Compound Efficacy Testing

Using Pathway and Mechanisms of Action (MOA) Analyses

InVivo Biosystems

Clients of InVivo Biosystems' include pharmaceutical companies that investigate chemically synthesized small molecules for efficacy on a specific gene target of interest and nutraceutical clients that wish to investigate the impact of naturally derived compounds or mixtures.

Quite often, our clients need to test and understand the effects of a compound, an ingredient, or a formulation on lifespan, oxidative stress, genetic pathways or transcriptional changes related to aging.

In this article, we will use sample data to illustrate the type of scientific data we typically produce for our clients. The data below demonstrate the data analysis using our platform to answer the following questions:

- Does compound/ingredient A and/or B increase lifespan in live animals? (Figure 1, Table 1, 2)
- Does compound/ingredient A and/or B increase quality of life (healthspan)? (Figure 2.1 - 2.3)
- What genes are up and down regulated when animals are treated with the compound or formulation? (Figure 3, Figure 4)
- What mechanisms of action and genetic pathways are impacted when animals are treated with compound/ingredient A? (Figure 5, Table 3)

Question 1: Does compound/ingredient A and/or B increase lifespan in live animals?

To measure lifespan and healthspan, precise movement data were automatically collected using our lifespan assay. Our longevity readout provides both a Kaplan-Meier estimate of survival (Fig. 1) and a hazard rate curve – two functions that are the industry standard for modeling survival and are widely used in worm, mouse, and human studies. We also provide statistical examination of the curves, including the Mantel-Cox Log-rank test (Table 1) and the Age in Days at Percent Mortality Milestones (Table 2).

To understand the physiological action and therapeutic potential of compounds targeting aging, it is crucial to use quantitative models. The analysis of lifespan data is grounded in the study of two mathematical functions: the survival curve and the hazard function. The survival curve (Figure 1) describes the fraction of a tested population that remains alive over time. The hazard function (Figure 2.1.2) is related to the survival curve and provides an intuitive measure of the risk of death. This function describes the probability that a typical individual who is currently alive will soon die, providing a clear visualization of the way a compound may change patterns in mortality.

To obtain high-resolution lifespan data and eliminate confounding factors such as worm handling and operator bias, lifespan data was collected using an Automated Lifespan Machine (ALM)7. Three biological replicates, derived from synchronizing three independently-maintained lines of N2 worms, formed a combined sample size of more than 450 synchronized worms per condition. Worms were exposed to compounds on day 1 of adulthood and placed in the machine two days later (adult day 3). Biological replicates were distributed across scanner instruments and images of the worms were collected for the next 40 days with no interruption or manipulations.

