

Stress-level determination using microarray analysis of northern green frog (*Rana clamitans melanota*) populations on reclaimed mine areas¹

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Retained settling ponds at the Powell River Project Education Center (PRP-EC) support frog populations. Species found calling at three such ponds at the PRP-EC during nightly surveys over the past three years include *Hyla chrysoscelis*, *Pseudacris crucifer*, *Rana catesbeiana*, *Rana clamitans*, and *Rana palustris* (Jansen, unpubl. data). The current health and sustainability of these populations in abandoned settling ponds is not known however. Specifically, the long-term influx and continued presence of elements normally found at low concentrations but released by the mining process into these settling ponds at higher concentrations has the potential to influence both larval and adult survivorship (e.g., Rowe et al. 2001). The present study intends to determine how stress differs in a population of the northern green frog *Rana clamitans melanota* (a frog species found commonly across several pond types at the PRP-EC) from an abandoned settling pond versus one from a relatively undisturbed pond. In doing so, the usefulness of microarrays in studies of population-based comparative environmental stress also will be illustrated.

A strong foundation for stress-differential studies in regard to varying habitat quality has been achieved using the stress-related hormone corticosterone as a measure. In a study of spotted salamanders (*Ambystoma maculatum*), Homan et al. (2003) found habitat degradation correlated with a decreased ability to respond to acute stressors, which is consistent with exposure to chronic stress. A comparison of southern toads (*Bufo terrestris*) which had been exposed to coal combustion waste to those from an unpolluted site showed that toads exposed to the polluted site were found to have increased circulating levels of both corticosterone and testosterone (Hopkins et al. 1997). While hormones have proved to be valuable tools in the study of physiological stress, the gene expression responsible for such hormonal changes can provide a more sophisticated profile of stress. Snell et al. (2003) suggested using PCR primers and cDNA arrays to examine genes that are known in model organisms to be upregulated in response to stress. This experimental model is also limiting, however, given the advancing state of genomics. Examining only certain genes leaves out potentially relevant genes for which a stress-correlation has not been previously established. Microarrays provide a far more complete profile of transcriptional activity during stress and allow advancements in understanding stress-response. For this reason, microarrays are being used to determine the extent of stress in *R. c. melanota*.

A pilot study is currently underway to determine the extent of stress levels in populations of *R. c. melanota* as a result of their presence or absence in an abandoned settling pond. The transcriptional activation of stress-related genes will be determined by analysis using GeneChip[®] *Xenopus laevis* Genome Arrays from Affymetrix (Santa Clara, CA), which can be used to study approximately 14,400 *X. laevis* transcripts. Microarrays have been used successfully for *X. laevis* specimens to profile differences in transcription based on developmental stages and the location of tissue samples using *X. laevis* genome data (Altmann et al. 2001). For this study, the same

concept is at work, although the parameters under study are quite different. The assumption will be made that the genes of the South African clawed frog *X. laevis* and *R. c. melanota* share the same function. This potential source of error is unavoidable, however, as the *R. c. melanota* genome has not been sequenced.

Two ponds have been used for this study: one retained settling pond at the PRP-EC and one pond not impacted by mining, located at the southwestern end of Powell Valley, Wise County, Virginia. Five frogs from each of the two ponds were captured at night and euthanized quickly (within one minute of capture) in the field using a double-pithing technique (see NRC 1992). The majority of the liver was removed and placed in a 15 ml tube, immediately flash-frozen using liquid nitrogen, and placed on ice until returning to the laboratory where the samples were placed in a -80°C freezer. RNA was extracted, total RNA was isolated, and RNA quality was assessed on an agarose gel. mRNA was hybridized to cDNA for microarray preparation, and the hybridization process is currently underway.

Differences in transcriptional activation between the two populations (mined and unmined) of frogs will be analyzed using analysis of variance (ANOVA; Kerr and Churchill 2001). While a microarray-based study leaves open the possibility for genes of previously unknown significance to stress to be identified, those genes known to be active in stress-response will be of primary interest. Genes associated with corticotrophin-releasing hormone, a 41 amino acid polypeptide which is integral to regulating stress-response (Valverde et al. 2001), will be of particular interest. There are large numbers of proteins generated as a result of eukaryotic stress, but there are three major transcriptional regulatory systems related to stress, as determined in *Saccharomyces cerevisiae*, with Msn2p/Msn4p being the most involved in stress-response (Estruch 2000). Genes associated with this system will also be of interest during microarray assessment and interpretation.

Stress-level data for *R. c. melanota* populations in abandoned settling ponds and undisturbed areas should provide insight into the health of local amphibian populations and the impacts of mine-related habitat alterations. Furthermore, stress levels of local populations may one day be considered and incorporated into mine reclamation efforts as an important indicator of reclamation success. The assessment of reclamation based on the presence and diversity of species alone could be succeeded by a more advanced understanding of the effects of environmental stressors on long term survival and evolution.

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