ATTACHMENT C *EVOSTC Annual Project Report Form*
Form Rev. 9.14.17

1. Project Number:

Project 17170115

2. Project Title:

Genomic mechanisms that underlie lack of recovery of Prince William Sound herring following the 1990's collapse

3. Principal Investigator(s) Names:

Andrew Whitehead

4. Time Period Covered by the Report:

June 29, 2017 to January 31, 2018

5. Date of Report:

March 01, 2018

6. Project Website:

None

7. Summary of Work Performed:

We have performed work on four tasks in this first year of the project.

1) Oil and pathogen challenge experiments. This preliminary range-finding experiment was designed to test whether early-life exposure to oil affects later-life ability to mount effective immune defenses in response to viral challenge. Starting in March 2017 we initiated a 6-month animal experiment, where herring embryos were exposed to very low concentrations of oil (plus no-oil control exposures) from one day post-fertilization through organogenesis. Fish were then transferred to clean water until hatch, then reared in clean conditions through the larval and early juvenile stages, during which time the immune system matures. Fish were then challenged with viral hemorrhagic septicemia virus (VHSV), and infection-induced mortality was recorded. Our intended high-oil dose ended up being a very low-concentration exposure. That is, the total PAH concentration was barely above GC-MS detection limit: 140 parts per trillion (pptr). Despite this extremely low dose, we still detected effects on heart development and fish length at hatch compared to controls, including significantly induced CYP1A gene expression. To our knowledge, this is the lowest concentration to yet show such effects in developing fish embryos. We have yet to discover the no-observable-effect level for PAH impacts on developing herring embryos. After six months of development post-

hatch, we challenged juveniles with VHSV. Unexpectedly, we found that very low-concentration exposure during embryogenesis seemed to protect juveniles against VHSV-induced mortality compared to controls that were not exposed to oil during embryogenesis. We are designing the current year's experiments to repeat such low-concentration exposures in an attempt to replicate these surprising findings. We will also include higher doses that may exert the expected immunosuppressant effects. During this experiment we collected tissues for genome-wide gene expression analysis at multiple timepoints during development. We are currently preparing these samples for expression profiling. We will use these data to characterize the time-course of immune system maturation, and to test how very low-concentration oil exposures may modulate the development of immune maturation.

2) Reference genome sequencing. We have completed a draft reference genome sequence for Pacific herring. Our reference individual was sourced from Prince William Sound, AK. We were able to optimize very high molecular weight DNA extractions. This was used as input for 10X genomics library preparation at the UC Davis Genome Center, and libraries were subsequently sequenced on the Illumina 4000. We achieved over 120X genome coverage. We recently completed our first draft assembly that is adequate in contiguity for the goals of this project. However, further refinement of our assembly algorithm, and planned inclusion of more data, should improve assembly contiguity. This is an ongoing effort. However, our assembly contiguity is currently good (N50 scaffold = 559 kb).
3) Reference transcriptome sequencing. We have sequenced a reference transcritptome from five tissues from a single fish sampled from the Prince William Sound population. We used the PacBio Sequel platform to achieve full-length transcript reads to optimize assembly of the transcriptome. It took some time to optimize RNA purification and library preparation for this new technology, but we have completed this, and have recently received the sequencing data. The reference transcriptome is currently in assembly.
4) Population genomics. We received tissue samples from four time periods, spanning three decades, from each of three Alaskan herring populations, including Prince William Sound, Togiak Bay, and Sitka Sound (only 3 time periods from Sitka). We have also collected tissues from 2017 captures from three additional populations that span the latitudinal distribution of the species (Central British Columbia Canada, Puget Sound WA, and San Francisco Bay CA). We have extracted DNA from 1,257 fish total. We have implemented a new protocol for inexpensively indexing (barcoding) the DNA of each individual fish to enable accurate estimates of allele frequencies, and to enable genotype likelihood estimates. We are preparing ~64 samples to be multiplexed together for sequencing. And ~20 sets of these multiplexed samples will be sequenced using one lane of Illumina HiSeq 4000 per set. This should yield ~1X full genome coverage per sample. We have sequenced an initial set of 64 individuals, which yielded the expected 1X genome coverage per individual. We are now proceeding with indexing, pooling, and sequencing the additional 19 sets.

