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Determining Master Regulatory Genes of Muscle Senescence in the Hawk Moth, Manduca sexta

Leah J. Naasz
University of South Dakota

DETERMINING MASTER REGULATORY GENES OF MUSCLE SENESCENCE IN THE HAWK MOTH, *MANDUCA SEXTA*

By: Leah Naasz

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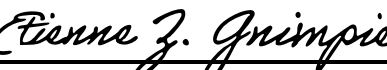
The members of the Honors Thesis
Committee designated to examine the thesis
of Leah Naasz find it satisfactory and
recommend that it be accepted.

Bernie Wone, Ph. D.
Assistant Professor of Biology
Director of the Committee

Beate Wone, M. S.
Instructor of Biology



Etienne Z. Gnimpieba, Ph. D.
Research Assistant Professor of the
Biomedical Engineering Program

ABSTRACT

DETERMINING MASTER REGULATORY GENES OF MUSCLE SENESCENCE IN THE HAWK MOTH, *MANDUCA SEXTA*

Leah Naasz

Director: Bernie Wone, Ph.D.

Skeletal muscle exhibits a gradual deterioration of its functional capabilities as it senesces. While the adverse effects of muscle aging are well-known, the molecular trigger of this degenerative process is unknown. Here, I aim to identify master regulatory genes (i.e., transcription factors) that might be involved in the initiation of the muscle senescence process in our muscle aging model *Manduca sexta*. This invertebrate adult moth was chosen as the model organism due to its relatively short lifespan, similarity to the vertebrate muscular system, and relatively low-cost to rear. Master regulatory genes are genes of a particular signaling pathway that is expressed at the foundation of specific biological pathways including growth, development, or disease manifestation. Time series RNA-Seq data can be used to construct gene regulatory networks to determine master regulatory genes. Here, I used the *corto* package in *Rstudio* to infer regulatory gene networks and create a regulon from the time series transcriptomics dataset from muscle tissue of *Manduca sexta*. *Corto* inferred a regulon of 118 candidates ($r > 0.74$). The regulon was visualized by Cytoscape to determine highly interconnected genes as possible master regulator genes of muscle senescence. Further research into the validation of top candidate genes is needed using qRT-PCR or knock out approaches. Discovering the master regulatory genes in *Manduca sexta* will help identify biomarkers involved in the upregulation of the muscle aging process.

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INTRODUCTION

Muscle aging is a dynamic biological process whose underlying molecular regulatory mechanisms are essentially unknown despite its negative effects on whole organism metabolism and physiology. In mammals, the impairment of muscle function is a key component of the overall aging process (Hindle 359). As muscles senesce and myofibrils decrease, the loss of force-generating capacity results in sarcopenia and decline in muscle function (Evans 6, Walston 623). Furthermore, muscle senescence can also lead to other detrimental conditions including osteoporosis, osteoarthritis, and muscular dystrophy (Baar 148). However, there is no unifying hypothesis for muscle senescence. While several hypotheses of skeletal muscle aging have been proposed, such as an increase of apoptosis, decrease in the number of satellite cells (Etienne), and mitochondrial fusion (Wone et al. b) what molecular mechanism triggers the aging process remains unknown due to the dynamic, yet universal, changes associated with aging. In fact, the unifying hypothesis of muscle senescence may be a combination of the hypotheses mentioned above. Previous studies into muscle senescence have demonstrated that significant transcriptional changes occur during the aging process (Lin). Therefore, further analysis into these transcriptional changes is necessary to determine the specific molecular mechanisms that initiate muscle senescence.

The development of biological processes or diseases is controlled by a transcription factor, or a group of transcription factors known as transcriptional master regulators (Sikdara and Datta). Therefore, identifying the master regulators of muscle senescence that initiate muscle aging will further our understanding of the process of aging itself and possibly the diseases or conditions associated with it. New correlation-

based gene regulatory network inference pipelines make it possible for master regulators to be identified from RNA-Seq data (Mercatelli 3917). Here, I determine the master regulator genes underpinning muscle senescence using the *corto* algorithm in *Rstudio* (Mercatelli), from a time series RNA-Seq dataset in muscle tissue of the hawk moth, *Manduca sexta*. Determining the master regulators of muscle senescence allows for the identification of the molecular mechanism that regulates transcriptional changes, alters gene expression, and ultimately, determines the molecular source that initiates skeletal muscle aging. By understanding the regulatory controls underlying the initiation of age-related musculoskeletal decline, the possibility to restore muscle function and prolong health expectancy in old age might be possible (Baar 153).

