**Additional File 1.** Materials and Methods, Supplementary Information

*Sample Preparation for Proteomics Analysis.* The BALF was filtered through sterile gauze and centrifuged at approximately 300 x *g* for 10 minutes. The cell-free supernatant was stored at -80 oC in 1 mL aliquots until further analysis. Protein concentration in the BALF supernatant was measured with a BCA Assay,[[1]](#endnote-1) according to manufacturer’s protocol. Protein standards were prepared using bovine serum albumin (BSA) included in the BCA Assay kit; sodium chloride (0.9% in dH2O) was used as a blank. Measurements were performed in triplicate. The three measurements for each sample were averaged, and this value was used as the absorbance for that sample. The absorbance of the blank was subtracted from each standard and sample. A standard concentration curve was generated using the BSA protein standards. The linear equation of the trendline was calculated, and the concentration of each sample was calculated from this equation.

For each BALF sample, the volume of fluid containing 100 µg of protein was mixed with cold acetone (three times the volume of sample) and incubated at -20 oC for 30 minutes to precipitate proteins. The samples were centrifuged for two minutes to concentrate the protein, and the supernatant was discarded. The samples were lyophilized to complete dryness (approximately 15 minutes). Trypsin digestion was performed according to standard laboratory protocol. Denaturation solution (10 µL of a solution of 8M urea with 10mM dithiothreitol in 10 mL dH2O) was added to each sample and incubated for 90 minutes at 37 oC. Ammonium bicarbonate(100 mM, 2 µL) and reducing cocktail (10 µL of a solution of 195 µL acetonitrile, 1 µL triethylphosphine, and 4 µL 2-iodoethanol) were added to each sample and incubated at 37 oC for 90 minutes. The samples were lyophilized overnight to complete dryness. The following day, each sample was resuspended in 80 µL of 100 mM ammonium bicarbonate. Trypsin was added at a ratio of 1 gram trypsin to 50 grams protein (2 µg of 0.5 µg/µL trypsin) to each sample, and incubated overnight at 37 oC. On the final day, 1 µL of 10% trifluoroacetic acid (TFA) was added to each sample to stop the digestion after approximately 15-18 hours.

Each sample was desalted over a C18 column,[[2]](#endnote-2) according to standard laboratory procedure. Each C18 column was placed in a 1.5 mL tube and equilibrated by washing with 500 µL acetonitrile and centrifuging at 100 x g for 3 minutes. The filtrate was discarded. The column was washed twice with 500 µL dH2O, centrifuged at 100 x g for 3 minutes, and the fluid was discarded. The entire sample (approximately 80 µL) was added to a column, and centrifuged at 100 x g for 2 minutes; the filtrate was discarded. The column was washed twice with 200 µL 0.01% TFA in dH2O, centrifuged at 100 x g for 2 minutes, and the filtrate discarded. Each column was placed in a new 1.5 mL tube and washed twice with 50 µL 80% acetonitrile, centrifuged at 100 x g for 2 minutes, and the filtrate was retained (sample). After the final column wash, the samples were lyophilized overnight to complete dryness. All samples were reconstituted in 100 µL of 0.01% TFA (final protein concentration estimated 1 µg/µL) before mass spectrophometric analysis. Samples were processed in batches, and the peptide solutions were stored at -20 oC until all samples were processed.

*LC-MS Analysis.* The peptides were separated on a nanoLC-Chip system.[[3]](#endnote-3) After injection of 1 µg of sample, the peptides were concentrated in the on-chip 300SB-C18 enrichment column and washed with buffer A (5% acetonitrile, ACN/0.01% TFA) at a flow rate of 4 μl/min for 5 minutes. The enrichment column was switched into the nano flow path and further separated with the on-chip C-18 reversed phase column (0.075 µm x 43 mm) coupled to the electrospray ionization (ESI) source of the ion trap mass spectrometer (XCT Plus). The column was eluted with a 55 minute linear gradient from 5%-35% buffer B (100% acetonitrile, 0.01% TFA) at a rate of 600 nl/min, followed by a 10 minute gradient from 35%-100% buffer B. The column was re-equilibrated with an isocratic flow (5% buffer B) at 600 nl/min. ChemStation softwarec was used to control the system. LC-MS chromatograms were acquired in positive ion mode under the following conditions: a capillary voltage of 1850 V and an end plate offset of 500 V. The dry temperature was set at 300°C. Dry gas flow was maintained at 4 L/min. Acquisition range was 350-2200 m/z with 0.15 second maximum accumulation time and scan speed of 8100 m/z per second.

