Evaluating gene expression in CWD-infected white-tailed deer W-178-R

Final Report FY 2018

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FINAL REPORT – FY2017-2018
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W-178-R-1

EXECUTIVE SUMMARY

Objectives

1. Evaluate post-mortem intervals of ≥25 hunter-harvested white-tailed deer for estimating RNA stability by 31 March 2017.
2. Determine (e.g., identify) differentially expressed genes in 10 CWD-infected and 10 uninfected white-tailed deer by 31 March 2017.
3. Create a genetic profile of CWD-infected white-tailed deer by 31 March 2017.
4. Evaluate the efficacy of genetic (transcriptome) profiling for identifying CWD-infected white-tailed deer by 31 March 2017.

Methods

We obtained tissue samples from hunter-harvested deer in Hancock County, Illinois, USA during the 2015 and 2016 annual firearm seasons. At the end of each sampling day, all harvested WTD were transported to a central location for tissue and biopsy collection. Biopsies were collected in field conditions (e.g., exposure to ambient temperatures, precipitation, no preservation) to determine effects of field conditions and intrinsic factors (i.e., body temperature) on PMI. We rinsed tissues using. Prior to collecting tissue samples, we extracted retropharyngeal lymph nodes (RPLN) and brain stems (BS) from all animals. Additionally, we rinsed tissues using double-distilled water (ddH2O) and removed excess body fluids (e.g., blood) before collecting biopsy samples (using 6 mm Miltex biopsy punches; Ref. Num. 33–36) from multiple tissues (i.e., liver [LV], BS, RPLN). We extracted samples at predetermined PMIs (i.e., 2, 8, 16, 24, 36, 48 hrs.), placed them in 1.5 mL centrifuge tubes, and immediately fixed them with liquid nitrogen prior to storage at –80º C. We attempted to collect tissue samples immediately (i.e., PMI = 0 hr) upon death, though this was not logistically feasible for hunter-harvested animals.

A 2 mm × 2 mm tissue biopsy was placed into a 1.5 mL tube with 350 µL of Qiagen RLT Buffer (Cat No. 79216) and 1% β-mercaptoethanol. Using a Kontes pellet pestle motor (ThermoScientific, Cat. No. K749515) and a disposable pestle, we homogenized RNA and centrifuged tissue lysates at full speed (e.g., 14,000 g) for 3 minutes. Supernatant was transferred to a clean tube and RNA was extracted from tissue lysate with the Qiagen RNeasy Mini Kit (Cat No. 74104); RNA concentrations were determined using a NanoDrop 1000 (ThermoScientific). We assessed RNA integrity using a 2200 TapeStation system and RNA ScreenTape (Agilent, Cat No. 5067-5576). TapeStations use an algorithm to assign a RNA Integrity Number (RIN) between 1 and 10 (i.e., 1 = completely degraded, 10 = completely intact) to each sample (Padmanaban 2012).

Prior to analyses, we screened all predictor variables for collinearity using Pearson's correlation coefficients ($|r| > 0.5$) and used quantile plots to evaluate assumptions of normality;

we used only 1 variable from a set of collinear variables for modeling (Jacques et al. 2011). Potential effects of body and daily temperature were collinear with PMI and thus, not included as covariates in analyses. We used a two-stage least squares regression model to evaluate potential effects of tissue type on RIN. Specifically, we calculated average rates of change in RIN (ΔRIN) for each individual tissue combination and fit a linear model with ΔRIN as the response variable. We used the estimated slope and variance for ΔRIN to fit a weighted mixed model; weights were the inverse variances of the estimated ΔRIN effects. By using weights, we up-weighted data points resulting from strong estimates and down-weighted data points resulting from weak estimates (A. Martin-Schwarze, Iowa State University, personal communication). The fitted model tested whether the slope of the RIN trend differed between tissue types. We conducted analyses using Program R (R Core Team 2015); statistical tests were conducted at $\alpha = 0.05$.

Liver and retropharyngeal lymph node (RPLN) samples were collected from 380 deer euthanized by Illinois Department of Natural Resource (IDNR) wildlife managers during annual population reduction (e.g., sharpshooting) and disease monitoring efforts. Deer were euthanized according to the IDNR CWD management plan. This program takes place throughout the chronic wasting disease (CWD)-endemic area of northcentral Illinois January–March 2015. Before sample biopsy, tissues were rinsed using double distilled water (ddH_2O) and any blood removed. Retropharyngeal lymph nodes were then extracted from all adult (≥ 1.5 yrs. old) deer. Samples were biopsied from multiple tissues (e.g. liver [LV], RPLN) using 6 mm Miltex biopsy punches (Ref. Num. 33-36). Livers were biopsied in approximately the same location every time, while one RPLN was randomly sampled. Biopsied tissues were placed into 1.5mL centrifuge tubes and stored using 1.5mL RNAlater (ThermoFisher Scientific, Cat. No. AM7020) per the manufacturer's recommendations. We placed all tissue samples in a refrigerator for 24 hrs at 2º C after which, they were placed in a freezer (–10º C) on site. Each week, we transported all tissue samples to Western Illinois University, at which time they were placed in a deep freezer (–20º C) until transported to the Core Genomics Laboratory at University of Illinois Chicago for sequencing.

We compared our samples to those IDNR biologists submitted to the Animal Disease Laboratory in Galesburg, IL, USA for disease testing (i.e., immunohistochemistry; IHC). Once disease state was determined (i.e., CWD prions observed or absent in tissue), a subset of male and female CWD-infected deer were randomly chosen and paired with uninfected deer of similar sex and age classes (i.e., adult) and harvest location. We used RPLN and LV tissue samples from 10 (5 CWD-positive [treatment group], 5 CWD-negative [control group]) adult female deer for RNA-Seq analyses.

Using a Qiagen RNeasy Mini Kit (Cat. No. 74104), we extracted RNA from each sample according to the manufacturer's instructions. We examined RNA integrity and quantity using a NanoDrop 1000 and a 2200 TapeStation system using RNA ScreenTape (Agilent, Cat. No. 5067-5576). A total of 1 µl RNA (RIN > 7) was used for RNA-Seq library construction. Additionaly, cDNA library was prepared with the TruSeq Stranded mRNA LT Sample Prep Kit- Set A (Illumina, Cat. No. RS-122-2101) and amplified using polymerase chain reaction (PCR). Specifically, we used the Illumina HiSeq 2500 Sequencing System (Illumina Inc., San Diego, CA, USA) with a HiSeq SBS sequencing kit. Resulting reads were 100 nt in length and sequenced on one lane from each end for 101 cycles. Data files (i.e., FASTQ) were generated

and demultiplexed with the bcl2fastq v1.8.4 Conversion Software (Illumina). We used RNA-Seq to analyze LV and RPLN tissue samples from infected and uninfected deer.

Cleaned reads were used for the reference transcriptome assembly based on Trinity version 2.06 with paired-end mode (Grabherr et al. 2011). Transcripts from liver and lymph node tissues were separately *de novo* assembled, then a reference transcriptome was generated by merging assemblies from both tissues, and reductant transcripts were filtered by CD-HIT software (Li and Godzik 2006). To filter out any misassembled transcripts, raw sequenced reads were mapped to assembled reference transcriptomes using Bowtie 1.0.0 (Langmead et al. 2009). Transcript abundance was estimated using RSEM software (Li and Dewey 2011), fragments per kilobase per transcript per million mapped reads (FMPK) values were calculated and transcripts with FPKM <1 were filtered out (Li and Godzik 2006). Filtered transcripts were used as the deer reference transcriptome for downstream analysis (Grabherr et al. 2011).

Assembled transcriptomes were annotated using BLASTX against NCBI-NR and UniProt protein databases, with a cutoff E-value of $<1e^{-6}$. We imported BLASTX results into BLAST2GO software (Conesa et al. 2005) and Gene Ontology (GO) terms, EC numbers, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were annotated by BLAST2GO software. Protein-coding DNA sequence region (CDS) were predicted using TransDecoder implemented in Trinity software. Sequences with a corresponding protein length greater than 100 were retained for further analysis.

Merged and filtered transcriptome generated through *de novo* assembly was used as a reference transcriptome for differential expression analysis (Grabherr et al. 2011). Raw reads generated from liver and lymph node tissues were mapped to the reference transcriptome, and FPKM values were calculated by RSEM software (Li and Dewey 2011). The resulting data matrix that contained FPKM expression values for liver and RPLN tissues was generated by "rsem-generate-data-matrix" script. This data matrix was imported into edgeR 2.14 to identify differentially expressed genes (DEGs; 2-fold increase or decrease in expression pair-wise comparison between genes, $P < 0.05$) with $P < 0.001$ for FDR (Robinson et al. 2010). Differentially expressed gene FPKM values were first normalized by $\log_2$, median centered, then cluster analysis was performed using Trinity Perl script and a hierarchical cluster method based on Euclidean distance (Grabherr et al. 2011). Gene Ontology enrichment analysis of the DEGs detected was conducted by DAVID function annotation tool (Huang et al. 2008)

Two assays were designed for each region using PrimerQuest Tool (Integrated DNA Technologies, Inc.). A total of 40 assays were used, with one assay repeated twice. Additionally, Flex Six BioMark chip (Fluidigm, Inc.) and Eva Green RT-PCR (Bio-Rad Laboratories, Inc.) assays were used. Samples were treated with DNase I (Zymo DNase I set, E1010) followed by column purification (i.e., Qiagen RNeasy Micro, Qiagen Cat. ID 74004). Samples were then analyzed using a 2200 TapeStation (ThermoScientific, Cat. No. 4368814) to verify removal of gDNA. Conversion of RNA to cDNA was accomplished using 1 μg of total RNA per reaction and a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Complimentary DNA was target-specific pre-amplified according to a gene expression pre-amp protocol by Fluidigm. We used 12 amplification cycles in the thermal cycling step. Final products were diluted 5-fold and each sample was analyzed in 3 technical replicates and 5 biological replicates (i.e., 5 infected, 5 uninfected). BioMark reactions were set up as per Fluidigm's quick reference protocol (Fluidigm 2015). We performed RT-PCR cycling and signal acquisition on the BioMark System and analyzed data using Fluidigm RT-PCR analysis software (Spurgeon et al. 2008).

Major Accomplishments and Findings

We analyzed 238 samples (i.e., 97 LV, 70 BS, 71 RPLN) from 17 individuals (i.e., 9 males, 8 females) over 6 PMIs (i.e., 2, 8, 16, 24, 36, 48 hrs.). Mean RIN was 4.52 (SE = 1.34), though ranged from 1.7 to 7.6. Our analysis revealed a significant difference ($P = 0.013$, $\beta = 0.022$, SE = 0.007) in RIN values between liver ($\bar{x} = 4.04$, SE = 0.16) and brain stem tissues ($\bar{x} = 5.01$, SE = 0.13). Similarly, we noted differences (P = 0.007, $\beta = 0.028$, SE = 0.007) between RPLN ($\bar{x} = 4.69$, SE = 0.10) and brain stem ($\bar{x} = 5.01$, SE = 0.13) tissues. In contrast, RIN was similar ($P = 0.715$, $\beta = 0.005$, SE = 0.007) between liver ($\bar{x} = 4.04$, SE = 0.16) and RPLN ($\bar{x} = 4.69$, SE = 0.10) tissues.