BACKGROUND

The term master regulators, or master regulator genes, was previously defined as a "gene that is at the top of the regulatory hierarchy" and by definition, "is not under the regulatory influence of any other gene" (Chan). However, this definition is no longer absolute. Current research published by Chan describes a new definition for the term master regulator as "a gene or signaling pathway that is expressed at the foundation of a developmental lineage or cell type, participates in the specification of that lineage by regulating multiple downstream genes, and when mis-expressed, has the ability to respecify the fate of cells destined to form other lineages." (Chan). Other studies have shown that some master regulator genes can be regulated by others (Cai). The action of master regulators triggers large-scale transcriptional cascades that are responsible for complex cellular processes, such as apoptosis, cell differentiation, and proliferation,

DNA-repairing processes, and disease manifestation (Hanahan and Weinberg, and Cai). I hypothesize that muscle aging lies under the control of the molecular action of master regulators. These master regulators lie at the top of the transcriptional regulation hierarchy because they control most of the regulatory activities of other transcription factors and associated genes (Sikdar and Datta).

Furthermore, master regulators have been shown to play a key role in multiple signal transduction pathways. Signal transduction pathways allow cells to sense specific signals, produce a cellular response, and serve an important role in information integration (Tapia-Carrillo). Specific transcription factors (TFs) can modulate the transcription of groups of genes that participate in these signal transduction pathways and can also be termed master regulators (Tapia-Carrillo). The analysis of the gene regulatory networks of signal transduction pathways that contain TFs and their target genes, along with generated co-expression data from transcriptomic data (Hansen), allows for the identification of master regulators that have the greatest influence over expression differences (Tapia-Carrillo).

The use of bioinformatic approaches that infer gene regulatory networks may help elucidate the specific molecular interactions involved in muscle senescence. Currently, most of the current tools for gene network inference that perform a master regulator analysis require a high amount of RAM and/or a computer cluster to be computed (Mercatelli 3916). As a result, the use of the lightweight *Rstudio* package *corto* for gene network inference and master regulator analysis can be used to determine master regulators and calculate the enrichment of the TF-centered network on a user-selected signature (Mercatelli 3917). A recent study revealed the master regulators behind the

SARS-CoV-2 virus by comparing infected and mock samples of bronchi epithelial cells in a MERS dataset and a SARS dataset (Guzzi et al. 982). Their results showed a decreased expression of the ACE-2 protein receptor and thus a candidate as a master regulator, allowing for the future development of specific therapies against the SARS-CoV-2 virus (Guzzi et al. 982). The ability of the *corto* algorithm to produce such results shows its potential in determining master regulators.

Manduca sexta was chosen as the model organism for examining muscle senescence based on previous research performed by Wone et. al (a and b). As shown previously, *M. sexta* has a relatively short lifespan of about ten days, making *in vivio* studies of aging readily observable. They quickly reproduce, supplying large populations with ease in comparison to its mammalian counterparts (Del Grosso 6). The housing and rearing of *M. sexta* are also relatively inexpensive. *Manduca sexta* also possesses a muscular system that is similar to vertebrates (Del Grosso 6). Specifically, the synchronous contraction of the endothermic, dorsolateral flight muscles of *M. sexta* during neural stimulation make it a reliable model organism (Yuan et. al, Marden 167, and Heinrich 232). The use of this invertebrate model will be useful in determining the molecular underpinnings of muscle senescence, allowing for further translational applications.