8. Coordination and Collaboration:

A) Projects within a Trustee Council-Funded Program: This project is a formal collaboration with my research group at UC Davis and that of Dr. Paul Hershberger at USGS Marrowstone. Animal experiments are being conducted by Dr. Hershberger's group

(Project #17120111-E) at the Marrowstone facility. Furthermore, a Ph.D. student and a postdoc from my research group travelled to Marrowstone to participate in pathogen challenge experiments in September 2017. Members of Dr. Hershberger's group also collected fresh tissues from Sitka Sound and Prince William Sound in March 2017 for reference genome sequencing and population genomics sequencing. In the past year I have travelled to the Seattle area twice for face-to-face logistics and project coordination meetings with Paul Hershberger and his group. My Ph.D. student also joined us for one of those meetings.

B) Projects not within a Trustee Council-Funded Program: Not applicable

C) With Trustee or Management Agencies: I am happy to report that a number of Alaska State Agency scientists, herring fishery industry personnel, and NOAA Fisheries scientists, have enthusiastically embraced this project, and have already contributed in important ways. NOAA Fisheries scientists John Incardona and Nathaniel Scholz (NOAA Northwest Fisheries Science Center, Seattle) are collaborating in animal exposure experiments, since they have research goals that include exposure impacts on growth and development. These measurements were easily added to tasks associated with our (Whitehead and Hershberger) 2017 exposure experiments and planned 2018 exposure experiments, so it made sense to join forces and leverage the collective experience, personnel, and equipment of our groups – the most important of these being the outstanding aquatics systems for rearing live herring at Marrowstone. A crucial source of historic tissue samples is the frozen tissue collection maintained by the Alaska Department of Fish and Game. Collections director Chris Habicht and staff members Judy Berger and Heather Hoyt were extremely helpful in facilitating the selection and transfer of hundreds of tissues from their collection to my lab at UC Davis. Also at Alaska DFG, Sherri Dressel is the Statewide Herring Fisheries Scientist. She and her group, including Katie Sechrist in particular, were instrumental in arranging for fresh tissue samples from 100 herring to be sent to my lab from the Bering Sea (Togiak Bay) with the enthusiastic participation of Ben Cain at Silver Bay Seafoods (Bristol Bay/Naknek plant). Outside of Alaska, other groups have graciously contributed samples for genetics analysis, including personnel from the Department of Fisheries and Oceans Canada (Kristen Daniel and Jaclyn Cleary) and California Department of Fish and Wildlife (Kathy Hieb). I have also been in touch with herring geneticist Sharon Wildes (NOAA Federal, Alaska Fisheries Science Center). She is enthusiastic about the project, and consulted on our choice of populations for genetics analysis.

9. Information and Data Transfer:

Nothing yet to report

10. Response to EVOSTC Review, Recommendations and Comments:

Science Panel Comments and Responses on Revised FY17-21 Proposal, September 2016

This innovative proposal complements the Herring Research and Monitoring Program by conducting a retrospective (pre-spill to present) analysis of genome diversity and the potential impacts of oil exposure on immune deficiency, as well as an assessment of the ability of current genetic diversity to cope with ongoing disease issues. The current Herring Program is focused primarily on stock assessments and current factors affecting the lack of recovery (e.g., whale

predation, disease monitoring, and recruitment issues). The Science Panel is supportive of the proposal because of the potential to answer important questions about the cause of the herring population crash as well as important genetic factors that may inhibit recovery. Notably, this project combines genome (Whitehead) and disease (Hershberger) expertise, and makes use of valuable genetic samples archived by ADFG pre-spill to present.

The Panel is quite enthusiastic about this new approach and opportunity to assess the evidence for mechanistic ties between oil and herring immune deficiency by bringing genomic expertise to bear on herring disease issues. The PI has an excellent track record of productivity and expertise. A major strength of the proposal is the utilization of fish tissues samples that have been archived for almost 30 years at ADFG. This work draws upon ADFG's existing tissue collection, in combination with advanced genomic techniques, to provide a unique (and possibly unparalleled) view into the population, genetic and evolutionary history of Alaskan herring before, during and after the oiling event. This unique opportunity to utilize ADFG samples, collected and archived across decades, will facilitate a novel approach to the pressing problem of lack of herring recovery and result in valuable information regarding the PWS herring genome.

The PI builds a strong case in support of the hypothesis that oil exposure has suppressed the immune response of herring to disease thereby contributing to the crash and slowing recovery of PWS herring. The PI is uniquely positioned to address this question given that he has found strong evidence that exposure to PAHs and oil on the Atlantic and Gulf Coasts respectively has suppressed immune responses of killifish. The PI works with Paul Hershberger, who has produced internationally groundbreaking herring disease work supported by EVOSTC funding. The second tier of experiments will rear disease-naïve herring embryos from PWS and two other stocks, expose embryos to oil, and determine if there is a difference in response and in genome diversity with disease response genes. Rearing and exposure of fish will take place in the laboratory of Paul Hershberger, who has vast experience in producing disease naïve fish. This research on herring immune deficiency will be valuable in determining the potential of PWS herring to resist disease after exposure to oil compared to other stocks and will be an important contribution to understanding the dynamics of PWS herring, as well as the potential for fish stocks in general exposed to other spills elsewhere. In addition, the research is valuable regardless of the outcome (i.e., whether the link between oil and herring immune deficiency is supported mechanistically and whether or not there is a genetic diversity bottleneck effect) as the proposed work has the potential to contribute significantly to our understanding of both the causes of herring decline and the failure to recover to date – key issues to the mission of the EVOSTC.

