aronia Research Institute

Curriculum Vitae

Name: Kathrine R. Springman

Mailing address:
P.O. Box 315
Littleriver, CA 95456

Phone: Office: (707) 937-6212
Mobile: (530) 400-4141

E-mail: krspringman@gmail.com

Education: B.S. *cum laude,* University of Texas, Austin
Ph.D., Texas A&M University (toxicology)

Publications:

Kathrine R. Springman, Jeffrey W. Short, Mandy R. Lindeberg, Jacek Maselko, Colin Khan, Peter V. Hodson, Stanley D. Rice. Semipermeable membrane devices link site-specific contaminants to effects: Part I – Induction of CYP1A in rainbow trout from contaminants in Prince William Sound, Alaska (in review).

Jeffrey W. Short, Kathrine R. Springman, Mandy Lindeberg, Jacek Maselko, Colin Khan, Peter Hodson, Margaret Krahn, Stanley D. Rice. Semipermeable membrane devices link site-specific contaminants to effects: Part II – a comparison of lingering *Exxon Valdez* oil with other potential sources of cytochrome P4501A inducers in Prince William Sound, Alaska (in review).

Kathrine R. Springman, Jeffrey W. Short, Mandy Lindeberg, Stanley D. Rice. Evaluation of bioavailable hydrocarbon sources and their induction potential in Prince William Sound, Alaska. *Marine Environmental Research* (in press).

John M. Emlen and Kathrine R. Springman. (2007). Developing methods to assess and predict the population and community level effects of environmental contaminants. *Integrated Environmental Assessment and Management* 3 (2): 157-165.

Jeffrey W. Short and Kathrine R. Springman. (2006). "Identification of Hydrocarbons in Biological Samples for Source Determination," Chapter 12. In: *Oil Spill Environmental Forensics - Fingerprinting and Source Identification*, Zhendi Wang and Scott A. Stout, eds. Elsevier Science.

Rice, S. D., J.W. Short, M.R. Lindeberg, J.M. Maselko, and K. R. Springman. (2006). Evaluation of lingering Exxon Valdez oil compared to other sources as the dominant source of CYP1A inducers in Prince William Sound 15 years after the spill. Exxon Valdez Oil Spill Trustee Council Restoration Project Final Report (Restoration Projects 060740). Auke Bay Laboratory, National Marine Fisheries Service, Juneau, Alaska.

Kathrine R. Springman, Gael Kurath, James J. Anderson, John M. Emlen (2005). Contaminants as viral cofactors: assessing indirect population effects. *Aquatic Toxicology*, 71(1): 13-23.

Collaborators since 2004:

Jim Anderson: University of Washington
Gary Cherr: University of California, Davis; Bodega Marine Lab
Dave Crane: California Dept. of Fish and Game, Water Pollution Control Lab
John Emlen: USGS, Western Fisheries Research Center
Kyle Garver: USGS, Western Fisheries Research Center; University of Washington
Peter Hodson: Queen's University
Larry Holland: NOAA, Auke Bay Lab
Colin Khan: Queen's University
Margaret Krahn: NOAA, Montlake Lab
Gael Kurath: USGS, Western Fisheries Research Center
Marie Larsen: NOAA, Auke Bay Lab
Mandy Lindeberg: NOAA, Auke Bay Lab
Abdou Mekebri: California Dept. of Fish and Game, Water Pollution Control Lab
Jacek Maselko: NOAA, Auke Bay Lab
Jeep Rice: NOAA, Auke Bay Lab
Jeff Short: NOAA, Auke Bay Lab
Catherine Sloan: NOAA, Montlake Lab
Scott Stout: NewFields Environmental Forensics Practice
Carol Vines: University of California, Davis; Bodega Marine Lab
Zhendi Wang: Environment Canada, Oil Spill Research

BUDGET JUSTIFICATION

Personnel: Ann Riddle, Laboratory Research Manager, will dedicate 2 months in FY08 and 6 months each in FY09 and FY10. Responsibilities include management of laboratory operations, including sample cataloging and recordkeeping. She will also manage and participate in all laboratory procedures, including cell isolation, cell characterizations, cell production for bioassays, and laboratory bioassays. The monthly cost of $4.4k in FY08 includes an annual rate of $42.0k plus a 25% fringe benefits rate. A cost of living increase of 3% is included between FY08 and FY09.

The PI and co-PI work with the Alaska SeaLife Center on a contractual basis, and their contributions and expenses are discussed below.

Travel: Prince William Sound field travel in FY08 will consist of two roundtrips from Seward to Cordova via Anchorage for co-PI Kathrine Springman and two roundtrips from Juneau to Cordova for Mandy Lindeberg (NOAA) for deploying and collecting the SPMDs.

Roundtrip travel for the Laboratory Research Manager from Seward, Alaska to Madison, Wisconsin is requested in FY09 for cell culture technique collaboration.

Travel to EVOS review meetings in Anchorage from Seward is included in FY08, FY09 and FY10 (two one-day trips in each year).

Travel for the PI and co-PI to attend the Marine Science in Alaska Symposium is included for FY09 and FY10.

Co-PI Kathrine Springman will need to travel from California to Seward to conduct laboratory work. Two roundtrips are included in FY08, and four roundtrips are included in both FY09 and FY10.

Travel to an out-of-state conference is requested for the PI, co-PI and Laboratory Research Manager in FY10 to present results of the project.

Contractual: We are requesting $1.0k in both FY08 and FY09 to support collaborations with aviculturists to obtain source materials for cell line development as specified in the project plan.

In FY08, we are requesting $2.5k for charter aircraft out of Cordova ($500/day for 5 days) to deploy and collect the SPMDs.