The weighted mixed model indicated brain stem (least squares means [LSM] = 0.008, SE = 0.006, 95% Confidence Interval [CI] = –0.005–0.020) RIN values were significantly different from liver (LSM = –0.015, SE = 0.006, 95% CI = –0.028 to –0.002) and RPLN (LSM = –0.020, SE = 0.006, 95% CI = –0.032 to –0.009). However, RPLN and liver were not significantly different from one another. Least squares means indicated high variation in brain stem tissue RIN values, but less variation in RPLN with a negative relationship between RIN and PMI. In contrast, we did not observe significant changes in RIN values between PMIs.

A total of 488,145,350 (243,310,654 from LV and 244,834,696 from RPLN) clean pair-end reads were generated through RNA-Sequencing of 10 deer. Assembled transcripts ranged in size from 320 bp to 25,965 bp. When LV and RPLN samples were merged (number of N50 transcripts = 14,877, N50 length = 3,204 bp, mean length = 2,108 bp), 74,479 transcripts were assembled; 51,647 transcripts were assigned genes in NCBI-NR and 47,292 transcripts were assigned genes in UniProt. In total 51,661 (69.36%) were assigned to a gene. *Ovis aries*, *Bos taurus*, *Bubalus bubalis*, *Bos mutus*, and *Capra hircus* were the top species associated with BLAST hits against the NCBI-NR database. These species accounted for 49.08% (20,980) of the BLAST hits whereas the "others" category accounted for 8,696 BLAST hits. There were 2,496 genes specific to LV, 6,633 specific to RPLN, and 7,899 shared between LV and RPLN.

Genes were analyzed using Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG); 40,308 transcripts were assigned at least one term and 37,853 transcripts were assigned at least one pathway. Gene Ontology analysis showed transcripts successfully mapped to 20 GO biological processes (level 2). Most transcripts were related to cellular process, metabolic process, or single-organism process. Additionally, transcripts were mapped to 11 cellular components and 11 molecular functions (level 2). Most (60.8%) transcripts assigned to a cellular process were related to cells and organelles. Similarly, most (80.9%) transcripts mapped to molecular functions were related to binding and catalytic activity. Top KEGG pathways include purine metabolism, biosynthesis of antibiotics, pyrimidine metabolism, glycerophospholipid metabolism, phosphatidylinositol signaling system.

We identified a total of 59 genes as differentially expressed in CWD–infected deer liver and RPLN tissues. Among these, 36 were found in liver tissue (16 up-regulated, 20 down-regulated) and 23 (12 up-regulated, 11 down-regulated) in RPLN tissue; 29 of these genes have a known function when compared to Uniprot and NCBI databases. Of 59 genes, 33 genes passed validation, 14 failed, and 12 should be interpreted with caution. Function of genes that passed validation include sodium channel proteins, endogenous retrovirus proteins, and cell death activators. Genes that passed validation and were unannotated are candidates for further study.

These genes could have implications for the transmission or replication of infectious prion proteins. Even genes that did not pass validation or that should be interpreted with caution may benefit from testing with additional primer sets. Differentially expressed genes associated with LV and RPLN tissues included top functions assigned by Gene Ontology associated with cellular membranes, binding, apoptosis, metabolic processes, cellular processes, catalytic activity. Furthermore, we identified several DEGs (i.e., ERVK13-1, ERVK-24) which had been assigned a Gene Ontology cellular component of plasma membrane, which are up-regulated in the disease state.

Prior to this study, evaluations of post-mortem RNA degradation had not been conducted in deer; such evaluations are necessary to ensure the collection of high quality RNA used in creating deer transcriptomes for differential gene analysis. In addition, a paucity of genetic information exists at the transcriptome level in deer. This lack of basic genetic information leads to difficulties in answering questions that require comprehensive genetic backgrounds of a species or individual (i.e., studies focused on epidemiology or differential gene expression on an organismal scale). We sequenced RNA from liver and retropharyngeal tissue samples collected from 10 deer to create transcriptomes for differential gene expression evaluation. Functions of DEGs identified in this study lend support to relationships identified between misfolded PrP and post-translational processes, cellular membranes, and internal cellular components. Identification of differentially expressed genes involved in the pathogenesis of CWD may enable researchers and wildlife managers throughout Illinois to detect the status of deer in earlier stages of infection using gene expression (transcriptome) profiles developed from this study. Additionally, genes identified in this study may be important in the conversion of PrP into PrPSc and subsequent transmission of CWD as a posttranslational process may be responsible for this structural change. Our study is important and to our knowledge, the first evaluation of CWD-infected white-tailed deer genetic profiles using next-generation sequencing (NGS).

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NARRATIVE

STUDY W-178-R: EVALUATION OF POST-MORTEM RNA DEGRADATION IN HUNTER-HARVESTED WHITE-TAILED DEER (ODOCOILEUS VIRGINIANUS) IN WESTCENTRAL ILLINOIS, USA

Objectives: *1) Evaluate post-mortem intervals of ≥25 hunter-harvested white-tailed deer for estimating RNA stability by 31 March 2017.*

INTRODUCTION

Genetic research can be an integral tool for wildlife managers as comprehensive genetic profiles allow researchers to understand organisms on a molecular level, thus allowing them to make improved management decisions (Sarre and Georges 2009). Genetic evaluation of tissues, individuals, or populations can provide an extensive database to answer genetic-based questions at relatively lower cost than traditional methods (Morozova et al. 2009). Multiple analyses (i.e., RNA-Seq, microarray, Northern Blot, Western Blot) use RNA for a wide range of molecular applications ranging from disease status monitoring to evaluating gene expression. A key advantage of RNA is that it can provide researchers with large amounts of genetic data from just micro-grams (< 25 ng) of biological tissues (Tang et al. 2009, Pan et al. 2013). However, quality of intact RNA must be high (RNA integrity number; RIN ≥ 7.0; Bahar et al. 2007) for high throughput genomic sequencing (e.g., RNA-Seq) analyses. Nevertheless, collecting high quality RNA samples has proven difficult due to a wide range of logistical constraints, including sampling protocols that lack standardization, multidisciplinary organization (i.e., field personnel, molecular biologists, laboratory personnel), and multiple processes (i.e., tissue collection and preservation, RNA processing and extraction; Copois et al. 2007). Transcriptome-level based

genetic research uses RNA and has traditionally focused on humans and model organisms (e.g., mice, rats, rabbits, *Drosophila* spp.) in laboratory settings. Annotating a complete transcriptome using older technologies (e.g., microarray, Sanger sequencing) requires previous knowledge (e.g., transcript start sites, polyadenylation sites, exon-intron structures, splice variants; Morozova et al. 2009). Newer technologies (e.g., next-generation sequencing; NGS) permit annotation of novel transcriptomes without prior knowledge required by older techniques (i.e., use of a reference genome of a similar species or *de novo* assembly; van Delft et al. 2012). Thus, use of RNA in high-throughput sequencers allows researchers to achieve high coverage of full transcriptomes efficiently (Morozova et al. 2009, Tang et al. 2009). While older technologies are useful for specific transcripts, the discovery of novel transcripts allows for the creation of more comprehensive and informative transcriptome analysis.

White-tailed deer (*Odocoileus virginianus*; hereafter deer) are a non-model organism that provide a wide range of benefits to wildlife users and other wildlife species, thus maintaining healthy deer herds is a top concern to wildlife managers (Hewitt 2011). Examining non-model organisms in field-based settings (e.g., deer) make logistics of tissue sample collection, evaluation, and quality control difficult (Murphy et al. 2002, Murphy et al. 2007). Samples collected in the field may be relatively limited by availability of tissues or individuals from which samples were obtained. In addition, samples may not be immediately biopsied at location of harvested animals, thus high quality samples are critical (Bustin and Nolan 2004). Consequently, subsequent time delays may compromise quality of tissue samples used in RNA analyses. Additionally, it is not always feasible or possible to control environmental (e.g., ambient temperature, weather conditions) or intrinsic (e.g., body temperature) factors during field-based studies, therefore the influence of these factors on RNA quality should be examined. While

effects of environmental and intrinsic factors may impact RNA quality, these potential effects and post mortem intervals (PMIs) for RNA in cervid species have been previously been quantified.

Because high RNA quality is essential to successful NGS, field-based studies using NGS technology may be contingent on their knowledge of RNA degradation (i.e., PMIs) in field settings. Previously, RNA quality PMI studies frequently focused on human diseases (Cummings et al. 2001, Durrenberger et al. 2010), forensics (Bauer et al. 2003, Bauer 2007, Koppelkamm et al. 2011, Sampaio-Silva et al. 2013) and model organisms (i.e., organisms not used as a research template for species with similar characteristics; Johnson et al. 1986, Noguchi et al. 1991, Inoue et al. 2002). To our knowledge, this is the first evaluation of RNA degradation across variable PMIs in deer. Therefore, standardizing PMI for a non-model organism like deer increases likelihood of successful tissue collection for high throughput sequencing. Thus, our primary study objective was to create a PMI for RNA that could potentially aid in the collection of tissues in free-ranging deer for high throughput sequencing.

METHODS

Tissue extraction and storage

We obtained tissue samples from hunter-harvested deer in Hancock County, Illinois, USA (Fig. 1) during the 2015 and 2016 annual firearm seasons. At the end of each sampling day, all harvested WTD were transported to a central location for tissue and biopsy collection. Biopsies were collected in field conditions (e.g., exposure to ambient temperatures, precipitation, no preservation) to determine effects of field conditions and intrinsic factors (i.e., body temperature) on PMI. We rinsed tissues using. Prior to collecting tissue samples, we extracted retropharyngeal lymph nodes (RPLN) and brain stems (BS) from all animals. Additionally, we rinsed tissues using double-distilled water (ddH_2O) and removed excess body fluids (e.g., blood)

before collecting biopsy samples (using 6 mm Miltex biopsy punches; Ref. Num. 33–36) from multiple tissues (i.e., liver [LV], BS, RPLN). We extracted samples at predetermined PMIs (i.e., 2, 8, 16, 24, 36, 48 hrs.), placed them in 1.5 mL centrifuge tubes, and immediately fixed them with liquid nitrogen prior to storage at –80^{o} C. We attempted to collect tissue samples immediately (i.e., PMI = 0 hr) upon death, though this was not logistically feasible for hunter-harvested animals.

RNA extraction and analysis of RNA integrity number (RIN)

A 2 mm × 2 mm tissue biopsy was placed into a 1.5 mL tube with 350 µL of Qiagen RLT Buffer (Cat No. 79216) and 1% β-mercaptoethanol. Using a Kontes pellet pestle motor (ThermoScientific, Cat. No. K749515) and a disposable pestle, we homogenized RNA and centrifuged tissue lysates at full speed (e.g., 14,000 g) for 3 minutes. Supernatant was transferred to a clean tube and RNA was extracted from tissue lysate with the Qiagen RNeasy Mini Kit (Cat No. 74104); RNA concentrations were determined using a NanoDrop 1000 (ThermoScientific). We assessed RNA integrity using a 2200 TapeStation system and RNA ScreenTape (Agilent, Cat No. 5067-5576). TapeStations use an algorithm to assign a RNA Integrity Number (RIN) between 1 and 10 (i.e., 1 = completely degraded, 10 = completely intact) to each sample (Padmanaban 2012).