The proposal's costs have been reviewed and are found to be appropriate for this level of technological capacity and typical for these types of advanced genomic techniques.

General Comments:
The PWS herring population collapsed several years after the spill and has not since had a sustained period of incremental growth. Scientific reports that describe potential causative linkages are matched by an approximately equal number of reports that describe alternative explanations for either the collapse, or lack of sustained recovery, or both. In short, even after several decades of research, we are still uncertain about whether there have been any long-term impacts of the spill on herring, or the herring collapse in 1993-94 and the lack of any

sustained recovery. This project has the greatest potential to have a retrospective look at the past in a scientifically meaningful way.

This proposal has an unprecedented capacity to apply novel, highly technical research on Alaskan herring genomics to actually test the hypothesis that exposure to oil during the egg (or embryo) and early larval stages has led to a decrease in the genetic capacity of PWS herring to resist naturally-occurring, endemic disease organisms. This retrospective genome determination from archived genetics samples would determine if present-day PWS herring would be detectably different than their ancestors residing in PWS prior to the spill, and from other Alaskan herring populations. The proposal consists of several tests. One would be based on a time-series analyses of archived samples of herring collected and stored annually since the spill to test for change in the frequency of alleles related to disease resistance or susceptibility in PWS versus areas that were not exposed to oil. A related test of differences in disease resistance of PWS herring from other herring would be based on laboratory experiments of reared herring from PWS and two other populations.

The proposal is important to EVOTC and the State of Alaska. It addresses the most fundamental question of the herring program: what is the impact of the spill on herring and what factors are now affecting recovery? This project builds off the current herring monitoring program, and, most importantly, builds off the unique collection of archived herring collections from ADFG, the work proposed in this proposal, regardless of the results, will reflect positively on the EVOSTC. Moreover, the proposed work will likely have worldwide implications and applications for coastal marine fishes.

Specific Technical Comments:
As is often the case with such novel, groundbreaking proposals, the Panel had a number of questions that the PI should address and submit to EVOSTC before reaching a final decision on the recommendation for funding the proposal. We are confident, given the expertise and track record of the investigators, that the PIs will submit appropriate details to these comments:

1. Add technical detail on pathogen exposure experiments. The Panel had several questions that need clarification. Which pathogens will fish be exposed to? Are these from purified sources that can be used at different times of exposure? Given the population differences and pathogen responses, this is a key detail that needs to be included. Will embryos/larvae from the different populations be tested simultaneously for oil and disease exposure in the lab? If not what assurances will be made that exposure (oil as well as pathogens) conditions are identical across populations? For example, how reproducible is the oiled gravel treatment and the pathogen challenge? What steps will be taken to ensure and verify this reproducibility? What will be the age of embryos at collection? That is, 10-14 day embryos may have a different transcriptome than 5-7 day embryos because they might have been exposed to environmental stressors such as UV, desiccation and salinity changes.

PI Response: Fish will be exposed to viral hemorrhagic septicemia virus (VHSV) Genogroup IVa, originally isolated from fish in the North Pacific. Experiments in the first year will include just the Puget Sound population, which will include range-finding experiments to choose the doses and timepoints appropriate for sampling, depending on what we learn about the timing of immune system development. The second year will then include all three populations tested in parallel. This will ensure consistent exposure conditions such that comparisons between populations are

robust. Embryos will be collected at hatch, larval post-hatch during immune system maturation (time points to be determined in year 1 experiments), and during juvenile exposures to pathogens.

