

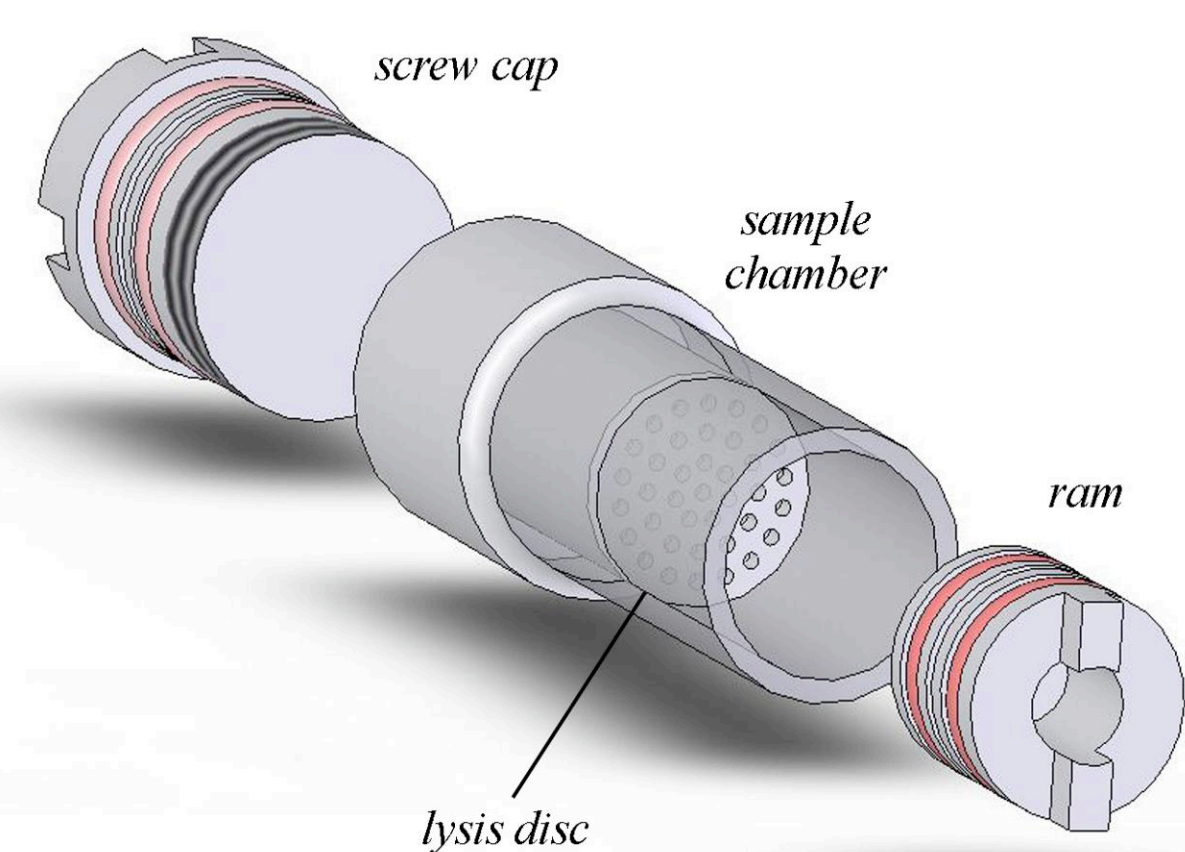
## 1. Abstract

*Daphnia* are parthenogenic microcrustacea belonging to the family Daphniidae. Under normal environmental conditions, *Daphnia* populations are exclusively female and reproduction is clonal. However, in response to adverse environmental stimuli, sexual reproduction is induced enabling genetic recombination and rapid adaptive response. Sexual daphnids produce resting eggs, termed ephippia, which can remain viable for centuries. Therefore, the analyses of Daphnids grown from ephippia isolated from layers of lake or stream sediment could potentially provide a chronology of environmental changes over several decades. Hence, it is of vital importance to be able to derive sufficient protein from a single *Daphnia* for such phenotypic analyses to be possible. Sample preparation involved using a pressure cycling technology (PCT) in which arthropods were disrupted at 35,000 psi maximum pressure, followed by ultrafiltrative exchange to remove non-proteinaceous components from the homogenate. Two-dimensional gel electrophoresis (2DGE) was capable of resolving differences between asexual and sexual phenotype from solitary *Daphnia magna*. For the smaller *Daphnia pulex*, 2DGE resolved  $904 \pm 7$  protein spots from a single organism, and  $1,267 \pm 3$  protein spots from a pool of five organisms. These data suggest the feasibility of using 2DGE for following phenotypic response to environmental stimuli such as hepatotoxin contamination during cyanobacterial blooms.