METHODOLOGY

Data Collection

The hawk moths, *Manduca sexta*, were obtained from the University of Arizona, Tucson, AZ, and were used for all experiments. An artificial diet was used to rear the larvae and was given *ad lib*. Then, adults were individually reared in a 299 cm^3 cage (BioQuip Products, Rancho Dominguez, CA, USA). Eggs, larvae, and adults were housed at 25 °C under a of 16-hour light / 8-hour dark cycle at 60% humidity. Artificial nectar was administered *ad lib* to the adult moths (Educational Science, League City, Texas, USA). Separation of the moths based on sex occurred when housing the adult moths to prevent mating. Dates for hatching and death were recorded for each individual. The lifespan of each adult was calculated in full days. Day 2 (D2) was identified as middle age and advanced age was defined as Day 5 (D5) post-eclosion (Wone et al. a). Female moths were identified as middle aged in Day 4 and advanced age was identified as Day 7 (Wone et al. a). A time series characterization of age-related changes in flight muscle across the moth lifespans was performed, sampling the moths at diel time and age. Moths were euthanized by decapitation. Beginning at middle age and continuing until advanced age, during photophase (0900 h) and scotophase (2100 h after 1 h of activity), dorso-longitudinal muscles of adults were collected (Wone et al. a). Each sex was sampled at eight consecutive time points. Males were sampled at time points D2 at photophase and N2 at scotophase, D3, N3, etc. until N5. During the sampling, males Age 2 (MA2) were classified as middle age and males Age 5 (MA5) were classified as advanced age. Females were sampled at time points D4 (day four at photophase), N4 (night 4 at scotophase), D5, N5, D6, N6, D7, and N7. Unlike the male classification, females Age 4

(FA4) were classified as “middle aged” and females Age 7 (FA7) were classified as “aged”. A total of 48 moths were sampled for RNA-Seq. Flight muscles were selected for sampling, dissected, and flash frozen in liquid nitrogen. Less than 90 seconds elapsed from time of death to flash freezing of dissected muscle tissue. The samples were ground to a fine powder under liquid nitrogen and stored at -80˚C until RNA extraction.

RNA extraction

Fifty mg of extracted muscle tissue was placed in 2 mL microcentrifuge tubes. 1 mL of TRIzol™ reagent was added to each tube and homogenized. After incubation for 5 min at room temperature, to dissociate the nucleoprotein complexes, 0.200 mL of chloroform was added to each tube, vortexed, and incubated at room temperature for 3 min. The samples were then centrifuged at room temperature for 15 min at 12,000x g. Approximately 500 µL of the aqueous phase was extracted and pipetted into a new 2 mL microcentrifuge tube. Chloroform was then added (0.200 mL) to each tube and was vortexed again. After centrifugation, the samples sat at room temperature for 3 min. Samples were then centrifuged for 15 min at 12,000x g at room temperature. The aqueous phase was extracted and placed in a fresh microcentrifuge tube. Isopropyl alcohol (500 µL of 100%) was added to the sample tubes, capped and inverted several times, then allowed to incubate at room temperature for 10 min. To pellet the RNA, samples were centrifuged at 12,000x g for 10 min at room temperature. The supernatant layer was pipetted from the sample tubes, rinsed with 500 µL of 75% ethanol, and centrifuged at 8,000x g at room temperature for 10 min. The supernatant layer was then removed once again, and the samples were washed again with the 500 µL of 75% ethanol. The samples were centrifuged again at room temperature for 10 min at 8,000x g.

After centrifugation, the supernatant layer was removed, and the precipitate was isolated for drying under a laminar flow hood until all remaining ethanol was evaporated. To reform the precipitate, 20 μL of molecular grade water was added to it and stored in a freezer at -80 ˚C.

Time Series RNA Sequencing

RNA sequencing (RNA-Seq) was performed on *Manduca sexta* flight muscles across diel time, ages, and sex. Novogene (Novogene, Sacramento, CA, USA) prepared cDNA libraries and performed RNA Sequencing (RNA-Seq). The Illumina NovaSeq 6000 platform sequenced RNA-Seq libraries as 150-nt paired-end reads (Illumina, San Diego, CA, USA) with 18 samples per high-output flow cell. A total of 16 cells were used to generate about 20-25 M reads per sample. After several quality control checks, such as trimming, removal of primer, and removal of low-quality reads, high-quality readers were obtained. Next, the filtered high-quality reads of all samples were mapped on the Bombyx mori reference genome, since only a draft genome is currently available for *Manduca sexta*. To count read numbers mapped on each gene FeatureCounter v1.5.0-p3 was used. In total, male *Manduca sexta* RNA-Seq analysis produced 1,501,272,784 raw reads and 1,424,653,808 clean reads. Upon further analysis, approximately 84.6% of total clean reads were mapped to the *Bombyx mori* reference genome. For the female *Manduca sexta,* RNA-Seq analysis produced 1,520,863,862 raw reads and 1,437,381,790 clean reads in total. Approximately 87.2% of the clean reads generated were mapped to the *Bombyx mori,* silkworm reference genome (Wone UnPub Data).