2. Aim 3 needs more details on replication, exposure duration and intensity.

PI RESPONSE: Adult pre-spawn herring (n ≈ 100 females and 50 males) will be collected by cast net, gill net, dip net, and / or hook-and-line. Fish will be euthanized immediately after capture by immersion in an overdose of buffered MS-222 and carcasses will be transported to the Marrowstone Field Station where gametes will be dissected. Embryos will be fertilized in vitro, afterwhich eggs will be adhered onto Nitex mesh sheets. Oil exposures to the fertilized eggs and developing embryos will be accomplished using the oiled gravel column method (Incardona et al. 2012). Briefly, gravel coated with crude oil will be packed into 4" x 24" PVC columns that receive continuously flowing seawater. Effluent from the columns will be collected in 2-gal aquaria. Nitex sheets with herring embryos will be suspended in tanks supplied with three dilutions of the effluent until 1-2 d prior to hatch. Nitex sheets will then be transferred to tanks supplied with clean (unoiled) seawater, where hatching and rearing will occur. The study will include 4 treatments; 3 oil doses (< 4ppt) and an unoiled control. After exposure, approximately 40,000 embryos from each of the four treatments will be transferred to four tanks (4ft diameter) supplied with clean (unoiled seawater). Larval hatch from the eggs will occur 1-2d later. Larvae from these four treatment groups will be reared in duplicate tanks (760L) used to fulfill the experimental objectives.

Controlled laboratory exposure studies will be performed to determine whether early life stage exposure to PAH's impacts the susceptibility of herring to VHS and their ability to mount a protective immune response. Under typical (unoiled) conditions, Pacific herring susceptibility to VHS is inversely related to temperature, with decreasing susceptibilities occurring at elevated temperatures. This temperature response is likely mitigated through an enhanced (earlier and more pronounced) innate immune response involving upregulation of antiviral genes at warmer temperatures. Additionally, survivors of a VHS epizootic typically develop adaptive immunity that is protective against future episodes of the disease. We will investigate whether early life stage exposure to PAH's compromises the innate and /or adaptive immune responses of Pacific herring, thereby affecting host susceptibility to the disease and their subsequent host resistance to the disease. Replicate tanks (triplicates for survival + one for subsampling) containing juvenile herring (N=100 / tank) from each of the oiled treatment groups will be exposed to VHSV (< 5x104 PFU / mL) by waterborne immersion at each of two temperatures (11, and 16 ^{o}C) to assess their susceptibility to VHS relative to unoiled control groups. Individuals (n = 10 / sampling day) will be periodically subsampled from one tank daily to assess the upregulation of select innate immune response genes. The molecular pathways and systems pathways will be further examined in a portion of these subsampled individuals using genome-wide gene expression in blood and liver tissues. Survivors of these VHSV initial exposures will be re-exposed to virus 60d later to assess whether their ability to mount a protective immune response was adversely affected by embryonic exposure to PAH's. Replicate tanks (triplicates for survival) containing herring (n=50 per tank) that are either naïve or have survived VHSV from each of the treatment groups (no oil, low, medium or high oil) will be exposed to VHSV at each of three temperatures (as above). Experimental fish will be monitored daily and moribund individuals will be euthanized if / when they appear. The experiment will proceed for 21d, after which all survivors will be euthanized and sampled. Subsamples will be collected periodically during the 21d experimental

period and gene regulation will be assessed similarly to that described during the first experiment period. All fish (subsampled + survivors) will be examined for the presence and titers of neutralizing antibodies to VHSV.

3. Functional annotation of genes. It would be useful to mention existing genomic resources for similar species to assure the Panel that these genes and others of potential relevance can be identified and the genome annotated.

PI RESPONSE: We may use transcripts from Atlantic herring to assist in annotating the genome. However, given our experience we predict that the best approach will be to annotate the reference genome with RNA sequence from the same individual and the one used for genome sequence and assembly. Transcriptome assembly has highest contiguity when from a single individual because the impacts of heterozygosity are minimized. Mapping of transcripts to the reference genome is also maximized for the same reason. For the best genome annotation possible, we will focus on this approach. We will also use evidence from orthology, which is drawn from annotations of other closely related fish species, to add another layer of evidence for building gene models. In summary, we will use the NCBI Eukaryotic Genome Annotation Pipeline as a guide for annotation of the Pacific herring assembly, which includes both expressed sequence data and orthology data. This is how our recently published Fundulus heteroclitus genome was annotated.

4. Add detail on retrospective population genomics sampling. Please provide information on where fish were sampled and the age classes of collected fishes to clarify how the longitudinal time series will be interpreted. For example, age 3 fish collected in 1993 would not have been exposed to oil, but age 8 would have been. Additional information is needed to ensure that samples were representative of the population at the time of sampling and that sample numbers are sufficiently large and were preserved in such a way that genomic level data can be recovered from the samples.

PI RESPONSE: Fish were not aged before tissues were taken for contribution to the ADFW collection. EVOS oil exposures of the greatest consequence will have been those to developing early life stages. Pacific herring recruit at ~4 years of age. So fish caught and contributed to the ADFW collection should be 4 years or older. So we figure that starting in 1993, some of the adult fish caught could have been exposed to oil during early life. Before 1993, no adult fish caught would have been exposed to EVOS during early life. By 1996, many more of the fish caught could have been exposed during early life. 1996 is our earliest post-collapse sample. We also have samples from 2007, and we just secured another set of samples (2017).