## 2 Materials and Methods

### 2.1 *D. pulex* cultures

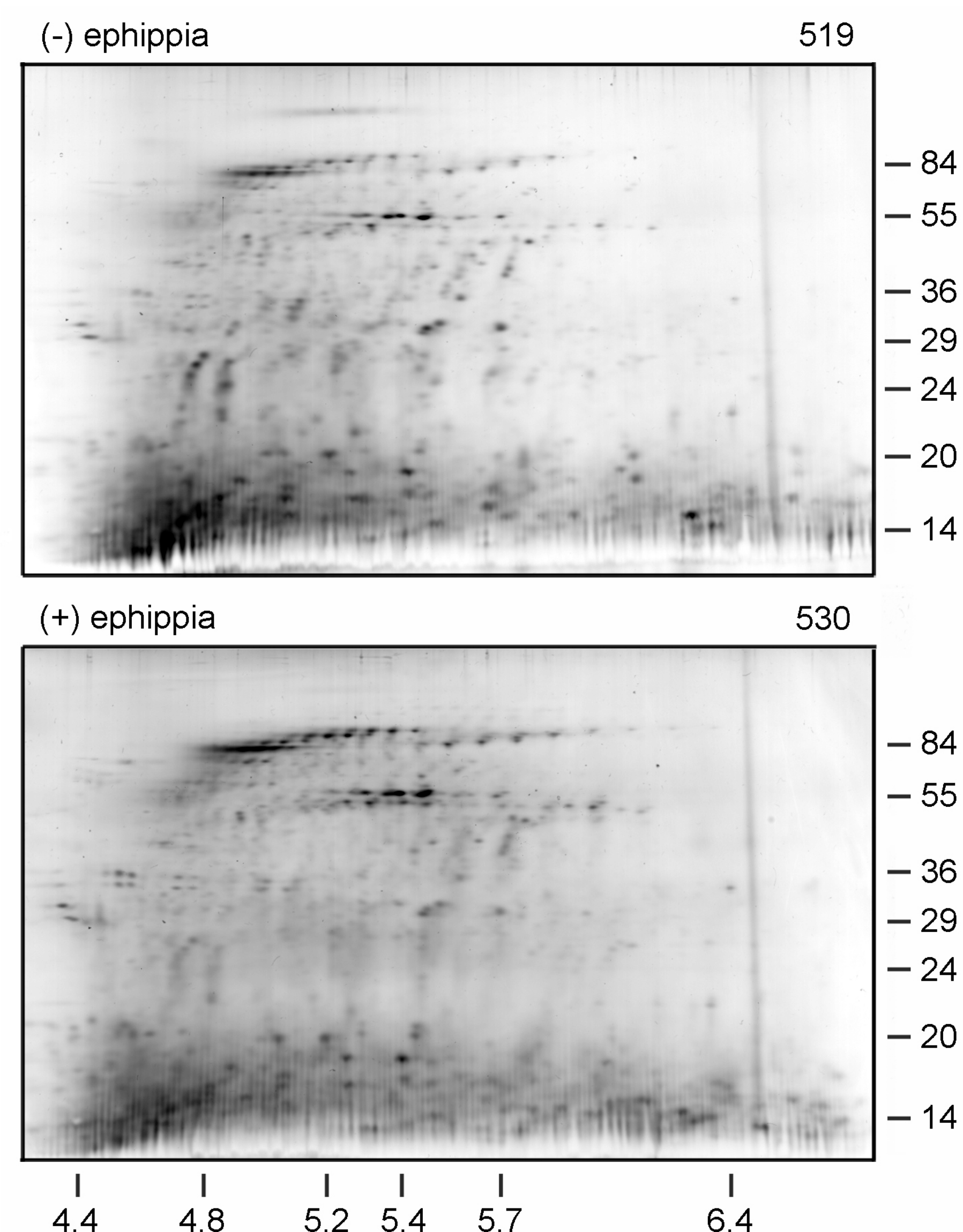
Cultures were maintained in 8 L of modified COMBO media [1] at a density of 30 individuals/L. Daphnids were cultured at  $20^\circ \pm 1^\circ\text{C}$  under a 16:8 hours light:dark photoperiod of low intensity. Cultures were fed daily with 1mg C/L of the green algae *Ankistrodesmus falcatus* obtained from The Culture Collection of Algae at (University of Texas, Austin, TX, USA). Daphnid gut contents were minimized by allowing the microcrustaceans to feed on copolymer microspheres of 4.3 micron mean diameter (Duke Scientific, Fremont, CA, USA) for one hour prior to harvesting. Microspheres were fed at a concentration equal to the number of algal cells previously supplied. *D. pulex* were harvested by filtration through 250 um Nitex mesh (Sefar America, Depew, NY, USA).



