

Annual Progress Report Form - Oil Spill Recovery Institute

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P.O. Box 705 - Cordova, AK 99574 - Fax: (907) 424-5820 - E-mail:
frontdes@pwssc.gen.ak.us

Deadline for this report: This report is due within 45 days of the anniversary of the effective date of the grant.

Today's date: April 21, 2004

Name of awardee/grantee: Merav Ben-David; Fellowship for Heidi Hansen

Project title: Development of long term monitoring protocols to assess potential effects of future oil spills on coastal river otters in Prince William Sound, Alaska

Dates this progress report covers: November 1, 2002 – March 31, 2004

PART I - Progress Report on Activities

A. Abstract or summary of project work to date.

On 24 March 1989, the super tanker *Exxon Valdez* ran aground on Bligh Reef spilling 39,000 metric tons of North Slope crude. Numerous marine organisms were injured as a result of the *Exxon Valdez* oil spill (*EVOS*), including coastal river otters (*Lontra canadensis*). Coastal river otters, fish predators that feed near the apex of the trophic pyramid, readily accumulate high levels of pollutants. Consequently, this mustelid serves as a sentinel species for monitoring acute and chronic effects of toxins, including biomagnification of heavy metals, and accumulation of petroleum hydrocarbons. In addition, coastal river deposit aquatically-derived nutrients at latrine sites that are located along the coast. Plants growing on latrine sites used by river otters incorporate marine-derived nitrogen (N) from otter feces, urine, and anal-gland secretions. This N fertilization (up to $0.16 \text{ kg m}^{-2} \text{ y}^{-1}$) has a substantial effect on diversity of the community of the beach-fringe forest.

Because of their role as keystone and sentinel species of the land-margin, population estimates of river otters are necessary for monitoring ecosystem health of both marine and terrestrial environment. Estimating numbers of river otters has proved extremely difficult in the past, however, due to their secretive nature and their immunity to re-captures. In this project, we are developing monitoring protocols for coastal river otters based on individual identification with microsatellite DNA analysis of feces coupled with mark-resight modeling.

Using methods for extraction and amplifications of DNA from otter feces optimized by Dr. P. Groves (University of Alaska Fairbanks), we obtained DNA yield lower than expected (less than 10% of samples). Therefore, we developed new protocols for extraction and amplification of DNA from otter feces. The new protocol increased extraction success from 10 to 86% and successful amplifications are at 59%. In addition, multiple amplifications (5.45 per sample with one locus, and 4.62 with a second locus) yielded unequivocal genotyping for 14 samples (13%). Future work will include DNA analysis of all archived samples and development of mark-resight models.

B. Brief review of the objectives as described in original proposal and progress report related to these objectives.

Objectives/Goals

1. To develop monitoring protocols for coastal river otters based on individual identification with microsatellite DNA analysis of feces coupled with mark-resight modeling.
2. Through modeling derive the cost-effective protocol that results in the most accurate population estimates.

Results to date

The correct identification of individuals from fecal DNA analysis is central to this project. At the onset of this project we anticipated using methods already optimized by Dr. P. Groves (University of Alaska Fairbanks) to obtain DNA profiles for archived

fecal samples collected in Prince William Sound, Alaska, in previous years. Unfortunately, these methods were inadequate as DNA yield from the archived samples was less than 10%. With such low yield genotyping of individual samples was impossible. Therefore, we were forced to develop new protocols for extraction and amplification of DNA from otter feces.