5. Ignoring alleles with less than 5% frequency. While this makes sense, with N=50 individuals, this means that genotypes with fewer than 3 individuals will be discarded. Depending on the degree of polymorphism, if diverse populations have large numbers of rare genotypes, this could result in many genotypes being ignored. This is a question, especially if disease perhaps maintains diversity via negative frequency dependent selection. It would be helpful if the PI could address this potential issue.

PI RESPONSE: These are valid points. We will assess our data once collected. We will do our best to filter the data to balance the need to purge genotypes with low confidence (e.g., may be spurious because of sequencing error) with the desire to retain genotypes that are real but

segregate at low frequency.

6. Clarify Hershberger's role and budget needs. There appears to be considerably more effort from Hershberger than indicated by the total dollar request. We assume that this is the result of "in-kind" contributions, but it would be good to document the source of those funds so that we can both be assured that they will happen and to account for any leveraging of funds. The Panel noted that this sort of in-kind contribution might be time sensitive and this is another very good reason to support funding the project in this cycle.

PI RESPONSE: It is acknowledged that the contribution of Hershberger, his team of scientists, and the capabilities at the USGS – Marrowstone Marine Field Station are critical to the success of this project. It is further recognized that the planned contributions of his team far outweigh the remuneration requested in this proposal. Recognizing the relatively high costs involved with the advance molecular work, we were concerned that the inclusion of the actual costs for Hershberger would price the project out of the realm of reasonable consideration. In the interests of advancing science and facilitating the success of this project, Dr. Hershberger has agreed to make the appropriate arrangements to accommodate the vast majority of the Marrowstone costs as in kind contributions. The USGS Marrowstone costs will be leveraged against EVOS TC Project #17120111-E (EVOS Herring Disease Program).

7. Add additional detail on the budget. Please clarify budget details for each objective to allow the reviewers and Trustees to know what the cost for each piece of the work would be and to assess what funds from other projects (both those funded by EVOSTC and others) might be being already leveraged in this proposal (see #6).

PI RESPONSE: Objective 1) The reference genome will be sequenced using Pacific Biosciences long read technology. Given an estimated genome size of 0.85Gb, for 65X coverage will require ~56 SMRT cells, and including library preparation will cost ~$22k. This also includes funds for sequencing and assembly of a reference transriptome from the same individual as that which was the source of DNA for the reference genome. Genome re-sequencing of population samples will include 450 samples. We estimate that library costs plus sequencing will cost ~$220 per sample (for 10X coverage per individual, genome size 0.85 Gb), summing to ~$100k for all samples. Objectives 2 and 3) These require exposure experiments and transcriptomics. Exposure experiments are now being performed in partnership with John Incardona's group at NOAA. This is a wonderful partnership because Incardona's group contributes much expertise in oil exposure experiments, including in herring. His group's contribution is supported by NOAA funding and funding from the North Pacific Research Board. Gene expression experiments will include replicate individuals from three populations and multiple exposure conditions (oil and pathogen, full factorial design), and two tissues, summing to ~360 samples. We have designed in-house high-throughput and low-cost library preparation protocols that come to $30 per sample. We can multiplex 16 samples per lane of Illumina sequence for our target of 20M reads per sample. Given current sequencing costs, we estimate ~$66k for RNA sequencing. We also budget for a technician (Jen Roach who is highly experienced) to generate sequence libraries, manage sample inventories, and manage data archiving and oversee quality control. We budget for a postdoctoral research associate to manage the population genomics data set. We budget for a graduate student to assist with exposure experiments and manage the gene expression data sets.

Science Panel Comments and Responses on FY18 Work Plans, September 2017
The Panel was pleased to see the integration with Paul Hershberger's disease work, linking them to see if see if there is a genomic change in response to these different pathogens in the PWS herring population. The Panel appreciates that goals are being achieved ahead of schedule and cost-effectively, allowing for additional samples at other locations. The Panel approves the shift of funds from future years to FY18 to get the postdoc onboard to work with the data being generated. There are many great collaborations being made. The Panel is excited to have the entire genome and transcriptome for herring mapped for other studies, including the possibility of adding more value to herring stock responses in Southeast Alaska. There might be another source of archived samples in Pacific Northwest (Doug Hay - Barkley Sound?).

PI RESPONSE: NA

11. Budget:

Actual cumulative: $129,085.72