Statistical Analysis

Prior to analyses, we screened all predictor variables for collinearity using Pearson's correlation coefficients ($|r| > 0.5$) and used quantile plots to evaluate assumptions of normality; we used only 1 variable from a set of collinear variables for modeling (Jacques et al. 2011). Potential effects of body and daily temperature were collinear with PMI and thus, not included as covariates in analyses. We used a two-stage least squares regression model to evaluate potential

effects of tissue type on RIN. Specifically, we calculated average rates of change in RIN (ΔRIN) for each individual tissue combination and fit a linear model with ΔRIN as the response variable. We used the estimated slope and variance for ΔRIN to fit a weighted mixed model; weights were the inverse variances of the estimated ΔRIN effects. By using weights, we up-weighted data points resulting from strong estimates and down-weighted data points resulting from weak estimates (A. Martin-Schwarze, Iowa State University, personal communication). The fitted model tested whether the slope of the RIN trend differed between tissue types. We conducted analyses using Program R (R Core Team 2015); statistical tests were conducted at $\alpha = 0.05$.

RESULTS AND DISCUSSION

We analyzed 238 samples (i.e., 97 LV, 70 BS, 71 RPLN) from 17 individuals (i.e., 9 males, 8 females) over 6 PMIs (i.e., 2, 8, 16, 24, 36, 48 hrs.). Mean RIN was 4.52 (SE = 1.34), though ranged from 1.7 to 7.6 (Fig. 2). Our analysis revealed a significant difference ($P = 0.013$, $\beta = 0.022$, SE = 0.007) in RIN values between liver ($\bar{x} = 4.04$, SE = 0.16) and brain stem tissues ($\bar{x} = 5.01$, SE = 0.13). Similarly, we noted differences ($P = 0.007$, $\beta = 0.028$, SE = 0.007) between RPLN ($\bar{x} = 4.69$, SE = 0.10) and brain stem ($\bar{x} = 5.01$, SE = 0.13) tissues. In contrast, RIN was similar ($P = 0.715$, $\beta = 0.005$, SE = 0.007) between liver ($\bar{x} = 4.04$, SE = 0.16) and RPLN ($\bar{x} = 4.69$, SE = 0.10) tissues.

The weighted mixed model indicated brain stem (least squares means [LSM] = 0.008, SE = 0.006, 95% Confidence Interval [CI] = –0.005–0.020) RIN values were significantly different from liver (LSM = –0.015, SE = 0.006, 95% CI = –0.028 to –0.002) and RPLN (LSM = –0.020, SE = 0.006, 95% CI = –0.032 to –0.009). However, RPLN and liver were not significantly different from one another (Fig. 3). Least squares means indicated high variation in brain stem

tissue RIN values, but less variation in RPLN with a negative relationship between RIN and PMI (Fig. 4). In contrast, we did not observe significant changes in RIN values between PMIs (Fig. 4).

Previous PMI studies suggest messenger RNA (mRNA) is stable in human and rat brains through 48 hrs (Johnson et al. 1986, Noguchi et al. 1991). In humans, ante-mortem factors (e.g., agonal state, medical history, age, toxicology, demographics) are taken into consideration when tissues are collected for tissue banks as they have been shown to potentially influence RNA quality across PMIs (Cummings et al. 2001).

Consistent ambient temperatures may be critical to optimal RNA quality (i.e., continual storage in liquid nitrogen even during transport to laboratory facilities). Thompson et al. (2007) suggested leaving samples at a stable temperature (e.g., room temperature) if proper preservation were not options at the time of tissue sampling. Studies examining model organisms in laboratory settings have documented no significant timeline associated with degradation (Harrison et al. 1995, Marchuk et al. 1998, Cummings et al. 2001) and most of these studies have been conducted for forensic purposes (i.e., to determine time of death in crime labs). Although other studies have developed PMIs for other species (Bauer et al. 2003), RNA stability appears to vary between tissue types (Sampaio-Silva et al. 2013, Bahar et al. 2007, Fleige and Pfaffl 2006). We observed similar results in this study (i.e, RIN between BS and LV, [$P < 0.05$]; BS and RPLN [$P < 0.05$]; LV and RPLN [$P > 0.05$]).

Though uncertain, differences between tissue types could be attributed to differences in chemical composition (i.e., pH) of the organs (Mexal et al. 2006, Vawter et al. 2006). While it would make intuitive sense that RIN values would decrease over time, we observed a decrease in RIN from 2–48 hrs, however, there was no apparent change between time intervals. This could

be attributed to a rapid decline in RIN immediately upon death. Previous studies have been conducted in laboratory settings and focused on PMIs starting at the moment of death (Harrison et al. 1995, Marchuck et al. 1998), typically on the scale of minutes rather than hours (Wilkes et al. 2010). For our purposes, these criteria were not applicable to field-based sampling (Copois et al. 2007). We were focused on longer PMIs (i.e., 2–8 hrs) for purposes of tissue sampling after collection of deer for routine disease monitoring and surveillance.

Quality of RNA was assessed as a precursor to tissue collection for RNA-seq analyses. Our results indicated that RIN values did not differ significantly between PMIs and that tissue collection soon after euthanasia of targeted animals is unnecessary. Tissue sampling conducted by field personnel would reduce tissue collection under potentially contaminating conditions (e.g., precipitation, lack of access to proper preservation) that may contribute to variation in RIN values. Sampling of tissues for RNA collection frequently occurs after a time delay; therefore demonstrating no significant difference in RIN between PMI suggests tissue samples may be collected during routine processing of deer.

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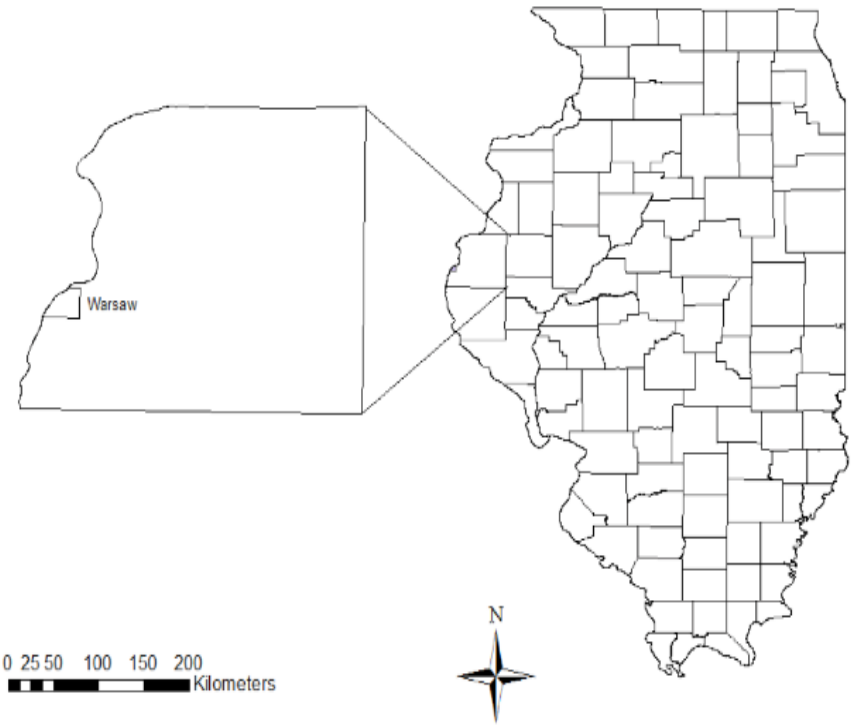
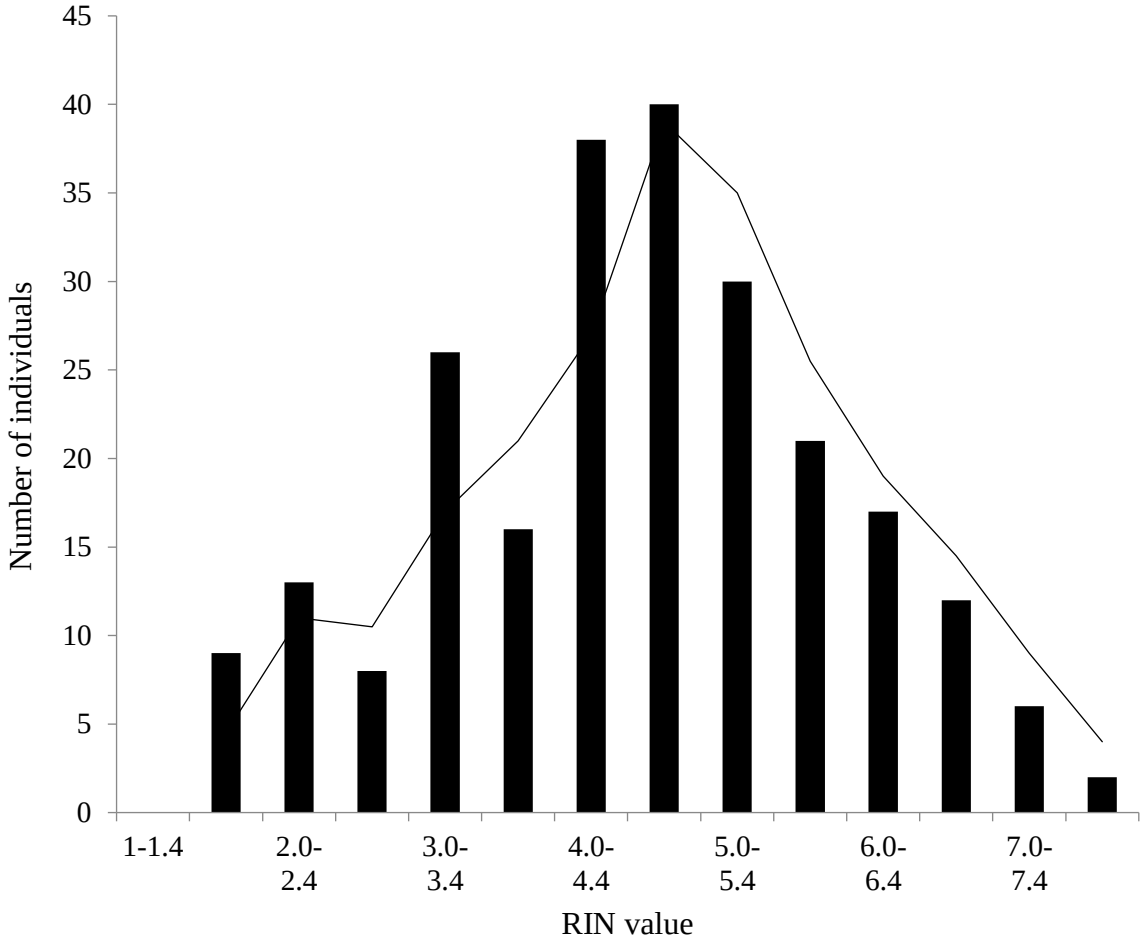