Validation of Differential Expression Genes (DEGs) using qRT-PCR

RNA-Seq results were validated from selected muscle-specific genes and these include *akirin, gelsolin, kelch-like protein 5*, and *titin* (see Del Grosso 34). Briefly, primers for the selected genes were designed and used for qRT-PCR (Table 1 from Del Grosso 34). The Luna® Universal One-Step qRT-PCR Kit (New England Biolabs, Ipswich, MA, USA) and the Applied Biosystems QuantStudio 3 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) were used for the qRT-PCR validation (Del Grosso 34).

Table 1. Forward and reverse primers designed for the selected genes for qRT-PCR validation (Del Grosso 34).

Gene	Forward Primer	Reverse Primer
Akirin	5' – TTA TGT TTC CCC ACC TGT CTG	5' – GAA CAC AAT TAT CCA GCG AAC C
Gelsolin	5' - CTA CAT TCT GGA CAC GGG AAG	5' – TGA AAC GTG TAC CCA GTT AGG
Kelch-like protein 5	5' – TTC CTT GCT GTT CTC CCA TAG	5' – AGT CCA AGT GTT TGT CCG TG
Titin	5' – TGA ACC CTA TTG AGT CTT GCT G	5'- GTG GCC TGA CAT GAA GTC TAG
Actin	5' – GCC AGA AAG ACT CCT ACG TTG	5' – TTC TCC ATG TCA TCC CAG TTG

Master Regulator Analysis

Using *corto* (Correlation Tool), a lightweight R package for Gene Network Inference and Master Regulator Analysis, a Master Regulator Analysis (MRA) was performed from gene expression data for the male hawk moths provided by the RNA-Seq analysis (Del Grosso 34). The female data was not used for this analysis because there were no identifiable point specific age-related changes in their RNA-Seq data (Del Grosso 54).

Therefore, using the male RNA-Seq data, a regulon was generated as the output of the *corto* function from an expression matrix, that featured genes as rows and samples as columns, and a list of centroids formatted into a character vector indicating which genes to consider as centroids. These centroids are otherwise referred to as master regulators. After the regulon was generated by the *corto* function, the regulon was loaded into Cytoscape—an open-source bioinformatics platform for visualizing molecular interaction networks and the integration of gene expression profiles. Loading the data into Cytoscape created a visual regulon network from which subnetworks were created and analyzed to see the connections and interactions of each gene. The genes with the most connections, *aka* the ones that were highly connected to other genes, were separated from the rest of the genes, colored, and then highlighted for visual examination purposes. The description of these genes was identified from the RNA-Seq matrix.

RESULTS

Using the *corto* function, an output object termed a regulon was generated and consisted of 118 centroid-based target genes ($r > 0.75$) from a list of 5,820 transcription factors. Loading the regulon into Cytoscape produced a visual network (Figure 1) consisting of three subnetworks, as well as other much smaller gene interactions between 2-3 genes.