**Figure 1.** Exploded view showing the components of the PULSE Tube FT-500. Under high pressure, the ram forces tissue and fluid through the perforated lysis disc. Upon return to ambient pressure, the ram retracts pulling in solvent from the other chamber.

PDF available at [www.pressurebiosciences.com](http://www.pressurebiosciences.com)

Daphnia Genome Consortium Meeting, Bloomington, IN, July 7-9, 2007. Poster No. \_\_\_\_\_



**Figure 2.** Phenotypic differences in *D. magna* (+) or (-) ephippia displayed by 2DGE. A single organism of each phenotype was processed by PCT for each analysis. The number of protein spots in each gel is indicated (upper right). Protein molecular weight and isoelectric point (pI) are estimated (ordinate and abscissa, respectively). Estimates of pI assume linearity of the IPG.

### 2.2 *D. magna* cultures

*D. magna* starter cultures were obtained from Sachs Systems Aquaculture (St. Augustine FL, USA). Stabilized cultures were maintained in 8 L of 25% mineralized water (Vermont Spring Water Company, Brattleboro, VT, USA) at a density of 60-120 individuals/L. Daphnids were cultured at  $22^\circ \pm 1^\circ\text{C}$  under constant illumination with standard fluorescent bulbs. Cultures were maintained at pH 7.0-7.4 by the addition of 100 g/L crushed coral (Tideline, Inglewood, CA, FL, USA) supplied in nylon bags. Starter cultures were fed daily with 1 mL/L of *Nanochloropsis* microalgae liquid concentrate (Reed Maricultures, Campbell, CA, USA) for the first four weeks, followed by 0.1 mL/L thereafter. Average mass of adult *D. magna* was  $1.37 \pm 0.46$  mg fully hydrated and  $0.23 \pm 0.06$  mg when dehydrated ( $n = 64$ ) indicating a 90.6% water content.

### 2.3 Pressure Cycling Technology (PCT)

The NEP-3229 Barocycler and PULSE Tubes were from Pressure BioSciences (West Bridgewater, MA, USA). Daphnids collected in PULSE Tubes were suspended in 500 uL of 7M urea, 2M thiourea, and 4% CHAPS (IEF reagent) supplemented with 100 mM dithiothreitol (DTT) and protease inhibitor cocktail (Sigma Aldrich Chemicals, St. Louis, MO). An additional 900 uL of mineral oil to meet the minimum volume requirement of the PULSE tube. Tubes were processed for 60 pressure cycles, each cycle consisting of 10 seconds at 35,000 psi followed by rapid depressurization and hold for 2 seconds at atmospheric pressure. Following PCT, the mineral oil was removed.