Beginning February 2003 we have adopted and optimized new methods for DNA extraction for otter feces. In the new protocol, otter feces are strained through a series of sieves, fish bones are removed and cleaned for future diet analyses, and remaining fecal material is rinsed in fresh EtOH. This EtOH is then evaporated at room temperature. A QIAamp DNA Stool Mini Kit is then used for purification of DNA from fecal samples (QIAGEN 2002). Approximately 180 to 220 mg (~200 μ l) of feces are measured into 2.0 ml microcentrifuge tubes for each sample, avoiding cross-contamination by using separate spatulas that have been sterilized in bleach, EtOH and deionized water for each sample. At least one tube without feces (negative control) is included with each extraction process to ensure that the samples are not contaminated. In the first steps of the protocol, fecal samples are lysed in Buffer ASL (1.6 ml per sample). After lysis, an InhibitEX tablet is added to each sample in order to remove DNA-damaging substances and PCR (polymerase chain reaction) inhibitors. After the InhibitEX tablet has absorbed these, the reagent will be pelleted by centrifugation. The DNA in the supernatant is then purified by using the QIAamp spin columns. DNA is absorbed onto the QIAamp silica-gel membrane in a series of binding steps using 25 μ l of Proteinase K, 0.6 ml of Buffer AL, and 0.6 ml of EtOH. The resulting lysate will be centrifuged 3 times, 0.6 ml of lysate each time in order to ensure maximum DNA binding on the membrane. With each centrifugation, a new 2 ml collection tube is used to collect the filtrate, which is then discarded. DNA bound to the membrane in the spin columns will then be washed in two centrifugation steps using two wash buffers, 0.5 ml Buffer AW1 and 0.5 ml Buffer AW2, respectively. The resulting purified and concentrated DNA is eluted from the membrane with 200 μ l Buffer AE and collected in 1.5 ml tubes. These are stored at 4°C or -20°C until PCR is run. To avoid contamination, extraction and PCR preparation steps is carried out in a laboratory exclusively dedicated to this purpose.

Amplifications of DNA microsatellites from fecal DNA will be done in a PTC-0200 DNA Engine Peltier Thermal Cycler (MJ Research, Inc.). Positive (blood samples from river otters with known genotypes) and negative controls are included with each PCR run. To avoid contamination, post-PCR steps are carried out in a separate laboratory. Stop dye is added to PCR products, which are then loaded into a LICOR DNA Analyzer Gene Reader 4200 for sizing. A molecular weight marker is loaded alongside the PCR products as an internal size standard. The data is sized in base pairs and analyzed using V3.00 Gene ImagIR software.

These new protocols were performed to date on 106 archived fecal samples that were preserved in EtOH. Successful extraction was achieved for 91 of these samples (or 86%). Such high extraction success is more than 8 times higher than we had with the previous protocol. Of these, 63 samples had non-degraded DNA (or 59%). For initial genotyping we used 2 primers (RO701 and RO733; Blundell et al. 2002b, *in press*, Dallas and Piertney 1998, Fleming et al. 1999). Successful amplification was achieved for 45% of the samples with RO701 and 25% for RO733 (Table 1). Complete genotypes as determined by the program GENEPOP (Raymond and Rousset 1995) are currently

available for 14 samples for the RO701 locus and for 4 samples for the RO733 locus (Table 1).

Table 1. Summary of effort (number of amplifications and average PCR per sample) and success (Number of successful PCR, successful amplifications per samples, and number of positive genotypes) for 63 fecal samples of river otters.

Loci	Number of amplifications	Average PCR per sample	Number of successful PCR	Successful amplifications per samples (percent)	Number of unequivocal genotypes
701	316	5.45	143	45	14
733	268	4.62	67	25	4
Total	584		210	36	

Currently we are analyzing additional samples as well as including additional primers to obtain complete genotyping of archived samples. We are also comparing genotyping data from blood and feces of known individual otters. These samples were obtained from a companion study at the Alaska Sealife Center in 1999 (Ben-David et al. 2002). Once the DNA “fingerprint” of each sample is obtained, recaptures will be established by comparing genotypes in different collection occasions.

Once captures and recaptures are assigned, capture histories for individuals will be constructed and input into the program NOREMARK (White 1996) for mark-recapture analyses. A resighting occasion using the DNA methodology will be defined as collection of feces from latrine sites within a narrow time interval (several days). Following the construction of the most comprehensive model, data will be curtailed both spatially and temporally with Jackknifing methods to evaluate the effects of sampling design and sample sizes on population estimates. All models will be re-run with different values of misidentified individuals based on observed variability in the 2 study areas. Using the program NOREMARK we intend to evaluate:

- (1) the minimum number of latrine sites required for obtaining an unbiased estimate.
- (2) the optimal number of latrine sites required for obtaining an unbiased estimate.
- (3) the optimal number of fecal samples per latrine site required for obtaining an unbiased estimate.
- (4) compare results of estimates based on all individuals with estimates based on females only.

For each model, cost of collection and analysis will be determined and an optimization modeling will be applied for selection of the most cost effective sampling design that yielded the most accurate estimates. Based on this selection a monitoring protocol will be written.