Figure 1.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35
30
25
20
15
10
5
0
Number of individuals
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2.0-2.4
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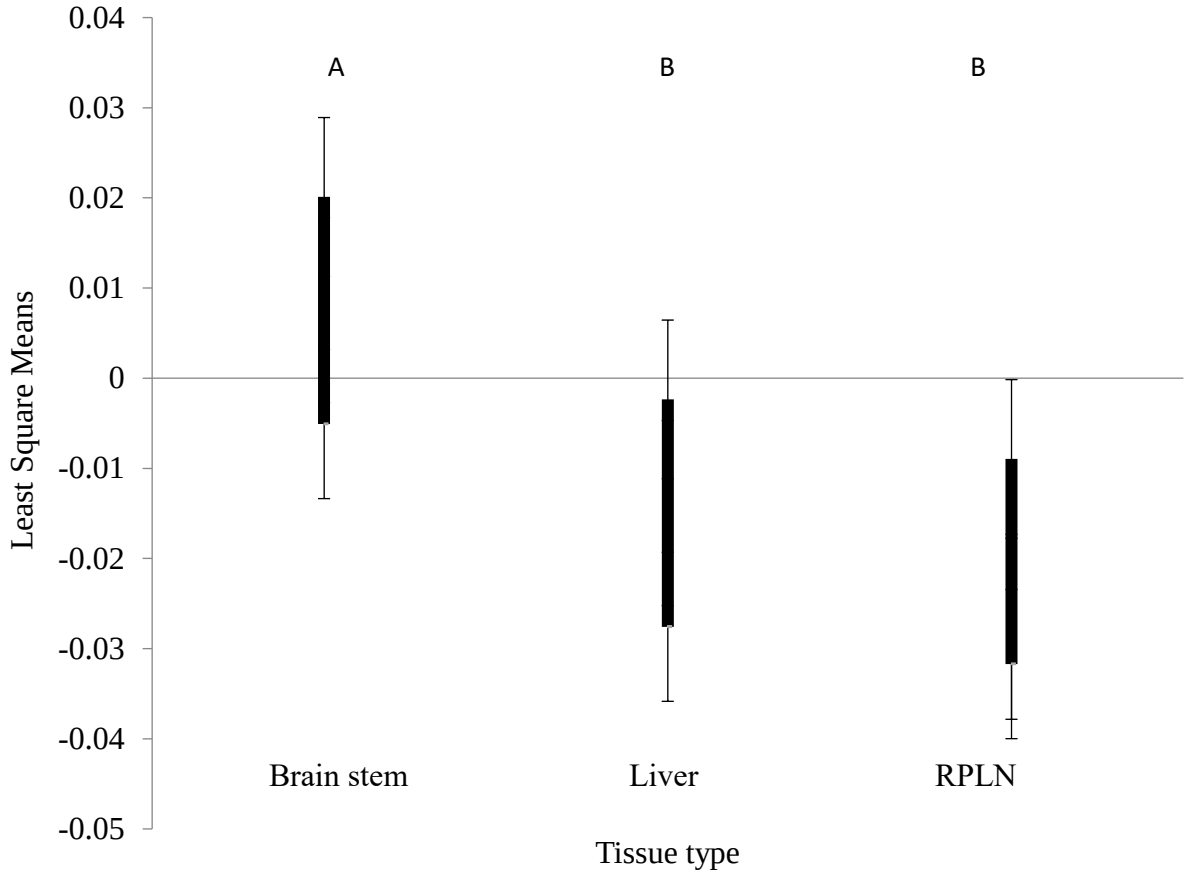
6.0-6.4
7.0-7.4
RIN value

Figure 2.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Least Square Means
A
B
B
Brain stem
Liver
RPLN
Tissue type

Figure 3.

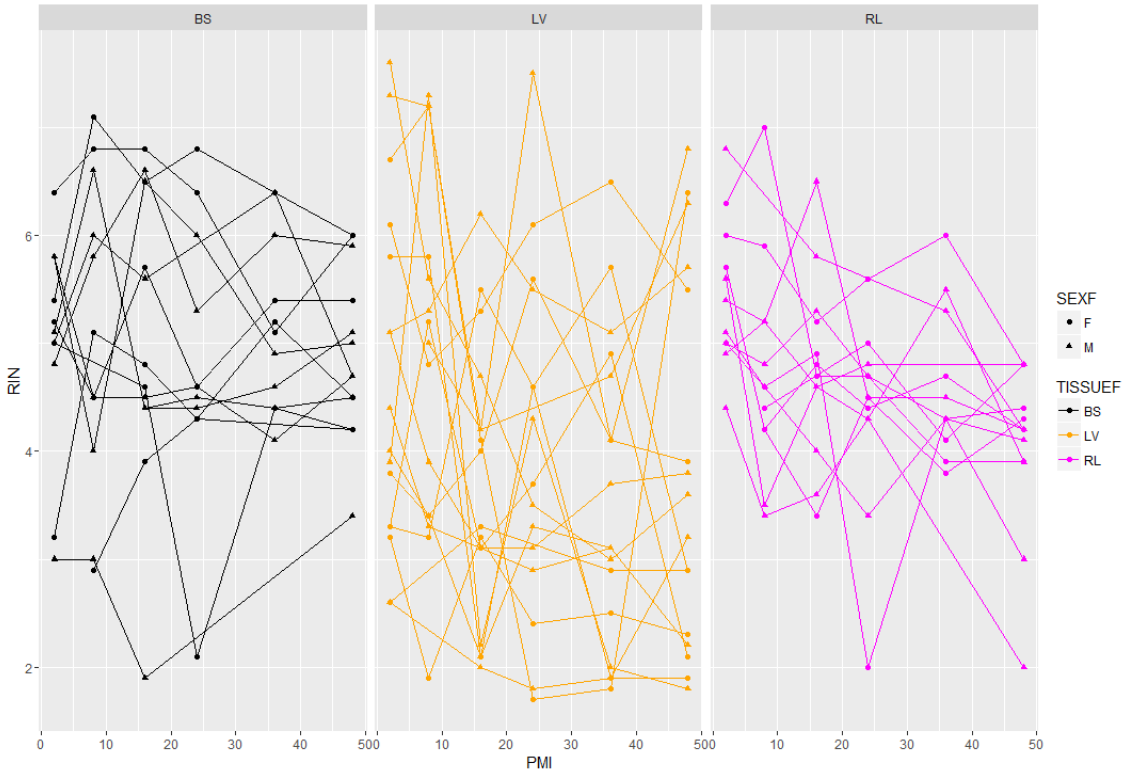

Figure 4.

STUDY W-178-R: DIFFERENTIAL GENE EXPRESSION IN CHRONIC WASTING DISEASE INFECTED WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*)

Objectives: *2) Determine (e.g., identify) differentially expressed genes in CWD-infected and uninfected white-tailed deer by 31 March 2017.*

3) Create a genetic profile of CWD-infected white-tailed deer by 31 March 2017.

4) Evaluate the efficacy of genetic (transcriptome) profiling for identifying CWD-infected white-tailed deer by 31 March 2017.

INTRODUCTION

Infectious transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative prion diseases (proteinaceous infectious particle; Prusiner 1982) that infect humans (Creutzfeldt-Jakob disease, kuru; Brown 2013), mink (transmissible mink encephalopathy; Hartsough and Burger 1965), sheep (scrapie; Prusiner, 1989), cattle (bovine spongiform encephalopathy; Hope et al., 1988), and cervids (chronic wasting disease [CWD]; Williams and Young 1980). Prion diseases are thought to be caused by conversion of naturally occurring protease-sensitive, cellular prion protein (PrP^{c}) into a conformationally altered isoform PrP^{Sc} (Griffith 1967, Prusiner 1982). These abnormal prions (i.e., amyloids) accumulate in the central nervous system (CNS) and peripheral lymphoid tissues (Kimberlin and Walker 1982, Caughey et al. 1988) of a host, are detergent insoluble, and partially proteinase K-resistant (Prusiner 1989, Pan et al. 1993). Post-translational alterations from PrP to PrP^{Sc} have been implicated as the causative factor leading to infection (Prusiner 1989).

Chronic wasting disease is of considerable interest and concern to wildlife managers throughout North America (Williams et al. 2002). Due to the potential risk of transmission to domestic species such as cattle, CWD poses an increased risk to human health (Basu et al. 2012).

Risk of cross species transmission is elevated by the ability of the prions to affect both captive and free-ranging animals (Williams et al. 2002) as captive individuals are more likely to come in contact with domestic species. Prevalence rates can reach as high as 50% in free ranging herds and 90% in captive herds (Gilch et al. 2011), though prions are difficult to diagnose in live cervids (Cheng et al. 2016). Tonsil, lymphoid, and third eyelid tissues may be biopsied to confirm infection in live individuals, though tissue biopsies require anesthesia so are impractical in free-ranging herds (Wild et al. 2002). Clinical signs are the most effective way to visually diagnose infected individuals, but are only apparent in final stages of disease progression (i.e., months to years after initial infection; Williams 2005, Gilch et al. 2011).

Little is known about prion transmission in native hosts (Saunders et al. 2012). Current research suggests underlying mechanisms of PrP^{Sc} formation may be dependent on the type of prion disease (i.e., infectious, sporadic, genetic; Harris, 1999). It is thought that CWD is transmitted primarily through oral and mucosal membranes (Safar et al. 2008) and contact with prions in the environment (e.g., through feces, blood, urine, saliva) via horizontal transmission (Saunders et al. 2012). Furthermore, prions are stable enough to withstand environmental changes (e.g., ultraviolet radiation, freeze-thaw cycles, bacterial and fungal enzymes; Gilch et al. 2011) and persist in soil even in the absence of infected deer (Johnson et al. 2006).

A single prior study identified gene expression changes in CWD-infected Rocky Mountain elk (*Cervus elaphus*) using microarray analysis and pre-determined transcripts (i.e., genes selected by researchers; Basu et al. 2012). This study provided evidence for the involvement of multiple genes (i.e., genes assigned to functional groups associated with biological regulation, metabolic process, and cellular process). Moreover, this previous research (Basu et al. 2012) identified pathways (i.e., calcium signaling, apoptosis and cell death, immune

cell trafficking, and inflammatory response) and novel genes (i.e., previously undescribed genes), which contributed to infection. However, confinement to known transcripts is a disadvantage of traditional microarray studies. Studies addressing hypothesis-driven questions related to the role of specific genes in facilitating or reducing disease infection in white-tailed deer (*Odocoileus virginianus*; hereafter deer) are difficult to conduct given the limited availability of annotated deer genomes and transcripts currently available in the literature.

Furthermore, researchers have evaluated the potential for CWD resistance due to sequence polymorphisms (Kelly et al. 2008) and potential genetic risk factors (Matsumoto et al. 2013), but have not previously examined potential roles of novel genes in disease resistance using high throughput next-generation sequencing (NGS). Next-generation sequencing allows for discovery of novel transcripts in a more rapid and comprehensive method than other current technologies available (Mardis 2008) at comparatively low costs (Metzker 2010). Discovery of novel genes using NGS does not require a priori knowledge of genes that may be present, thus mitigating ascertainment bias (i.e., previous knowledge of impacted genes). Thus, a need exists for NGS application (Basu et al. 2012) and discovery of novel transcripts to further gene expression studies in all TSE-impacted species. At the initiation of this research, gene expression evaluation in free-ranging deer using RNA-sequencing technology had not previously been conducted. We sought to identify differentially expressed genes in CWD-infected, free ranging deer to contribute to an increased understanding of molecular mechanisms involved in the pathology and replication of CWD in cervid species. To our knowledge, this is the first study evaluating gene expression in CWD-infected WTD using NGS to identify novel transcripts.