The PI and co-PI are supported through contractual arrangements with ASLC. They will work together on all aspects of the project, including cell line development, bioassay development, laboratory analysis of site-specific samples in bioassays, data analysis, reporting, and manuscript preparation. Tuula Hollmen will dedicate up to 1 month in FY08 and up to 3 months each in FY09 and FY10. Kathrine Springman will dedicate up to 3 months in FY08 and up to 6 months each in FY09 and FY10.

Facilities costs are not included in the ASLC indirect rate, but rather are charged as direct costs based on actual space utilization. This project will use a dedicated isolation room (125 square feet) for all cell culture work during the entire project period and 25% of an analytical laboratory (25% of 435 square feet) for sample preparation and biochemical and DNA analysis.

We are requesting that funding of $9.0k ($4.5k in both FY08 and FY09) be sent directly to Jeep Rice at NOAA's Auke Bay Laboratory (NOAA/NMFS/AFSC) for hydrocarbon analysis. While this amount is included in our budget, we have not included this line item in the ASLC indirect cost calculation.

Commodities: We are requesting $1.5k for field supplies and $6.8k for semipermeable membrane devices (SPMDs) to support field work in Prince William Sound in FY08. The majority of the commodities expenses will be for lab supplies, chemicals, reagents and biological source materials to complete specific laboratory tasks. These include:

Cell isolation ($10.0k in FY08 and FY09)
Cell characterization and preparation for assays ($1.0k in FY08, $6.0k in FY09 and FY10)
Reference cell lines ($1.0k in FY08 and FY09)
Cell viability and apoptosis ($3.0k in FY09 and FY10)
Enzyme induction – EROD ($3.0k in FY09 and FY10)
DNA damage assessment ($8.0k in FY09 and FY10)
Laboratory consumables ($2.5k in FY08 and FY09, $2.0k in FY10)

Equipment: Three pieces of equipment are needed to complete the project:

1. Egg incubator ($5.0k): dedicated incubator with required capacity and operating parameters to provide source materials for duck cell culture development.
2. Centrifuge ($7.7k): refrigerated centrifuge with operating conditions specific to cell bioassay protocols and dedicated to use in the isolation room.
3. Freezer -80°C ($12.0k): lockable ultra-low -80°C freezer dedicated to secure storage of all samples and biological materials collected, catalogued and analyzed for the project.

Indirect costs: The ASLC indirect rate is calculated as 27.08% of the modified total direct costs (excluding equipment greater than $5,000).

Data Management and Quality Assurance/Quality Control Statement

Data management and quality assurance/quality control will be the responsibility of the PI and Co-PI. We will use required MetaLite freeware and FGDC compliant protocols when developing metadata records for the project

1. This experimental study will be conducted under controlled laboratory conditions to analyze species-specific data as specified in the project plan. The dependent variables to be studied include a battery of bioassays to evaluate a series of endpoints to determine cellular response to contaminant exposure. Response variables include cellular pathology, cell viability, apoptosis, enzyme induction, and DNA damage.
2. Appropriate laboratory quality assurance/quality control measures will be included at all phases of testing and analysis to ensure data quality. Positive and negative controls and intra- and interassay references will be included in test panels to provide a measure of assay performance. Records of assay specifics and performance will be maintained for all laboratory analyses.
3.
    a.) Metadata will be provided if the proposal is funded.
    b.) Quantitative datasets will involve species-specific measurements. Fields associated with dataset:
        i. Cell viability and cell pathology
        ii. EROD activity
        iii. DNA damage
        iv. Chemical profile of materials eliciting responses. Other germane information (e.g. site location, oiling history, data from previous studies from same sites) will be assembled.
4. Described in project plan when applicable.
5. All samples will be collected, appropriately labeled, and frozen by personnel from the ASLC or by collaborators until analyzed. Samples will be stored -20C or -80C freezers, as appropriate, at the ASLC and catalogued to facilitate tracking and distribution. For field samples that are subsequently processed and analyzed at other locations, appropriate chain of custody forms will accompany the samples and be retained with all documentation at ASLC.
6. Calibration of analytical equipment will be performed by methods supported by the manufacturer of the equipment. For GC/MS and other analytical chemistry equipment, see Short et al. (1996)
7. Information collected from laboratory analyses is entered into Microsoft Excel or Access and stored on servers at ASLC. Statistical methods are described in the study plan. Statistical software will include SYSTAT and SAS, and TableCurve 2D will be used for curve fitting. All results will appear in annual reports and peer reviewed publications.

EXPORT FROM METALITE METADATA FILE

What does this data set describe?

Title:
Abstract:

1. **How should this data set be cited?**

   Online Links:

2. **What geographic area does the data set cover?**
3. **What does it look like?**
4. **Does the data set describe conditions during a particular time period?**
5. **What is the general form of this data set?**

6. **How does the data set represent geographic features?**
   a. **How are geographic features stored in the data set?**
   b. **What coordinate system is used to represent geographic features?**
7. **How does the data set describe geographic features?**

Who produced the data set?

1. **Who are the originators of the data set?** (may include formal authors, digital compilers, and editors)
2. **Who also contributed to the data set?**
3. **To whom should users address questions about the data?**

Why was the data set created?

How was the data set created?

1. **Where did the data come from?**

2. **How were the data processed and modified?**

How reliable are the data; what problems remain in the data set?

How can someone get a copy of the data set?

Are there legal restrictions on access or use of the data?
Access_Constraints:
Use_Constraints:

1. **Who distributes the data set?** (Distributor 1 of 1)
2. **What's the catalog number I need to order this data set?**
3. **What legal disclaimers am I supposed to read?**
4. **How can I download or order the data?**

Who wrote the metadata?