### 2.4 IEF and 2DGE

Proteins were reduced and alkylated directly in the ultrafiltration devices as previously described [2]. Dried immobilized pH gradients (Bio-Rad, Hercules, CA, USA) pH 4-7 were hydrated for six hours with 200 uL of each sample. Isoelectric focusing (IEF) and 2DGE was performed as described [3]. Gels were stained with SilverQuest Silver Stain Kit (Invitrogen, Carlsbad, CA, USA). Images were analyzed using PDQuest™ Version 7.1 software (Bio-Rad, Hercules, CA, USA). Background was subtracted and protein spot density peaks were detected and counted. After background subtraction and spot matching, the total spot count was determined for each gel.

### 2.7 LC-Tandem MS protein identification

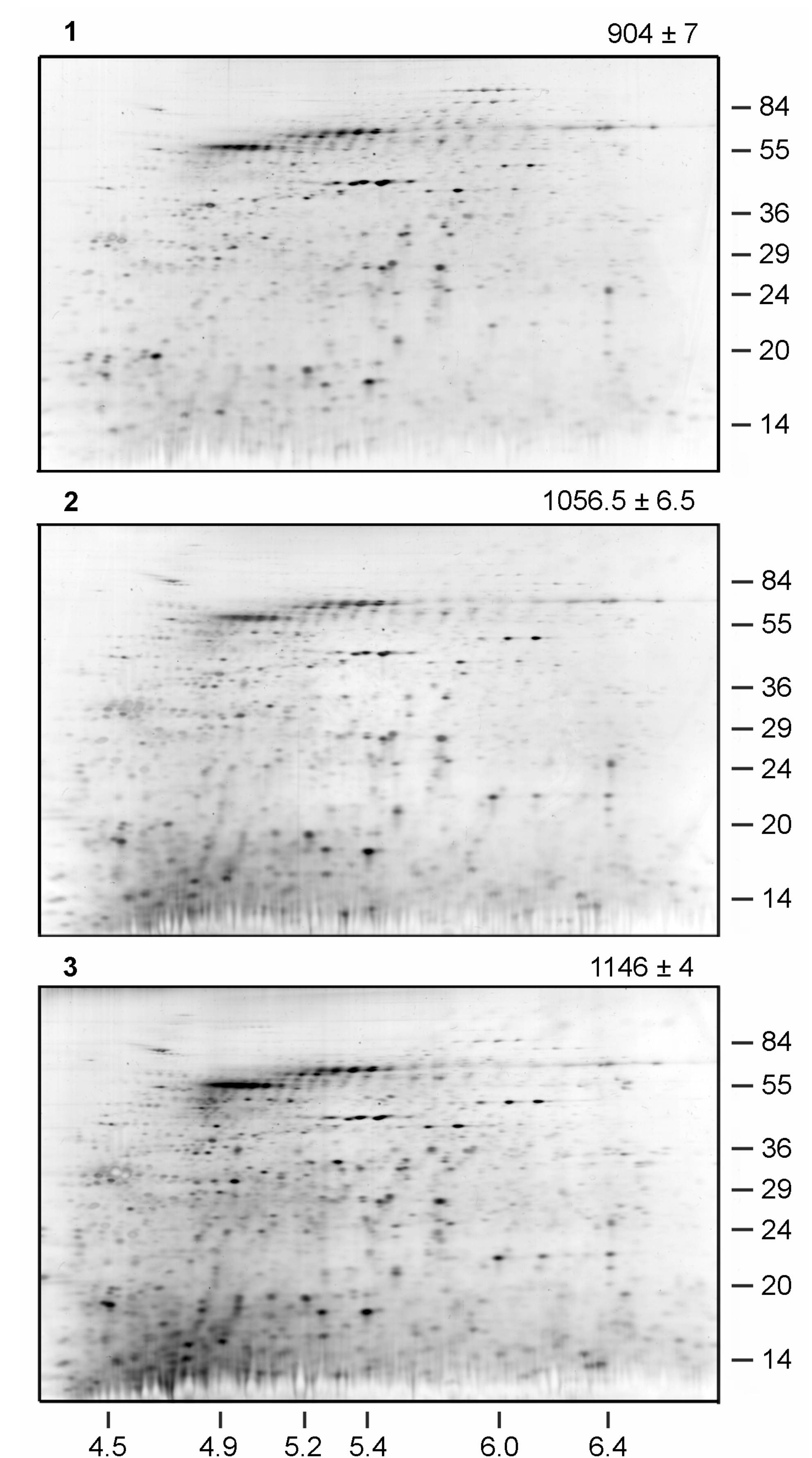
Protein bands were cut, destained in Farmer's reagent, and treated with trypsin (5 uL of 20 ng/uL trypsin in 50 mM ammonium bicarbonate) overnight at room temperature. The peptides that were formed were extracted from the polyacrylamide, evaporated to near dryness, and reconstituted in 30 uL of 1% acetic acid. The LC-MS system was a Finnigan LTQ ion trap mass spectrometer system. The HPLC column was a self-packed 9 cm x 75 um ID Phenomenex Jupiter C18 reversed-phase capillary

