Understanding how an organism responds to its environment on the molecular level is paramount to advancing the fields of molecular biology and medicine. The model system with both well-established ecological and molecular resources, Daphnia have long been used in ecological studies and now, with the development of vast molecular resources, including a complete genome sequence, stand as one of the few model systems with both components in place. Under ideal environmental conditions, Daphnia populations are exclusively female and reproduced is parthenogenetic. However, in response to environmental stress, the appearance of males and the shift to sexual reproduction facilitates genetic recombination. Using pressure cycling technology (PCT) in combination with two-dimensional gel electrophoresis (2DGE), we were able to reliably detect differences in protein expression from individual Daphnia of distinct genotypes and from individuals exhibiting distinct phenotypes. We conclude that this highly-sensitivity method of protein extraction and detection, reveals differences in protein expression that are biologically meaningful and is an important step in understanding individual variation and how that variation matters in the context of the natural environment.

INTRODUCTION
Understanding and predicting how individual organisms respond to the molecular level to environmental change will provide new insight into the evolution of complex biological systems. This insight will lead to the development of new predictive models of interacting organisms, environmental stress and community dynamics as a function of environment and genotype/phenotype (National Science Board, 2000), advancing the field of individualized molecular medicine.

Daphnia are a powerful model for understanding basic and applied genetic problem. They are rapidly maturing into a powerful model for understanding basic and applied genetic problem. They are species of freshwater cladocerans commonly known as water fleas (Tullberg, 1878). They have long been used in ecological studies and now, with the development of vast molecular resources, including a complete genome sequence, stand as one of the few model systems with both components in place. Under ideal environmental conditions, Daphnia populations are exclusively female and reproduction is parthenogenetic. However, in response to environmental stress, the appearance of males and the shift to sexual reproduction facilitates genetic recombination. Using pressure cycling technology (PCT) in combination with two-dimensional gel electrophoresis (2DGE), we were able to reliably detect differences in protein expression from individual Daphnia of distinct genotypes and from individuals exhibiting distinct phenotypes. We conclude that this highly-sensitivity method of protein extraction and detection, reveals differences in protein expression that are biologically meaningful and is an important step in understanding individual variation and how that variation matters in the context of the natural environment.

PRESSURE CYCLING TECHNOLOGY
Pressure Cycling Technology (PCT) has been shown to be effective for isolating proteins, nucleic acids, lipids, and small molecules from a wide range of cells, tissues, and small organisms. PCT uses alternating cycles of high pressure to efficiently disrupt cells and tissues. Rapid cyclic high and low pressure has been demonstrated to be more disruptive than sustained high pressure.

Our goal was to demonstrate that it is possible to conduct a comparative analysis of protein expression and to analyze biologically relevant variation from an individual Daphnia. Using pressure cycling technology (PCT) for sample preparation and two-dimensional gel electrophoresis (2DGE), we were able to detect individual variation from single Daphnia of distinct genotypes (lin1b vs. Xinb) and exhibiting distinct phenotypic differences (parthenogenetic vs. sexual). We were able to detect individual variation from single Daphnia of distinct genotypes (lin1b vs. Xinb) and exhibiting distinct phenotypic differences (parthenogenetic vs. sexual).

MATERIALS AND METHODS
Daphnia cultures Daphnia cultures were maintained in 8 L of COMBO media (Kilham and others, 1998) at a density of 30 individuals/L. Cultures were maintained at 20 ± 1°C under a 16:8 h light:dark photoperiod of low intensity. Cultures were fed daily with 1 mg Carbon/L of the green algae Ankistrodesmus falcatus (obtained from LIMEK Corporation, Danvers, MA). Prior to PCT, gut contents were minimized by allowing the microcrustaceans to feed on copolymer flakes. Components of PCT Shredder Pulse Tube. The screw cap (sc) is threaded into the base of the pulse tube. The polished stainless steel and the Barocycler 3229 were from Pressure Biosciences (Easton, MA). Individual Daphnia were transferred to PULSE Tubes and suspended in 550 mL of 100 mM Na+ST buffer supplemented with protease inhibitor mix. PCT was performed in 60 cycles at 36,000 psi maximum pressure. Following PCT, each PULSE tube was cooled to 4°C and spun at 10,000 RCF. The PULSE tube was removed and centrifugation continued for 4 minutes at 4000 RCF.