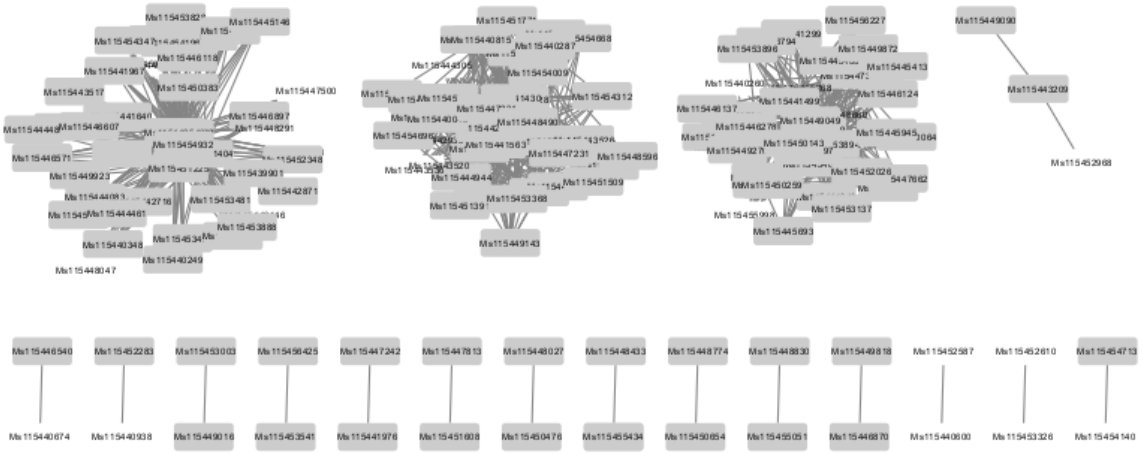

Figure 1. Visualization of the regulon consisting of 118 candidate genes that might be involved in muscle senescence of the hawk moth, *Manduca sexta*.

Further analysis of subnetworks of the regulon provided a list of 16 highly connected candidate genes of muscle senescence. The visualization of subnetwork A identified 6 genes that were highly connected to the other genes Ms115451404, Ms115442008, Ms115454932, Ms115442540, Ms115451225, and Ms115440962 (Figure 2). Visualization of subnetwork B identified 6 genes that were highly connected to the other genes Ms115455723, Ms115450289, Ms115454391, Ms115442113, Ms115453610, and

Ms115453934 (Figure 3). Visualization of subnetwork C identified 4 genes that were highly connected to the other genes Ms115453894, Ms115449049, Ms115445268, and Ms115445569 (Figure 4).

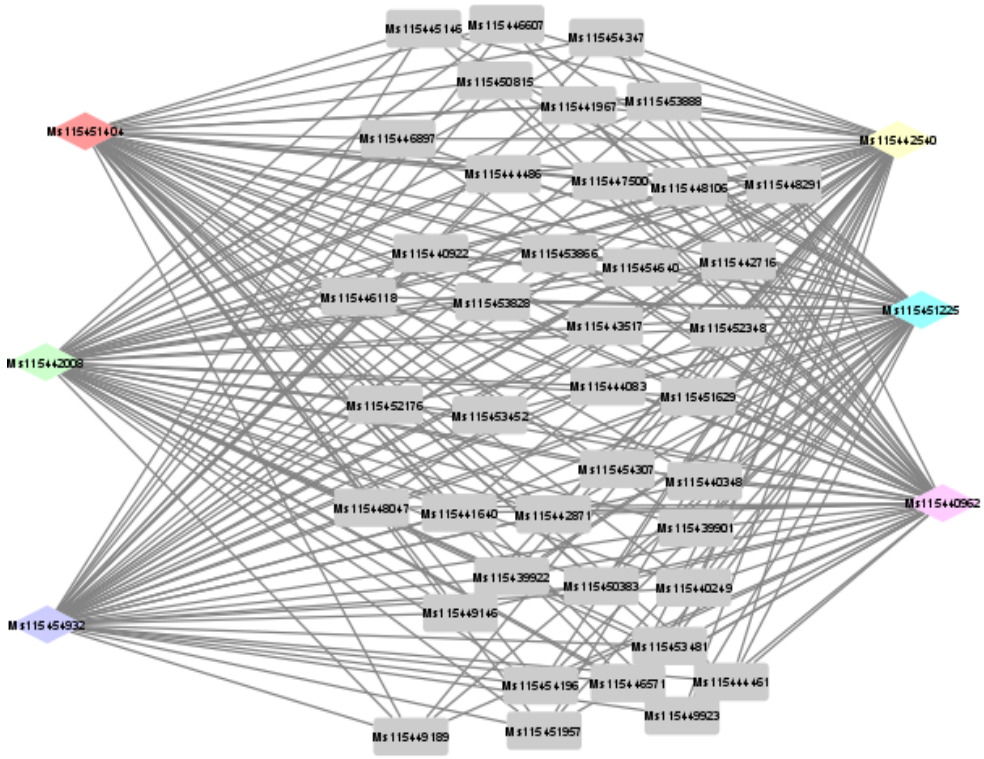

Figure 2. Visualization of subnetwork A showing 6 candidate master regulator genes Ms115451404, Ms115442008, Ms115454932, Ms115442540, Ms115451225, and Ms115440962 highly interconnected with other genes.