chromatography column. Two microliter volumes were injected and the peptides eluted from the column by linear acetonitrile gradient at a flow rate of 0.2 uL/min. The MS system used a data-dependent multitask capability that acquires a full scan mass spectra to survey the column eluate followed by 3 to 5 product ion spectra to determine amino acid sequence in successive scans. This mode of analysis produces approximately 2500 collisionally induced dissociation (CID) spectra, although not all CID spectra are derived from peptides. The data were analyzed by using all CID spectra collected in the experiment to search the NCBI non-redundant database with the search program Mascot. Each identification is verified by manual interpretation of at least two spectra.

## 3. Results and Discussion

### 3.1 Image analysis of 2D gels

Figure 2 shows silver stained 2D gels revealed 519 and 530 protein spots from single unephippiated and ephippiated *D. magna* organisms, respectively. Image analysis comparing the two phenotypes detected 60 mismatched proteins. These data demonstrate the feasibility of using 2DGE for following phenotypic response to environmental stimuli. For the smaller *Daphnia pulex*, 2DGE resolved  $904 \pm 7$  protein spots from a single organism, and  $1,267 \pm 3$  protein spots from a pool of five organisms. Figure 3 shows 2DGE of 1, 2, or 3 individual *D. pulex*. Figure 4 shows the number of