MATERIALS AND METHODS

Tissue extraction

Liver and retropharyngeal lymph node (RPLN) samples were collected from 380 deer euthanized by Illinois Department of Natural Resource (IDNR) wildlife managers during annual population reduction (e.g., sharpshooting) and disease monitoring efforts. Deer were euthanized according to the IDNR CWD management plan. This program takes place throughout the chronic wasting disease (CWD)-endemic area of northcentral Illinois January–March 2015. Before sample biopsy, tissues were rinsed using double distilled water (ddH_2O) and any blood removed. Retropharyngeal lymph nodes were then extracted from all adult (≥ 1.5 yrs. old) deer. Samples were biopsied from multiple tissues (e.g. liver [LV], RPLN) using 6 mm Miltex biopsy punches (Ref. Num. 33-36). Livers were biopsied in approximately the same location every time, while one RPLN was randomly sampled. Biopsied tissues were placed into 1.5mL centrifuge tubes and stored using 1.5mL RNAlater (ThermoFisher Scientific, Cat. No. AM7020) per the manufacturer's recommendations. We placed all tissue samples in a refrigerator for 24 hrs at 2º C after which, they were placed in a freezer (–10º C) on site. Each week, we transported all tissue samples to Western Illinois University, at which time they were placed in a deep freezer (–20º C) until transported to the Core Genomics Laboratory at University of Illinois Chicago for sequencing.

We compared our samples to those IDNR biologists submitted to the Animal Disease Laboratory in Galesburg, IL, USA for disease testing (i.e., immunohistochemistry; IHC). Once disease state was determined (i.e., CWD prions observed or absent in tissue), a subset of male and female CWD-infected deer were randomly chosen and paired with uninfected deer of similar sex and age classes (i.e., adult) and harvest location. We used RPLN and LV tissue samples from 10 (5 CWD-positive [treatment group], 5 CWD-negative [control group]) adult female deer for RNA-Seq analyses.

RNA extraction and sequencing

Using a Qiagen RNeasy Mini Kit (Cat. No. 74104), we extracted RNA from each sample according to the manufacturer's instructions. We examined RNA integrity and quantity using a NanoDrop 1000 and a 2200 TapeStation system using RNA ScreenTape (Agilent, Cat. No. 5067-5576). A total of 1 μl RNA (RIN > 7) was used for RNA-Seq library construction. Additionaly, cDNA library was prepared with the TruSeq Stranded mRNA LT Sample Prep Kit- Set A (Illumina, Cat. No. RS-122-2101) and amplified using polymerase chain reaction (PCR). Specifically, we used the Illumina HiSeq 2500 Sequencing System (Illumina Inc., San Diego, CA, USA) with a HiSeq SBS sequencing kit. Resulting reads were 100 nt in length and sequenced on one lane from each end for 101 cycles. Data files (i.e., FASTQ) were generated and demultiplexed with the bcl2fastq v1.8.4 Conversion Software (Illumina). We used RNA-Seq to analyze LV and RPLN tissue samples from infected and uninfected deer.

De novo assembly

Cleaned reads were used for the reference transcriptome assembly based on Trinity version 2.06 with paired-end mode (Grabherr et al. 2011). Transcripts from liver and lymph node tissues were separately *de novo* assembled, then a reference transcriptome was generated by merging assemblies from both tissues, and reductant transcripts were filtered by CD-HIT software (Li and Godzik 2006). To filter out any misassembled transcripts, raw sequenced reads were mapped to assembled reference transcriptomes using Bowtie 1.0.0 (Langmead et al. 2009). Transcript abundance was estimated using RSEM software (Li and Dewey 2011), fragments per kilobase per transcript per million mapped reads (FMPK) values were calculated and transcripts with FPKM <1 were filtered out (Li and Godzik 2006). Filtered transcripts were used as the deer reference transcriptome for downstream analysis (Grabherr et al. 2011).

Transcriptome annotation

Assembled transcriptomes were annotated using BLASTX against NCBI-NR and UniProt protein databases, with a cutoff E-value of $<1e^{-6}$. We imported BLASTX results into BLAST2GO software (Conesa et al. 2005) and Gene Ontology (GO) terms, EC numbers, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were annotated by BLAST2GO software. Protein-coding DNA sequence region (CDS) were predicted using TransDecoder implemented in Trinity software. Sequences with a corresponding protein length greater than 100 were retained for further analysis.

Differential expression analysis

Merged and filtered transcriptome generated through *de novo* assembly was used as a reference transcriptome for differential expression analysis (Grabherr et al. 2011). Raw reads generated from liver and lymph node tissues were mapped to the reference transcriptome, and FPKM values were calculated by RSEM software (Li and Dewey 2011). The resulting data matrix that contained FPKM expression values for liver and RPLN tissues was generated by "rsem-generate-data-matrix" script. This data matrix was imported into edgeR 2.14 to identify differentially expressed genes (DEGs; 2-fold increase or decrease in expression pair-wise comparison between genes, $P < 0.05$) with $P < 0.001$ for FDR (Robinson et al. 2010). Differentially expressed gene FPKM values were first normalized by $\log_2$, median centered, then cluster analysis was performed using Trinity Perl script and a hierarchical cluster method based on Euclidean distance (Grabherr et al. 2011). Gene Ontology enrichment analysis of the DEGs detected was conducted by DAVID function annotation tool (Huang et al. 2008)

Gene validation

Two assays were designed for each region using PrimerQuest Tool (Integrated DNA Technologies, Inc.). A total of 40 assays were used, with one assay repeated twice. Additionally, Flex Six BioMark chip (Fluidigm, Inc.) and Eva Green RT-PCR (Bio-Rad Laboratories, Inc.) assays were used. Samples were treated with DNase I (Zymo DNase I set, E1010) followed by column purification (i.e., Qiagen RNeasy Micro, Qiagen Cat. ID 74004). Samples were then analyzed using a 2200 TapeStation (ThermoScientific, Cat. No. 4368814) to verify removal of gDNA. Conversion of RNA to cDNA was accomplished using 1 μg of total RNA per reaction and a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Complimentary DNA was target-specific pre-amplified according to a gene expression pre-amp protocol by Fluidigm. We used 12 amplification cycles in the thermal cycling step. Final products were diluted 5-fold and each sample was analyzed in 3 technical replicates and 5 biological replicates (i.e., 5 infected, 5 uninfected). BioMark reactions were set up as per Fluidigm's quick reference protocol (Fluidigm 2015). We performed RT-PCR cycling and signal acquisition on the BioMark System and analyzed data using Fluidigm RT-PCR analysis software (Spurgeon et al. 2008).

RESULTS

A total of 488,145,350 (243,310,654 from LV and 244,834,696 from RPLN) clean pair-end reads were generated through RNA-Sequencing of 10 deer. Assembled transcripts ranged in size from 320 bp to 25,965 bp (Fig. 1). When LV and RPLN samples were merged (number of N50 transcripts = 14,877, N50 length = 3,204 bp, mean length = 2,108 bp), 74,479 transcripts were assembled; 51,647 transcripts were assigned genes in NCBI-NR and 47,292 transcripts were assigned genes in UniProt. In total 51,661 (69.36%) were assigned to a gene. *Ovis aries*, *Bos taurus*, *Bubalus bubalis*, *Bos mutus*, and *Capra hircus* (Table 1) were the top species associated with BLAST hits against the NCBI-NR database. These species accounted for 49.08% (20,980)

of the BLAST hits whereas the “others” category accounted for 8,696 BLAST hits. There were 2,496 genes specific to LV, 6,633 specific to RPLN, and 7,899 shared between LV and RPLN.

Genes were analyzed using Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG); 40,308 transcripts were assigned at least one term and 37,853 transcripts were assigned at least one pathway. Gene Ontology analysis (Fig. 3) showed transcripts successfully mapped to 20 GO biological processes (level 2). Most transcripts were related to cellular process, metabolic process, or single-organism process. Additionally, transcripts were mapped to 11 cellular components and 11 molecular functions (level 2). Most (60.8%) transcripts assigned to a cellular process were related to cells and organelles. Similarly, most (80.9%) transcripts mapped to molecular functions were related to binding and catalytic activity. Top KEGG pathways include purine metabolism, biosynthesis of antibiotics, pyrimidine metabolism, glycerophospholipid metabolism, phosphatidylinositol signaling system (Table 2).

We identified a total of 59 genes as differentially expressed in CWD–infected deer liver and RPLN tissues (Table 3). Among these, 36 were found in liver tissue (16 up-regulated, 20 down-regulated) and 23 (12 up-regulated, 11 down-regulated) in RPLN tissue; 29 of these genes have a known function when compared to Uniprot and NCBI databases. Of 59 genes, 33 genes passed validation, 14 failed, and 12 should be interpreted with caution (Table 4). Function of genes that passed validation include sodium channel proteins, endogenous retrovirus proteins, and cell death activators. Genes that passed validation and were unannotated are candidates for further study. These genes could have implications for the transmission or replication of infectious prion proteins. Even genes that did not pass validation or that should be interpreted with caution may benefit from testing with additional primer sets. Differentially expressed genes associated with LV and RPLN tissues included top functions assigned by Gene Ontology

associated with cellular membranes, binding, apoptosis, metabolic processes, cellular processes, catalytic activity (Tables 5, 6). Furthermore, we identified several DEGs (i.e., ERVK13-1, ERVK-24) which had been assigned a Gene Ontology cellular component of plasma membrane, which are up-regulated in the disease state.

DISCUSSION

Ersdal et al. (2009) suggested abnormal and misfolded prion proteins (i.e., PrP^{Sc}) cause plasma membranes to change as the aberrant protein accumulates on theses surfaces. Functions of PrP and DEG syntaxin-binding protein 5 (STXBP5) are overlapped. STXBP5 may regulate fusion and docking of presynaptic vesicles (Bennett et al. 1992), while PrP may play a role in synaptic function, maintenance, or structure and a regulatory role at both central and peripheral synapses (Westergard et al. 2007). Additionally, DEG IL-17 is responsible for communication between cells, specifically as an inflammatory response in diseased individuals (Huang et al. 2015). Misfolded PrP^{Sc} accumulates on cellular surfaces, thus associations between differentially expressed genes and cellular membranes, binding, and cellular processes suggest an interaction between the PrP molecule and internal cellular components. Caughey et al. (1991) suggested plasma membranes are where the conversion from PrP to PrP^{Sc} occurs. Therefore, an interaction critical to the successful conversion of PrP may take place on the plasma membrane. Blocking the specific site of conversion, may potentially prevent the misfolding of PrP into PrP^{Sc}.

Differentially expressed genes assigned a Gene Ontology cellular component of plasma membrane may suggest a change occurring in the plasma membrane due to CWD. Naturally occurring PrP^{C} is attached to the outer surface of the plasma membrane (Peters et al. 2003) and has been shown to be expressed during stress (Linden et al. 2008). Naturally occurring PrP has multiple binding partners involved in cytoskeletal processes (e.g., maintenance, cell growth;

Zafar et al. 2011) and its function has been linked to copper homeostasis, oxidative stress, cell survival differentiation, cell signaling, and cell proliferation. A number of effects have been observed in individuals deficient in PRNP (i.e., gene responsible for PrP), including depressive behavior (Gadotti et al. 2012, Beckman et al. 2015), cognitive deficits (Criado et al. 2005), increased susceptibility to oxidative stress (Brown et al. 2002), and peripheral myelin deficits (Schmitz et al. 2014). However, many biological functions associated with PrP and its intracellular partners are still uncertain (Río and Gavín 2016). Ambiguity of PrP function may be attributed to the number of different isoforms associated with the protein (Safar et al. 1993).