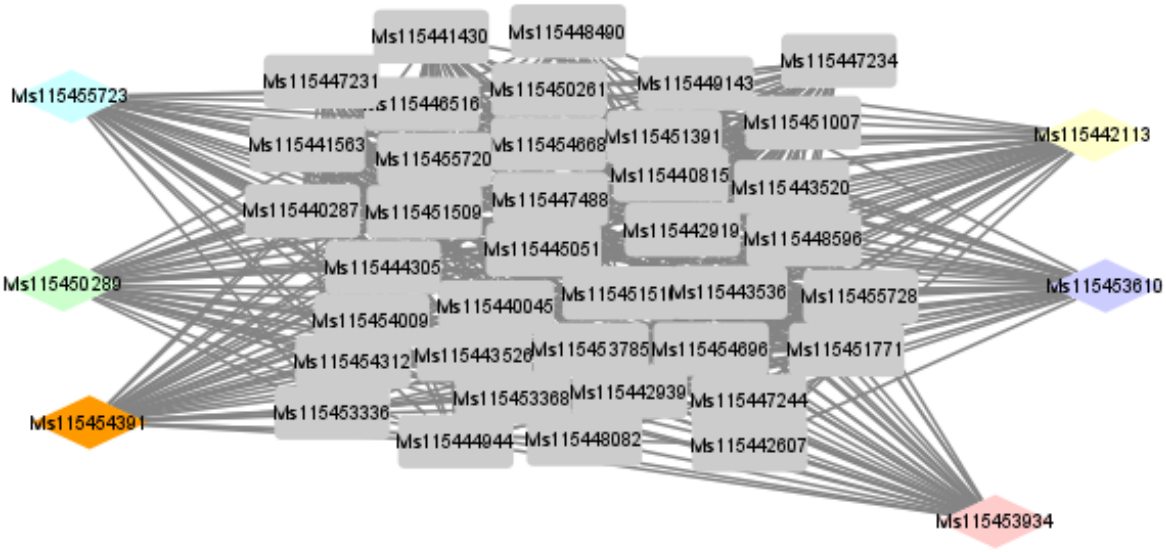

Figure 3. Visualization of subnetwork B showing 6 candidate master regulator genes Ms115455723, Ms115450289, Ms115454391, Ms115442113, Ms115453610, and Ms115453934 highly interconnected with other genes.

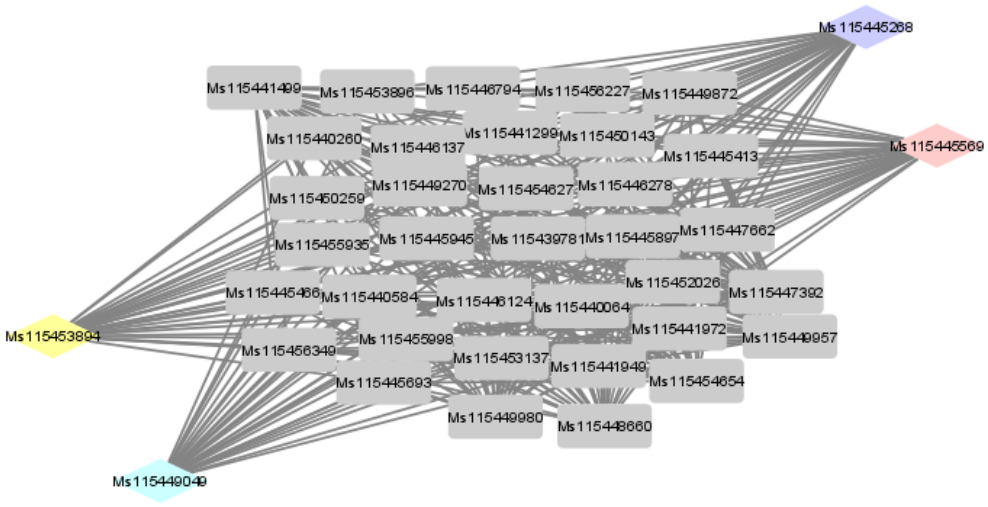

Figure 4. Visualization of subnetwork C showing 4 candidate master regulator genes Ms115453894, Ms115449049, Ms115445268, and Ms115445569 highly interconnected with other genes.