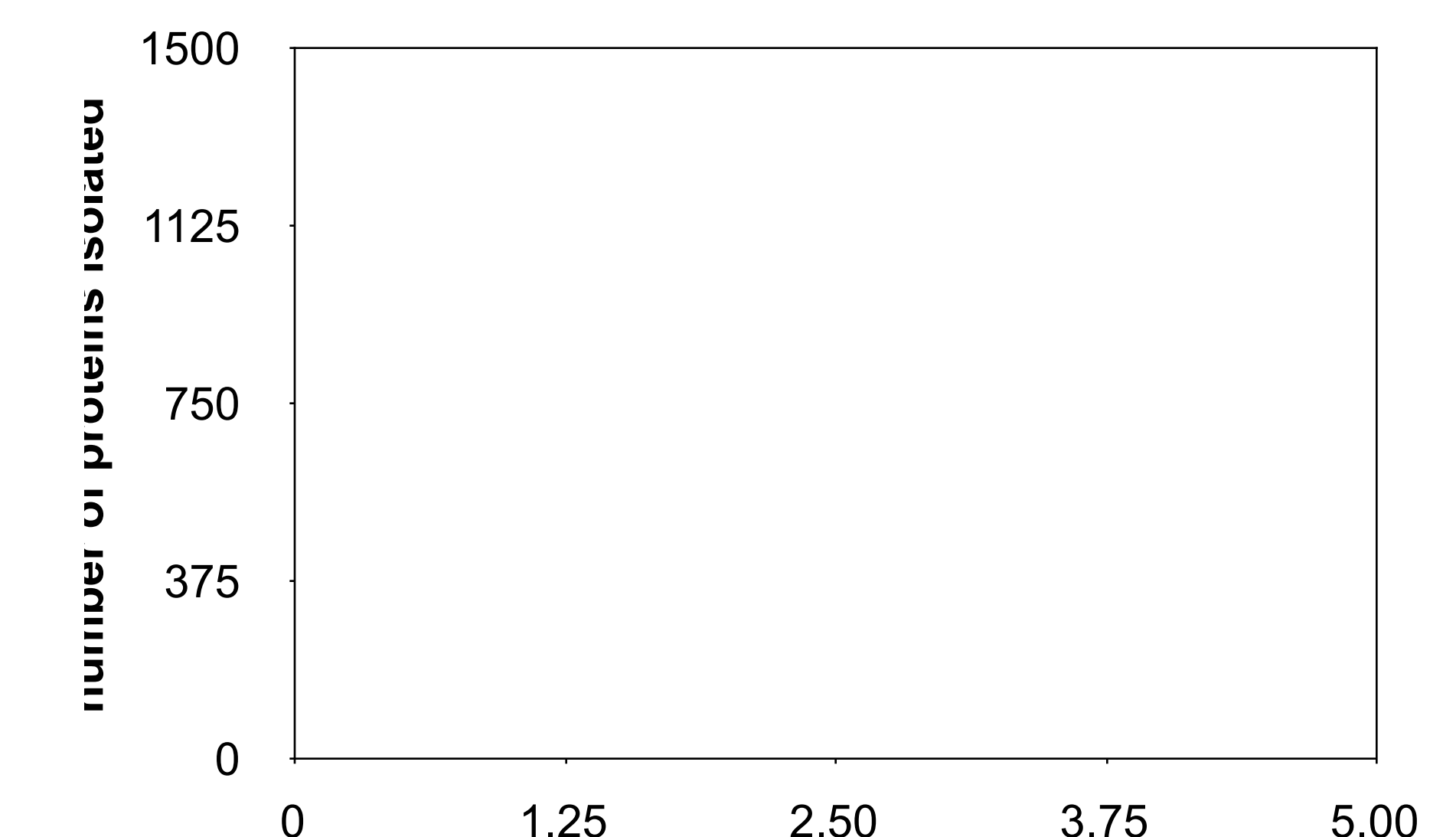


**Figure 3.** Representative silver stained 2D gels of 1, 2, or 3 individual *D. pulex* organisms. The number of protein spots (mean  $\pm$  SD) from duplicate gels are indicated (upper right).

protein detected as a function of the number of organisms. In duplicate gels, low coefficient of variation (CV) indicated the high degree of reproducibility.

### 3.2 Status of protein identification

We are using two overlapping approaches to identify the proteins in these experiments. Our first approach is the best way to identify selected proteins - by cutting them directly out of the gel being considered. For silver stained bands, however, the LC-tandem MS identification has a 50% success rate, so this approach has some practical limits. Therefore, we are also using a second approach in which parallel gels are run with higher protein loads specifically for the identification experiment. Our ultimate goal is to identify all proteins in the gel and annotate the gel in a manner that allows subsequent experiments to identify a protein based on its position in the gel.



**Figure 4.** The number of protein spots detected by silver staining as a function of the number of *D. pulex* and the reproducibility of 2DGE.

## 4. Conclusion

The fast, efficient, and accurate release of proteins from cells and tissues is a critically important initial step in most analytical processes, and is essential to reliable proteomic analyses. 2DGE can be an accurate representation of a proteome only if the entire protein constituency of cells is recovered during the sample preparation process. PCT uses alternating cycles of high and low hydrostatic pressure to effectively induce the lysis of cells and tissues. Previously, PCT has been shown to release high molecular weight proteins associated with the chitin present in exoskeleton [4]. In these experiments, two-dimensional arrays of the *D. pulex* and *magna* proteomes were elicited from single organisms by PCT. Downstream proteomic analyses, including the identification of proteins and their post-translational modifications, will ultimately improve our understanding of the biological processes involved in the adaptive response to adverse environmental changes.

## 4. References

- [1] Jahnke L.S., White A.L. (2003). *J. Plant Physiol.* 160, 1193-1202.
- [2] Smejkal G.B., et al. (2006). *J. Proteomic Res.* 5, 983- 987.
- [3] Smejkal G.B., et al. (2007). *Anal. Biochem.*, 363, 309-311.
- [4] Smejkal G.B., et al. (2006). AACC, Oakridge Conference, San Jose, CA.