The genes ERVK13-1 and ERVK-24 also were associated with retroviruses (ERVs). Furthermore, other genes (i.e., L_TR15492|c1_g1_i1, N_TR113697|c0_g2_i1) were also associated with retroviruses. Retroviruses may be activated in disease states (Lee et al. 2013) and studies suggest retroviruses play an integral part in prion infections (Leblanc et al. 2006), potentially increasing risk of transmission (Ashok and Hegde 2006). Additionally, naturally occurring PrP has been suggested to influence retroviral activity (Lötscher et al. 2007) in healthy individuals. Prions and retroviral cells may be localized in the same cellular compartments. Leblanc et al. (2006) suggested retroviruses may increase prion infectivity by acting as transport vectors in the spread of infective prions throughout an individual. Retroviral Gag was suggested to enhance the release of prion proteins in cellular culture when expressed (Leblanc et al. 2006) as was further demonstrated by Bian et al. (2010) using CWD prions. In our study, at least 2 DEGs are associated with Gag protein. In addition to Gag, Pol and Env proteins also are differentially expressed in CWD-infected deer. These three genes combined with Pro create the backbone of replicating retroviruses (Petropoulos 1997). Although Pro is not specifically listed in our DEGs, one of the unknown genes may be a form of the protein.

Retroviruses are found in all vertebrate genomes, however some are species- specific (Coffin 1992). Retrovirus genes we identified are potentially linked to a disease state and may act as a modifier (Krasemann et al. 2012), though further investigation is needed to describe these genes. If retroviruses do play a role in the infectivity of prions, specific ERVs may be linked to prion disease phenotypes (Krasemann et al. 2012). Consequently, species-specific ERVs may be linked to the species barrier present in many prion diseases. An additional candidate for further CWD-related studies is an endogenous gammaretrovirus (CrERVγ) found in mule deer (Elleder et al. 2012). White-tailed deer and elk genomes have been shown to contain a similar retrovirus (Elleder et al. 2012). If this retrovirus is specific to cervids and plays a role in CWD infectivity, a species barrier jump may not be as likely (Krasemann et al. 2012), thus reducing potential risks to humans.

Several differentially expressed genes are related to tumor necrosis factor (TNF), a cytokine that produces an immune response to help prevent the spread of infection. It induces fever, apoptotic cell death, and inhibits viral replication. Chronic exposure to TNF can cause shock-like symptoms including a wasting syndrome (Chu 2013). It also is important to maintaining follicular dendritic cell (FDC) networks (Sallusto and Lanzavecchia 1994). Kitamoto et al. (1991) suggested FDCs were important to the replication of prions in lymphoid tissues as early Prp^{Sc} accumulates on these cells. Specifically, we identified a gene (i.e., AdipoQ) that acts as a TNF antagonist (Masaki et al. 2004) up-regulated in liver tissues of positive deer and a monokine (i.e., CCL3) that is responsible for positive regulation of TNF production (Ramos et al. 2005). The CCL3 gene was down-regulated in positive deer, therefore it is possible that infected deer are down regulating TNF production at time of sampling. This is an expected immune response to a long-term infection and may indicate an individual in alter stages of

infection. It is possible the accumulation of TNF may cause clinical symptoms of the disease (i.e., wasting syndrome; Chu 2013).

Logistics of collecting and preserving many high quality tissue samples and transporting samples to laboratory settings for storage make field-based RNA studies difficult. However, these types for studies are important in eliminating confounding factors (i.e., exposure to artificially high concentrations of prions, inheritance of partial CWD resistance conferring PrP polymorphism at greater frequency than in natural settings) induced by captive breeding and evaluating a disease in a natural setting. Animals in captive facilities are exposed to higher concentrations of CWD prions over less space than their free-ranging counter parts. This may lead to higher infection rates in captive individuals and infections with higher concentrations of prions than their free-ranging counterparts (Miller and Wild 2004). A difference in prion concentration may affect gene expression and time of detection; thereby highlighting the importance of examining CWD gene expression in free-ranging naturally infected individuals. Therefore if tested too early in disease progression, an infected animal may not exhibit prion concentrations high enough for detection using traditional methods (Haley et al. 2009). By using differential gene expression to determine disease status, earlier detection in free-ranging animals may be possible.

Any potential role of the differentially expressed genes discussed in this study should be examined in normal prion proteins. Normal PrP function is ambiguous and DEGs identified in this study may further the understanding of PrP. While many normal prion protein functions have been described, underlying pathogenesis of TSEs is not well understood as amyloid deposits can be found in outwardly healthy individuals (Diack et al. 2016). It also remains unclear if conversion of PrP^{C} to PrP^{Sc} leads to a gain-of-function in PrP^{Sc} infected individuals (Collins et al.

2004) or a loss-of-function (Samaia and Brentani 1998). Additionally, DEGs associated with retroviruses warrant further investigation as they may influence the transmissibility of CWD. Future studies should build upon CWD associated DEGs identified in this study by examining DEGs in other tissues (i.e., brain stem, blood, rectoanal mucosa-associated lymph tissue; RMALT). Further genetic analyses at a transcriptome level could lead to a greater understanding of naturally occurring prion protein functions and thus aid in the understanding of disease causing prion transmission and formation mechanisms.

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Table 1. Species distribution of top BLAST hits of *de novo* assembled white-tailed deer transcripts against NCBI NR database.

Species	BLAST Top-Hits
Ovis aries	8904
Others	8696
Bos taurus	7361
Bubalus bubalis	5837
Bos mutus	4831
Capra hircus	2951
Pantholops hodgsonii	2771
Bison bison	2535
Homo sapiens	1150
Sus scrofa	582
Mus musculus	513
Balaenoptera acutorostrata	508
Camelus ferus	442
Physeter catodon	398
Orcinus orca	349
Myotis brandtii	291
Equus przewalskii	278
Equus caballus	277
Pteropus alecto	276
Lipotes vexillifer	275
Tursiops truncatus	272
Cricetulus griseus	271
Synthetic construct	252
Tupaia chinensis	251

Rattus norvegicus	250
Ursus maritimus	239
Ailuropoda melanoleuca	237
Chlorocebus sabaeus	222
Canis lupus	221
Macaca mulatta	207

Table 2. Top 40 KEGG pathways in the white-tailed *de novo* deer assembly transcriptome.

Pathway	Sequences in Pathway	Number of Enzymes
Purine metabolism	843	55
Biosynthesis of antibiotics	841	126
Pyrimidine metabolism	343	33
Glycerophospholipid metabolism	255	31
Phosphatidylinositol signaling system	250	22
Lysine degradation	244	19
Aminoacyl-tRNA biosynthesis	230	24
Glycolysis / Gluconeogenesis	228	26
Glutathione metabolism	199	19
Drug metabolism - cytochrome P450	194	7
Fatty acid degradation	186	15
Tryptophan metabolism	183	21
Glycerolipid metabolism	180	16
Metabolism of xenobiotics by cytochrome P450	178	8
Oxidative phosphorylation	178	7
Pyruvate metabolism	174	20
Amino sugar and nucleotide sugar metabolism	174	35
Glycine, serine and threonine metabolism	170	31
Carbon fixation pathways in prokaryotes	163	20
Inositol phosphate metabolism	162	21
T cell receptor signaling pathway	162	2
Thiamine metabolism	161	4
Valine, leucine and isoleucine degradation	159	24
Nicotinate and nicotinamide metabolism	157	16
Citrate cycle (TCA cycle)	156	17
Arachidonic acid metabolism	142	17

Sphingolipid metabolism	142	20
Cysteine and methionine metabolism	139	25
Pentose phosphate pathway	125	17
Drug metabolism - other enzymes	122	17
Methane metabolism	120	15
Steroid hormone biosynthesis	117	16
Butanoate metabolism	111	15
Arginine and proline metabolism	108	23
Retinol metabolism	105	10
Alanine, aspartate and glutamate metabolism	103	24
Starch and sucrose metabolism	102	18
Propanoate metabolism	101	16
Biosynthesis of unsaturated fatty acids	100	8

Table 3. Differentially expressed genes in chronic wasting disease-infected liver and retropharyngeal lymph node tissues from white-tailed deer collected in the chronic wasting disease endemic area of northern Illinois during annual population reduction, winter 2015.

Gene ID	logFC	PValue	FDR	Positive FPKM	Control FPKM	Annotation
Up-regulated in liver						
L_TR43469|c2_g3_i5	3.39	1.47E-10	6.12E-06	6.05	0.57	endogenous retrovirus group k member 25 env poly
L_TR63450|c1_g1_i1	5.28	7.55E-10	1.50E-05	6.29	0.16	uncharacterized protein loc105607204 isoform x1
L_TR45335|c0_g1_i1	2.97	2.80E-09	2.34E-05	3.9	0.49	Unknown
L_TR29095|c7_g3_i1	3.24	3.84E-09	2.67E-05	4.24	0.44	sodium channel protein type 11 subunit partial
L_TR56520|c6_g2_i1	4.64	2.04E-07	0.001065	2.15	0.08	Unknown
L_TR56520|c6_g3_i2	5.79	5.58E-07	0.002325	1.15	0.02	syntaxin-binding protein 5
L_TR41343|c0_g1_i2	4.28	1.32E-06	0.004595	3.61	0.18	uncharacterized protein loc102402433 isoform x1
L_TR49285|c0_g1_i1	5.16	3.65E-06	0.00801	2.73	0.07	Unknown
L_TR77350|c0_g1_i1	3.98	4.85E-06	0.00963	4.46	0.27	gag protein
L_TR47646|c1_g1_i1	2.32	2.14E-05	0.028849	4.67	0.92	Unknown
L_TR56520|c6_g1_i1	4.24	2.62E-05	0.030398	1.14	0.06	syntaxin-binding protein 5
L_TR53215|c2_g3_i1	7.08	3.83E-05	0.038974	1.71	0	Unknown
L_TR41343|c0_g1_i3	3.77	4.09E-05	0.040655	2.86	0.2	Unknown
L_TR79592|c7_g2_i1	3.36	4.46E-05	0.04327	1.8	0.17	interleukin-17 receptor a isoform x2
L_TR27390|c2_g1_i1	11.61	5.11E-05	0.046946	3.22	0	Unknown
L_TR41343|c0_g1_i1	5.01	5.22E-05	0.046946	4.06	0.12	Unknown

Down-regulated in liver

L_TR47259|c0_g1_i1	-9.76	1.23E-09	1.50E-05	0.03	34.22	uncharacterized protein loc105607204 isoform x1
L_TR10266|c1_g2_i3	-3.83	1.44E-09	1.50E-05	1.52	21.59	acyl- -binding protein
L_TR8752|c0_g3_i2	-2.05	1.48E-07	0.00088	28.5	117.84	zinc ion binding
L_TR28955|c0_g1_i1	-8.13	3.15E-07	0.001462	0.02	10.09	Unknown
L_TR30917|c0_g1_i1	-3.87	7.23E-07	0.002742	0.07	1.09	upf0545 protein c22orf39 homolog isoform x1
L_TR28955|c0_g2_i1	-6.93	2.88E-06	0.00801	0.05	8.13	ubiquinone biosynthesis protein coq4 mitochondrial isoform x5
L_TR29354|c0_g1_i1	-3.05	3.03E-06	0.00801	0.51	4.21	Unknown
L_TR5337|c0_g1_i1	-4.73	3.21E-06	0.00801	0.12	3.34	Unknown
L_TR60746|c4_g1_i1	-4.48	3.35E-06	0.00801	0.05	1.3	uncharacterized protein partial
L_TR74636|c14_g8_i1	-2.02	3.58E-06	0.00801	11.5	46.47	craniofacial development protein 2
L_TR26826|c4_g1_i1	-2.95	4.59E-06	0.009568	0.38	3.06	endonuclease-reverse transcriptase
L_TR22918|c2_g1_i1	-3.99	8.73E-06	0.015372	1.48	23.38	myomegalin isoform x14
L_TR43779|c0_g1_i2	-7.74	8.85E-06	0.015372	0	3.7	Unknown
L_TR62456|c0_g1_i1	-2.65	9.56E-06	0.015945	1.74	13.38	Unknown
L_TR69615|c0_g1_i1	-3.65	1.24E-05	0.019822	2.1	25.99	Unknown
L_TR3476|c0_g1_i1	-2.61	1.56E-05	0.023558	1.05	6.36	Unknown
L_TR46171|c0_g2_i2	-2.84	1.58E-05	0.023558	0.51	3.59	Unknown
L_TR15492|c1_g1_i1	-4.05	1.91E-05	0.027461	0.13	2.14	endonuclease reverse transcriptase
L_TR26463|c1_g1_i1	-4	2.39E-05	0.028849	0.93	15.15	Unknown
L_TR23471|c1_g1_i3	-2.45	2.42E-05	0.028849	0.42	2.31	cell death activator cide-3 isoform x2