DISCUSSION

The genes that showed multiple connections may be of importance for regulating other genes involved in muscle senescence. These candidate master regulator genes need to be verified using either qRT-PCR to quantify gene expression or knock-out approaches to validate they are of regulatory significance. Using qRT-PCR allows for the validation of reference genes in this experiment by comparing it to a foreign cRNA added to each RNA sample prior to qRT-PCR of younger hawk moths to normalize data for reference gene transcripts prior to assessment of their expression stability (Czechowski et al. 12). I hypothesize that if these genes are of significance, a gene knock-out approach will allow for genetic mutation of these genes to render them non-functional. Thus, the process of muscle senescence might be greatly inhibited. Analyzing the flight capabilities of adult hawk moths also provides useful since only younger hawk moths can fly, while aged hawk moths cannot. Therefore, if these candidate genes are indeed master regulators, advanced age hawk moths will be able to fly following knock-out of the genes.

The *corto* algorithm appeared to identify genes that likely do not have regulatory significance. Specifically, genes identified were at one time point with very low expression levels near advanced age. After all, high expression levels of a gene during early age might be an indicator that that gene is a master regulator (Lavenus et al. 1368–1388). However, our low expression levels of these genes at advanced age samples likely indicate that these genes identified by *corto* are not involved in the regulation of muscle senescence, nor are they master regulators. If they were master regulators, we would expect to see low expression levels at the younger age samples. Instead, these genes were only very lowly expressed at one time point in advanced age. The *corto* algorithm

was only able to find genes that had one expression at one time point within the time series data. When genes with only one expression time point were removed from the dataset (i.e., all genes with zero values for any time point), the *corto* function was unable to generate a regulon. This nonfunctioning *corto* command might be an error with either the coding or the algorithm itself. We have communicated with the developer of *corto* and have not heard back and thus resolve this issue (Per Comm F. Giorgi).

Because *corto* is a correlation tool and not a tool for temporal time expression data, it does not have the ability to consider the RNA-Seq data of *M. sexta* as dependent data points. Instead, *corto* measures the degree in which one variable is related to another by measuring the co-occurrence between variables (Mercatelli 3916). This measured co-occurrence is used as a means to infer regulatory mechanisms or gene functions (D'Haeseleer, Hansen). *Corto* treats each sample as an independent time point, whereas time series data expression is dependent on the data prior. Therefore, *corto* does not take prior data points into consideration. Perhaps because of this, the algorithm is not as powerful for identifying key regulators or genes if it does not consider what is expressed prior

To overcome the issues mentioned above, the use of another gene regulatory network algorithm is necessary. A gene regulatory network algorithm that might provide useful for analyzing this specific data is ARACNe: Algorithm for the Reconstruction of Accurate Cellular Networks or the Time Delayed Correlation algorithm (TDCor) in *Rstudio*. TDCor has successfully inferred linear gene regulatory network for a time-series transcriptomic data set of an *Arabidopsis* lateral root initiation (Lavenus et al. 1369 and 1372), showing its ability to counteract the problems encountered in this study with

corto and our time-series dataset. In the *Arabidopsis* study, TDCor reconstructed network topology from a subset of expression profiles to provide links in very early activated genes to late activated genes of lateral root initiation (Lavenus et al. 1373). The study indicated that TDCor is suitable for identifying master regulators of *Arabidopsis* lateral root initiation with a high level of confidence (Lavenus et al. 1375). The ability of TDCor to analyze transcriptomic data accurately while also creating topology links based on time of activation may help elucidate the master regulators that control muscle senescence in *Manduca sexta*.

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