Detection of differential protein expression between individual Daphnia by Pressure Cycling Technology (PCT) and two-dimensional gel electrophoresis

ABSTRACT
Reduction, alkylation, and ultraltrification
Samples were transferred to ULTRA-4 ultradry devices with 10 Kda MWCO (Millipore Corporation, Danvers, MA). Centrifugation assisted the ultradry and the samples were exchanged with fresh UTC until the final DTT concentration was 10 mM. Reduction and alkylation of the samples were performed directly in the ultradry devices using 5 mM tris(2-carboxyethyl)phosphine and 50 mM acrylamide as described (Smejkal and others, 2006a).

IEF and 2DGE
Two hundred µL of each sample was placed onto individual wells in IPG rehydration trays from Proteome Systems (Woburn, MA). Bio-Rad ReadyStrip®IPG strips with a pH range of 4-7 (Hercules, CA) were placed onto each sample, and the tray was placed into a humidifying chamber. Rehydration occurred over six hours until all the sample was visibly absorbed by the strip. At the termination of rehydration, strips were placed into isoelectric focusing tubes and ran for 10,000 volts in an accumulation of 400,000 volt-hours. Strips were equilibrated, then placed onto Criterion Tris-HCl 8.3% IPG+1 gel (Bio-Rad Laboratories, Hercules, CA) and ran at 120 V and 60 ma/gel.

Digital image analyses
The 24-bit images were analyzed using PDQuest™ software (Bio-Rad, v.7.1). Background was subtracted and protein spot density peaks were detected and counted. A reference pattern was constructed from one of the individual gels to which each of the gels in the matchset was matched. Numerous proteins that were uniformly expressed in all patterns were used as landmarks to facilitate rapid gel matching. After matching, the total spot count was determined in each gel.

Results and Discussion
Our goal was to demonstrate that PCT and 2DGE are effective methods of sample preparation to detect differences in protein expression between individual Daphnia of distinct genotypes. Individual D. magna from lin1b and Xinb genotypes were isolated, proteins extracted and analyzed in quadruplicate by 2DGE as described above. Silver staining detected an average of 689 ± 11 protein spots from the Xinb gels and 692 ± 14 protein spots from the lin1b gels. After normalization of the 2DGE image analysis for differential of Daphnia genotypes isolated in the same way under identical conditions, 678 spots were matched between the two gel images. A total of 136 spots showed a two-fold or greater difference in spot intensity. 79 of these were more abundant in Xinb and 57 were more abundant in the lin1b.

Protein variation between individual D. magna of distinct genotypes
We were also able to demonstrate that differences in protein expression could be detected between individual Daphnia with distinct phenotypes. Individual D. magna, with and without epiphyses, were isolated, proteins extracted and subjected to 2DGE as described above. Silver staining detected 524.5 ± 7.8 protein spots in 2D gels produced from single D. magna. After normalization of the gel images based on total intensity, 266 spots were matched between the two gel images. A total of 48 spots showed a three-fold or greater difference in spot intensity. Fifty-five of these were more abundant in the parthenogenetic (no epiphysis) animal, while 29 were more abundant in the sexual animal. In addition, eleven protein spots unique to the parthenogenetic phenotype, while 49 protein spots were unique to the sexual phenotype. This demonstrates the feasibility of 2DGE and image analysis for the differentiation of Daphnia genotypes isolated in the same way under identical conditions.

Conclusions
PCT, which rapidly cycles pressure, has been shown to be an effective means for isolating proteins from a variety of microorganisms, as well as many difficult to lyse samples such as Caenorhabditis elegans (Smejkal and others, 2007; Geiser and others, 2002; Smejkal and others, 2006b). PCT facilitated extraction of proteins from single Daphnia magna with distinct phenotypes (sexual vs. parthenogenetic) and with distinct genotypes (Xinb vs. lin1b) and from single Daphnia pulex. 2DGE and silver staining revealed that protein expression differences between individual Daphnia can be detected using single animals. Proteins from a single Daphnia genotype, while biased towards the most abundant proteins, represent a functionally diverse set of proteins. The highly sensitive technique of using PCT to extract proteins, coupled with 2DGE, represents an important step to a greater understanding of individual variation and genotype/phenotype expression, how individuals interact with their environment on a molecular level and is critical to advancing the field of individualized molecular medicine.

References