C. Describe problems or roadblocks encountered in project implementation.

The correct identification of individuals from fecal DNA analysis is central to this project. At the onset of this project we anticipated using methods already optimized by Dr. P. Groves (University of Alaska Fairbanks) to obtain DNA profiles for archived fecal samples collected in Prince William Sound, Alaska, in previous years. Unfortunately, these methods were inadequate as DNA yield from the archived samples

was less than 10%. With such low yield genotyping of individual samples was impossible. Therefore, we were forced to develop new protocols for extraction and amplification of DNA from otter feces.

The development of new protocols took over 8 months, and we are still testing and perfecting the methodologies. As is clear from Table 1, obtaining an unequivocal genotype for each sample requires multiple PCR reactions. Currently our efforts are concentrated in improving amplification success and reducing the average number of PCR reactions per sample. Also, we are developing criteria for early screening of samples to reduce the costs associated with each sample.

D. Highlight accomplishments, whether or not they were part of the original proposal.

The major achievement of this project our ability to increase the success of extraction and amplification of DNA from river otter feces. While we did not expect DNA extraction and amplification to be a problem, the low success using previously available protocols was a hindrance to the project success. Therefore, developing the solutions for this problem is a major accomplishment.

While working on developing the new protocols for DNA extraction and amplifications, Heidi Hansen has also analyzed a related, existing, dataset and produced a manuscript which is currently under review in *Animal Behavior*. In this manuscript, Heidi explores the factors that determine group formation and maintenance in coastal river otters. The dataset was obtained from 15 captive river otters that were held at the Alaska Sealife center between 1998 and 1999 (Ben-David et al. 2002). Portions of the data were obtained through a project funded by OSRI. Using DNA profiles and relatedness analyses, information on geographic distance pre-captivity and post-release (funded by OSRI), and data on behavioral interactions, Heidi was able to show that otter groups are not composed of close kin but rather familiarity is a driving force in shaping their social organization.

E. Conclusions to date.

With the new protocols for extraction and amplification of DNA from otter feces, we believe the original goals can be met despite the temporal setback we experienced. In addition, by analyzing an existing dataset and producing a publishable manuscript while working on improving lab procedures we were able to optimize the returns from Heidi's efforts.

F. References

- Ben-David, M., G. M. Blundell, and J. E. Blake. 2002. Post-release survival of river otters: effects of exposure to crude oil and captivity. *Journal of Wildlife Management* 66: 1208-1223.
- Blundell, G. M., M. Ben-David, P. Groves, R. T. Bowyer, and E. Geffen. 2002b. Characteristics of sex-biased dispersal and gene flow in coastal river otters:

- implications for natural recolonization of extirpated populations. *Molecular Ecology* 11: 289-303.
- Blundell, G. M., M. Ben-David, P. Groves, R. T. Bowyer, and E. Geffen (*in press*) Kinship and sociality in coastal river otters: are they related? *Behavioral Ecology*.
- Dallas, J. F. and S. B. Pieltney. 1998. Microsatellite primers for the Eurasian otter. *Molecular Ecology* 7:1247-1263.
- Fleming, M. A., E. A. Ostrander, and J. P. Cook. 1999 Microsatellite markers for American mink (*Mustela vison*) and ermine (*Mustela erminea*). *Molecular Ecology* 8: 1352-1354.
- QIAGEN. 2002. QIAamp DNA Stool Mini Kit Handbook. QIAGEN Inc. Valencia, CA, USA.
- Raymond, M., and F. Rousset. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity* 86:248-249.
- White, G. C. 1996. NOREMARK: Population estimation from mark-resighting surveys. *Wildlife Society Bulletin* 24: 50-52.

G. Appendix including copies of all written reports or publications completed or in progress, resulting from the project work.

Hansen, H., M. Ben-David, P. Groves and J. A. K. Maier, (*in review*). What determines group formation and maintenance in coastal river otters? *Animal Behaviour*.

See attached pdf file.

Part II - Annual Financial Statement

Year-to-date Balance:

Budget Category	Budget Expenses	Remaining
Direct Costs		
Personnel	18,170	417
Travel	1,201	-401
Contractual	0	0
Commodities	16	-16
Equipment	0	0
Subtotal Direct Costs	4,847	4,847
Indirect		
Project Total		