*LC-MS/MS Analysis.* Tandem MS (MS/MS) analysis was performed on one horse from each group from the first baseline and the exposure testing periods. To identify differentially expressed peptides, automated MS/MS spectra were acquired during the run in the data-dependent acquisition mode with the selection of the three most abundant precursor ions (0.5 min active exclusion; 2+ ions preferred). The MS/MS files acquired on the ion trap mass spectrometer were uploaded to Spectrum Mill protein identification softwarec and searches were performed using Spectrum Mill and the NCBI database.[[4]](#endnote-4) The search parameters were: no more than two tryptic miscleavages allowed, cysteine searched as ethanol cysteine, variable oxidized methionine, 2.5 Da peptide tolerance, and 0.7 Da mass tolerance. Only peptides with a score of 5 or higher were considered true positives.

*Peak Identification.* The LC-MS/MS data obtained from one heaves-affected horse and one control horse were uploaded into Spectrum Mill and searched against the NCBI non-redundant mammalian protein database and the NCBI non-redundant equine protein database. The PDP peak data and the Spectrum Mill peptide data were aligned by merging the output files from the PDP statistical analysis and Spectrum Mill peptide identification for each horse into one file, and a list of potential peptide identifications was made for the peaks based on m/z and retention time. As the LC-MS data (used for the PDP) and LC-MS/MS data (used for Spectrum Mill) are not exactly the same, the m/z and retention time data from the two output files were slightly different. First, peaks (PDP data) were aligned with potential peptide identifications (Spectrum Mill data) within ± 3 Da and ± 3 minutes for the RT. Each peak was then manually reviewed in the spectra and evaluated for charge. For each potential identification, the charge of the peptide (from the Spectrum Mill data) was compared with the charge of the peak (manual spectra review). Any peak with an incorrect charge was deleted. From this list, the peak with the same charge as the peptide and the closest m/z and retention time was chosen as the best match for that peptide. Finally, for any peaks that were matched to more than one peptide, a single peptide was selected based on the highest Spectrum Mill score and percent scored peak intensity (% SPI) and the lowest Spectrum Mill reverse score.

*Western blot.* All Western blots were performed according to the manufacturer’s recommendations.[[5]](#endnote-5) Western blots were performed using 2 µg of protein from each sample. Ten BALF samples were more dilute than 0.2 µg/µL, and 2 µg of protein from these samples was a larger volume than 10 µL (largest volume able to be used in the mini gels). These samples were concentrated on a 1K molecular weight cut-off filter.[[6]](#endnote-6) Samples were centrifuged at 2750 x *g* in order to achieve a concentration of approximately 0.2-0.3 µg/µL. Protein concentration was measured before and after sample concentration with a BCA Assay. Electrophoresis was performed at 200 Volts for approximately 30 minutes. The transfer was performed at 30 Volts for one hour. Following transfer, the nitrocellulose membrane was air-dried for 60 minutes, then blocked with 5% normal horse serum (NHS) for 45 minutes. The membrane was washed twice in Tris-buffered saline (TBS)-Tween20 (0.1%) for five minutes each time. The primary antibody was diluted in 19.9 mL TBS-Tween20 (0.1%) and 0.1 mL NHS to the appropriate dilution. The membrane was incubated in the primary antibody overnight at 4 oC with gentle rocking.

The following morning, the membrane was washed four times in TBS-Tween20 (0.1%) for 5 minutes, 5 minutes, 10 minutes, and 15 minutes. The secondary antibody was diluted in 19.9 mL TBS-Tween20 0.1% and 0.1 mL NHS to the appropriate dilution. The membrane was incubated in the secondary antibody for 60 minutes at room temperature. The membrane was washed four times in TBS-Tween20 (0.1%) for 5 minutes, 5 minutes, 10 minutes, and 15 minutes.

Western Blotting reagents[[7]](#endnote-7) were warmed to room temperature and used for development of the blot. The reagents were poured over the membrane and incubated for 60 seconds. The excess reagent was poured off the membrane, and the membrane was exposed to chemiluminescence film[[8]](#endnote-8) at room temperature. The films were developed with a standard x-ray developer. ImageJ softwared was used to quantify the density of the bands from the protein of interest on each Western blot.

1. Thermo Scientific Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Inc., Rockford, IL [↑](#endnote-ref-1)
2. C-18 Vydac, 300 A, The Nest Group, Southborough, MA [↑](#endnote-ref-2)
3. 1100 Series LC equipped with HPLC Chip interface; ZORBAX 300SB-C18 analytical column; Agilent, Santa Clara, CA [↑](#endnote-ref-3)
4. National Institutes of Health, Bethesda, MD [↑](#endnote-ref-4)
5. XCell SureLock™ Mini-Cell and XCell II™ Blot Module, Invitrogen, Carlsbad, CA [↑](#endnote-ref-5)
6. Pall Life Sciences, Ann Arbor, MI [↑](#endnote-ref-6)
7. Amersham™ ECL™ Western Blotting Analysis System Reagents, GE Healthcare, Piscataway, NJ [↑](#endnote-ref-7)
8. Kodak, Rochester, NY [↑](#endnote-ref-8)