Up-regulated in RPLN

N_TR92656|c3_g7_i1	3.15	3.58E-09	9.60E-05	3.82	0.4	Unknown
N_TR181264|c0_g1_i1	5.42	5.42E-09	0.000109	4.8	0.1	low quality protein: saoe class i histocompatibility a alpha chain-like

N_TR87145|c0_g1_i4	7.75	1.81E-08	0.000242	2.38	0	Unknown
N_TR101034|c0_g4_i11	2.03	2.93E-07	0.002067	4.01	0.99	Unknown
N_TR113697|c0_g2_i1	2.5	6.94E-07	0.00294	3.31	0.59	low quality protein: endogenous retrovirus group k member 25 pol
N_TR160975|c0_g1_i1	2.02	8.34E-07	0.003354	5.01	1.24	Unknown
N_TR122168|c0_g4_i1	2.25	3.49E-06	0.012216	5.26	1.08	Unknown
N_TR108513|c1_g2_i1	2.69	5.04E-06	0.016226	3.93	0.58	Unknown
N_TR100013|c5_g1_i1	2.31	6.28E-06	0.018102	2.91	0.58	Unknown
N_TR113697|c0_g5_i1	2.45	8.65E-06	0.022443	5	0.91	uncharacterized protein loc104969954 isoform x1
N_TR158607|c0_g2_i2	3.42	1.00E-05	0.02443	2.2	0.19	Unknown
N_TR164441|c0_g1_i1	2.46	1.77E-05	0.035534	6.64	1.19	Unknown
Down-regulated in RPLN						
N_TR151520|c0_g2_i1	-2.13	6.19E-11	4.98E-06	6.29	27.09	nascent polypeptide-associated complex subunit alpha
N_TR149140|c6_g1_i2	-8.29	3.06E-10	1.23E-05	0	3.72	Unknown
N_TR81328|c8_g2_i1	-8.58	8.84E-09	0.000142	0	4.69	Unknown
N_TR81328|c8_g5_i1	-7.3	2.22E-08	0.000255	0	2.33	Unknown
N_TR81328|c8_g1_i1	-6.65	7.86E-08	0.00079	0	1.69	Unknown
N_TR84619|c1_g1_i3	-2.03	2.84E-07	0.002067	1.38	5.52	uridine-cytidine kinase 1
N_TR93637|c1_g1_i1	-2.32	5.17E-07	0.002599	0.59	2.99	Unknown
N_TR41415|c0_g1_i1	-2.51	6.75E-07	0.00294	0.26	1.48	Unknown
N_TR43226|c0_g1_i1	-3.67	1.35E-06	0.005155	0.4	5.16	Unknown
N_TR164448|c1_g2_i2	-4.39	2.06E-06	0.007536	0.14	2.94	Unknown
N_TR97881|c0_g1_i1	-5.73	1.10E-05	0.026083	0.16	8.02	membrane-spanning 4-domains subfamily a member 8 isoform x1

Table 4. Validation status and known functions of differentially expressed genes collected from liver and retropharyngeal lymph node (RPLN) tissues from white-tailed deer in the chronic wasting disease endemic area of northern Illinois, winter 2015.

Sequence name	Sequence description	Validation status
Up-regulated in liver		
L_TR43469|c2_g3_i5	endogenous retrovirus group k member 25 env poly	Interpret with caution
L_TR63450|c1_g1_i1	uncharacterized protein loc105607204 isoform x1	Failed
L_TR45335|c0_g1_i1	Unknown	Passed
L_TR29095|c7_g3_i1	sodium channel protein type 11 subunit partial	Passed
L_TR56520|c6_g2_i1	Unknown	Passed
L_TR56520|c6_g3_i2	syntaxin-binding protein 5	Passed
L_TR41343|c0_g1_i2	uncharacterized protein loc102402433 isoform x1	Passed
L_TR49285|c0_g1_i1	Unknown	Interpret with caution
L_TR77350|c0_g1_i1	gag protein	Interpret with caution
L_TR47646|c1_g1_i1	Unknown	Failed
L_TR56520|c6_g1_i1	syntaxin-binding protein 5	Passed
L_TR53215|c2_g3_i1	Unknown	Interpret with caution
L_TR41343|c0_g1_i3	Unknown	Failed
L_TR79592|c7_g2_i1	interleukin-17 receptor a isoform x2	Passed
L_TR27390|c2_g1_i1	Unknown	Passed
L_TR41343|c0_g1_i1	Unknown	Failed

Down-regulated in liver

L_TR47259|c0_g1_i1	uncharacterized protein loc105607204 isoform x1	Failed
L_TR10266|c1_g2_i3	acyl- -binding protein	Interpret with caution
L_TR8752|c0_g3_i2	zinc ion binding	Passed
L_TR28955|c0_g1_i1	Unknown	Passed
L_TR29354|c0_g1_i1	Unknown	Passed
L_TR5337|c0_g1_i1	Unknown	Interpret with caution
L_TR60746|c4_g1_i1	uncharacterized protein partial	Passed
L_TR74636|c14_g8_i1	craniofacial development protein 2	Interpret with caution
L_TR26826|c4_g1_i1	endonuclease-reverse transcriptase	Passed
L_TR22918|c2_g1_i1	myomegalin isoform x14	Interpret with caution
L_TR43779|c0_g1_i2	Unknown	Failed
L_TR62456|c0_g1_i1	Unknown	Passed
L_TR69615|c0_g1_i1	Unknown	Passed
L_TR3476|c0_g1_i1	Unknown	Passed
L_TR46171|c0_g2_i2	Unknown	Passed
L_TR15492|c1_g1_i1	endonuclease reverse transcriptase	Failed
L_TR26463|c1_g1_i1	Unknown	Passed
L_TR23471|c1_g1_i3	cell death activator cide-3 isoform x2	Passed

Up-regulated in lymph

N_TR92656|c3_g7_i1	Unknown	Failed
N_TR181264|c0_g1_i1	low quality protein: saoe class i histocompatibility a alpha chain-like	Passed
N_TR87145|c0_g1_i4	Unknown	Passed
N_TR101034|c0_g4_i11	Unknown	Failed

N_TR113697|c0_g2_i1	low quality protein: endogenous retrovirus group k member 25 pol	Passed
N_TR160975|c0_g1_i1	Unknown	Passed
N_TR122168|c0_g4_i1	Unknown	Interpret with caution
N_TR108513|c1_g2_i1	Unknown	Passed
N_TR113697c0_g5_i1	uncharacterized protein loc104969954 isoform x1	Passed
L_TR30917|c0_g1_i1	upf0545 protein c22orf39 homolog isoform x1	Passed
L_TR28955|c0_g2_i1	ubiquinone biosynthesis protein coq4 mitochondrial isoform x5	Passed
N_TR151520|c0_g2_i1	nascent polypeptide-associated complex subunit alpha	Failed
N_TR149140|c6_g1_i2	Unknown	Failed
N_TR81328|c8_g2_i1	Unknown	Passed
N_TR81328|c8_g5_i1	Unknown	Failed
N_TR81328|c8_g1_i1	Unknown	Interpret with caution
N_TR84619|c1_g1_i3	uridine-cytidine kinase 1	Interpret with caution
N_TR93637|c1_g1_i1	Unknown	Passed
N_TR41415|c0_g1_i1	Unknown	Failed
N_TR43226|c0_g1_i1	Unknown	Failed
N_TR164448|c1_g2_i2	Unknown	Interpret with caution
N_TR97881|c0_g1_i1	membrane-spanning 4-domains subfamily a member 8 isoform x1	Passed

Table 5. Gene ontology classifications for differentially expressed genes in retropharyngeal lymph node tissue of chronic-wasting disease infected white-tailed deer collected in the chronic wasting disease endemic area of northern Illinois during annual population reduction, winter 2015.

Classification	GO terms	Number of transcripts
Cellular Component		
GO:0005623	cell	1
GO:0044464	cell part	1
GO:0043226	organelle	1
GO:0044464	cell part	1
Biological Process:		
GO:0008152	metabolic process	2
GO:0051179	localization	1
GO:0009987	cellular process	2
GO:0051234	establishment of localization	1
Molecular Function:		
GO:0030528	transcription regulator activity	1
GO:0003824	catalytic activity	1
GO:0005488	binding	2

Table 6. Gene ontology classifications for differentially expressed genes in liver tissue of chronic-wasting disease infected white-tailed deer collected during annual population reduction, winter 2015.

Classification	GO term	Number of transcripts
Cellular Component		
GO:0005623	cell	3
GO:0044464	cell part	3
GO:0032991	macromolecular complex	1
GO:0043226	organelle	3
GO:0044464	cell part	3
Biological process		
GO:0032502	developmental process	2
GO:0016043	cellular component organization	1
GO:0008152	metabolic process	3
GO:0016265	death	1
GO:0043473	pigmentation	2
GO:0051179	localization	1
GO:0032501	multicellular organismal process	1
GO:0009987	cellular process	3
GO:0051234	establishment of localization	1
GO:0065007	biological regulation	2
Molecular binding		
GO:0030234	enzyme regulator activity	1
GO:0003824	catalytic activity	1
GO:0005488	binding	3

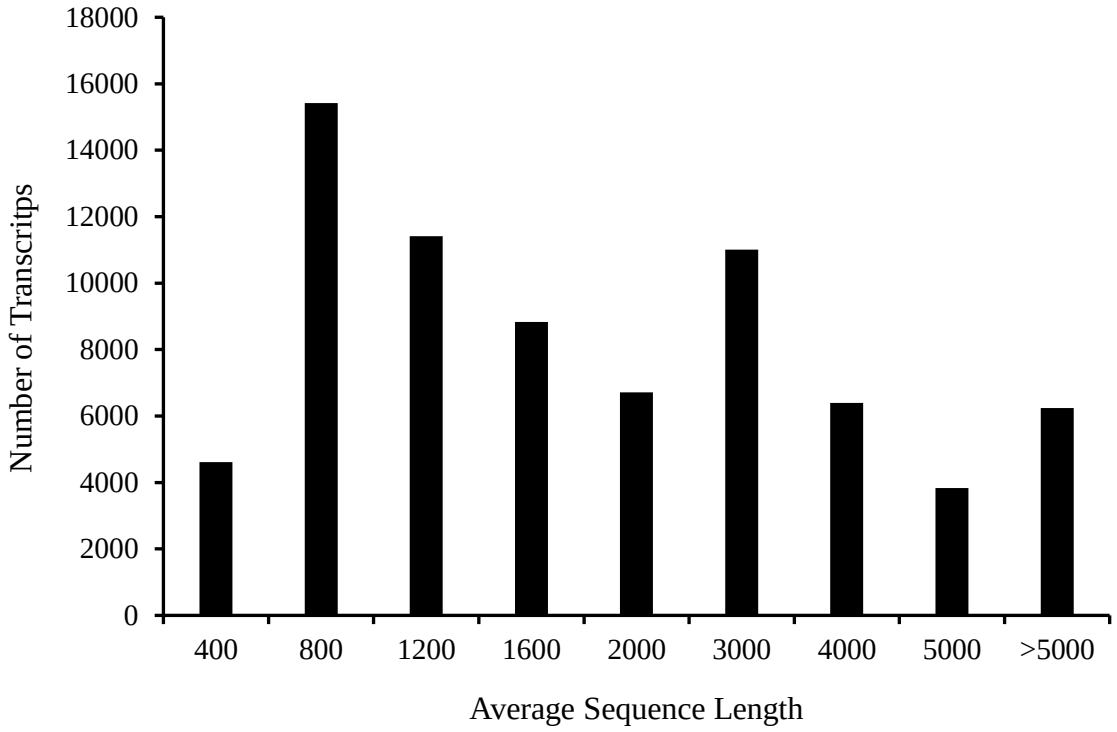
List of Figures:

Figure 1. Overview of average sequence length distributions for all the transcripts sequenced *de novo* using RNA-Seq from white-tailed deer collected in the chronic wasting disease endemic area of northern Illinois, winter 2015.

Figure 2. Number of white-tailed deer transcripts associated with Gene Onotology (GO) cellular component classifications.

Figure 3. Number of white-tailed deer transcripts associated with Gene Onotology (GO) biological process classifications.

Figure 4. Number of white-tailed deer transcripts associated with molecular Gene Onotology (GO) molecular function classifications.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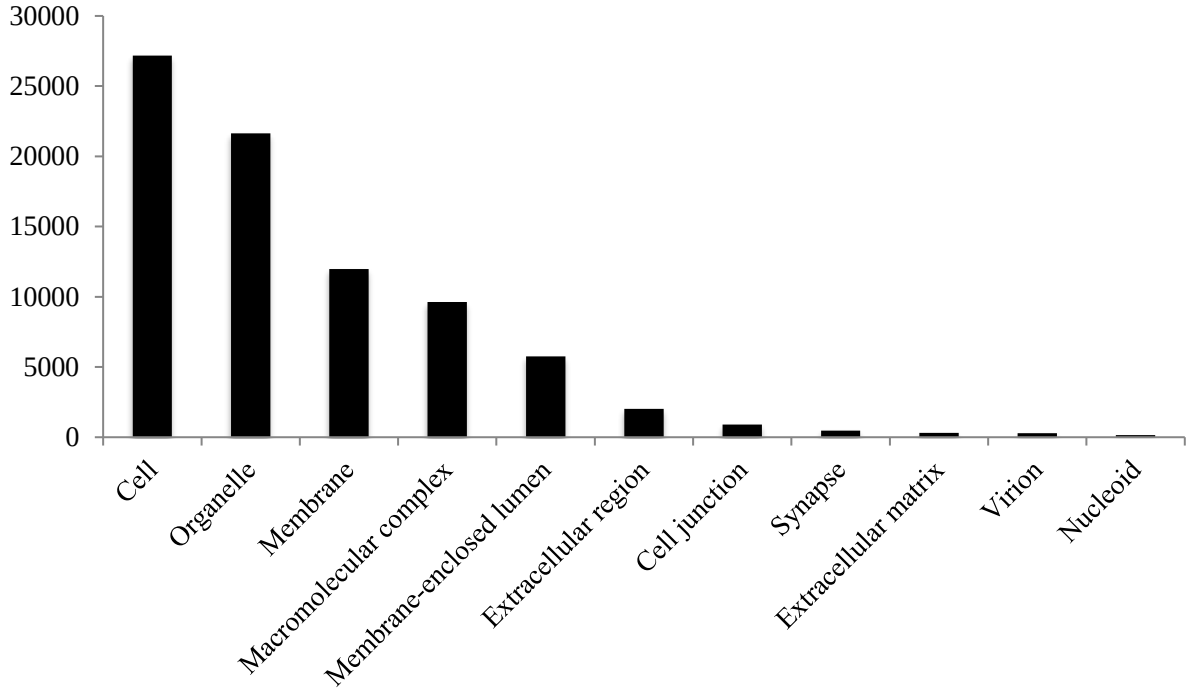
16000
14000
12000
10000
8000
6000
4000
2000
0
Number of Transcritps
400
800
1200
1600
2000
3000
4000
5000
>5000
Average Sequence Length

Figure 1.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25000
20000
15000
10000
5000
0
Cell
Organelle
Membrane
Macromolecular complex
Membrane-enclosed lumen
Extracellular region
Cell junction
Synapse
Extracellular matrix
Virion
Nucleoid

Figure 2.

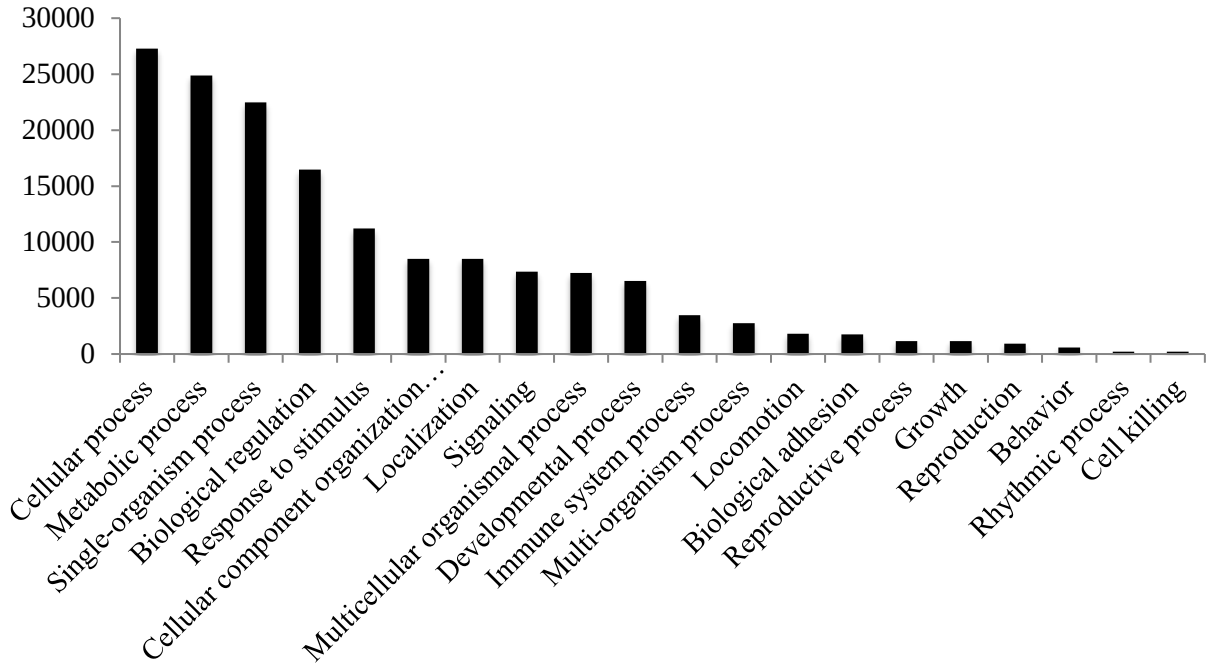
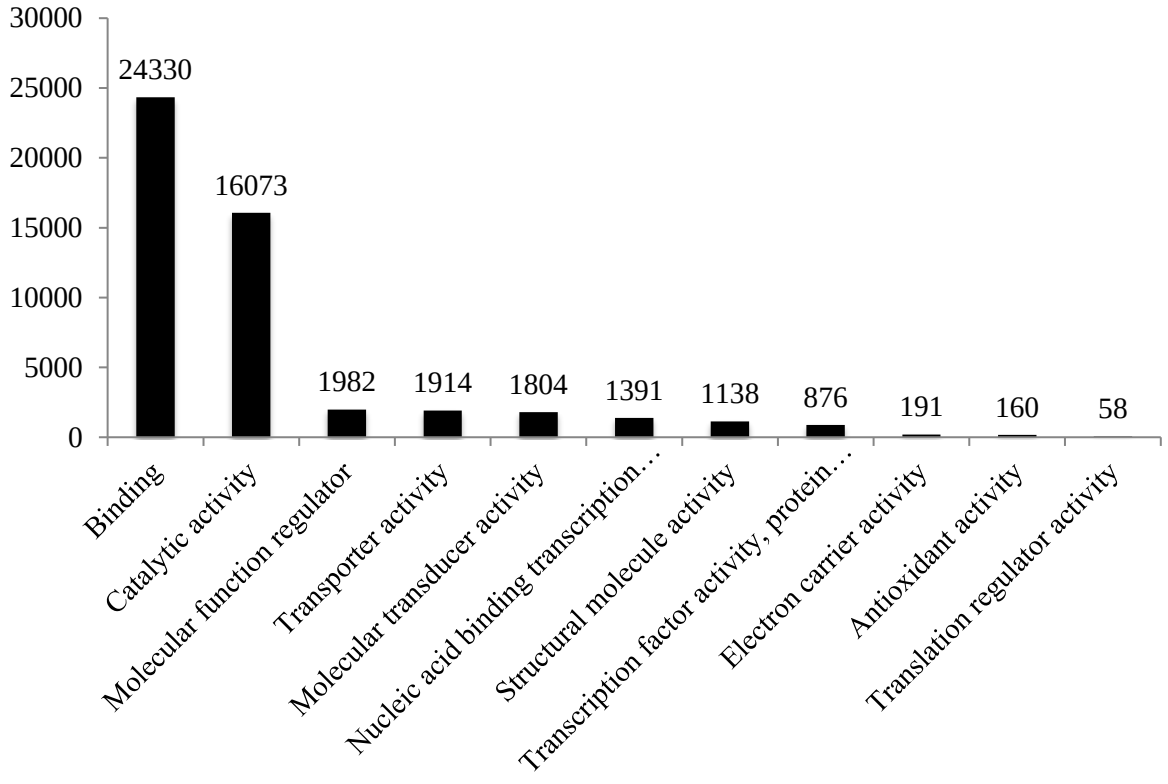

30000
25000
20000
15000
10000
5000
0
Cellular process
Metabolic process
Single-organism process
Biological regulation
Response to stimulus
Cellular component organization…
Localization
Signaling
Multicellular organismal process
Developmental process
Immune system process
Multi-organism process
Locomotion
Biological adhesion
Reproductive process
Growth
Reproduction
Behavior
Rhythmic process
Cell killing

Figure 3.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25000
20000
15000
10000
5000
0
24330
16073
1982
1914
1804
1391
1138
876
191
160
58
Binding
Catalytic activity
Molecular function regulator
Transporter activity
Molecular transducer activity
Nucleic acid binding transcription…
Structural molecule activity
Transcription factor activity, protein…
Electron carrier activity
Antioxidant activity
Translation regulator activity

Figure 4.