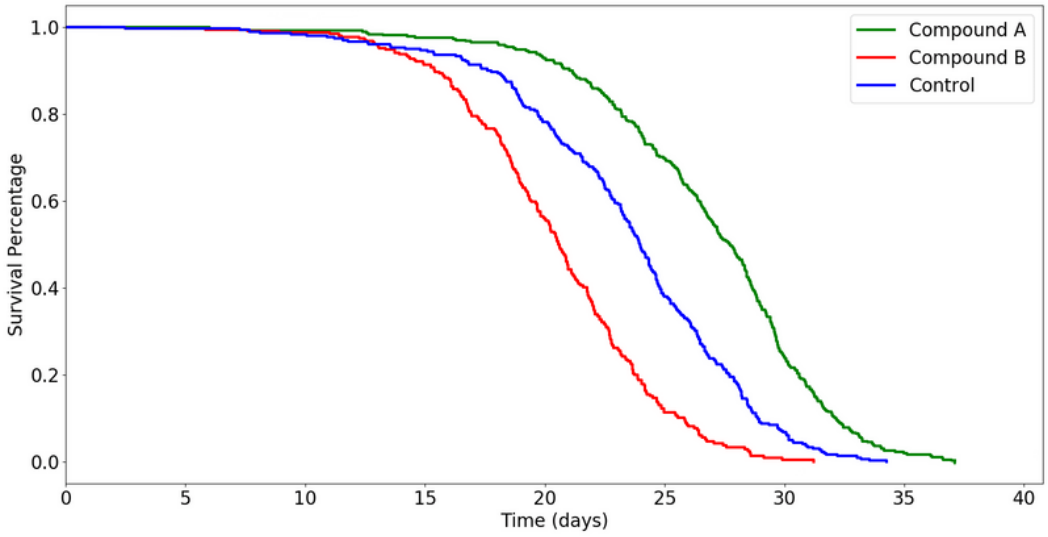

Figure 1. Kaplan-Meier curve showing the effects of two compounds on the lifespan of a population of animals. The curve depicts the decline in population as time progresses. The population is assessed 3 times each hour for 40 days. Animals treated with Compound A have a significantly longer lifespan than the animals treated with vehicle control. Animals treated with Compound B have a shortened lifespan.

Table 1. Mantel-Cox Pairwise statistical analysis of survival curves

Curve comparison	Test statistic (X^2)	Log-rank test P-value
Control vs. Compound A	138.97	<0.0005***
Control vs. Compound B	213.50	<0.0005***
Compound A vs. Compound B	203.55	<0.0005***

The Mantel-Cox Log-rank test is a non-parametric test that compares two survival functions across the duration of the lifespan.
Numbers and asterisks represent P-value and significance, respectively.

Table 2. Age in Days at Percent Mortality Milestones					
Treatment	25% mortality	50%	75%	90%	100%
Compound A	23.2	26.9	37.1	33.1	37.1
Control	19.5	23.1	25.8	27.6	32.3
Compound B	17.4	20.8	24.0	26.0	30.7

Age at percent mortality is the age in days at which the given percentage of worms are dead. Age at 50% mortality is equal to the median. These analyses are useful for examining early or late life-specific effects or when the survival curves are not parallel. Fisher's exact tests at these time points are included with the data supplement.

Question 2: Does compound/ingredient A and/or B increase quality of life (healthspan)?

To measure healthspan, active worms were identified using images from the lifespan assay. The worms' spatial location on the plate and their morphology were quantified throughout their lifespan to assess healthspan.

In this study, an Alzhiemer's model strain was used as the control to determine if the compounds A, B or A+B could positively impact healthspan.

Worm Activity serves as a proxy for animal health. Changes in spatial distribution of the worms between time points is used to derive **aggregate movement** for the population over time. Worm Activity is described by two complementary measurements of aggregate movement:

1. **Spatial Distribution** uses changes in worm contours between time points to measure the changes in the spatial distribution of the worms' bodies. The normalized distribution of these distances in the population provides a measure of how much the group of worms altered their positions and posture on the plate between two time-points.
2. **Centroid Distance calculates a geometric center for each individual worm, and then** measures the minimum collective distance that a group of worms moved between time points. It achieves measurement of the changes in spatial distribution between time-points.

For both of these Activity metrics, a plot with higher numbers indicates good health, and all measures are normalized to fall between 0 and 1.

Worm morphology is measured from the worm contours obtained detected in the images. In the process of aging, worms become shorter and stouter over time and their shape is an indicator of their overall health and biological age. The Length is calculated from the central spline fitted to the worm contour and Width is measured from each worm's widest point. The worms' posture also changes with age as they lose the ability to maintain an elongated position. Average Circularity measures how close the shape and posture comes to being enclosed by a circle.

Each of these measures are obtained by averaging data for all active worms detected on a plate, then averaging across different replicate plates of the same condition. All measurements are based on worms that are still alive and moving at the time of quantification. All measures start when worms were placed on the scanner at day 5 of age (120 hours of age, day 3 of adulthood).

Healthspan Results:

Aggregate Movement. The Alz. Model Control curve is shifted far to the left indicating that it had the weakest movement and the most rapid decline. The same model strain treated with Compound A + B had a much greater degree of movement. Compound A shows partial rescue in that it was consistently more active than the Alz. Model Control for a longer duration. The Compound B treatment showed a level of aggregate movement intermediate between the Alz. Model Control and the Compound A treated strain (Figure 2.1 A, B).

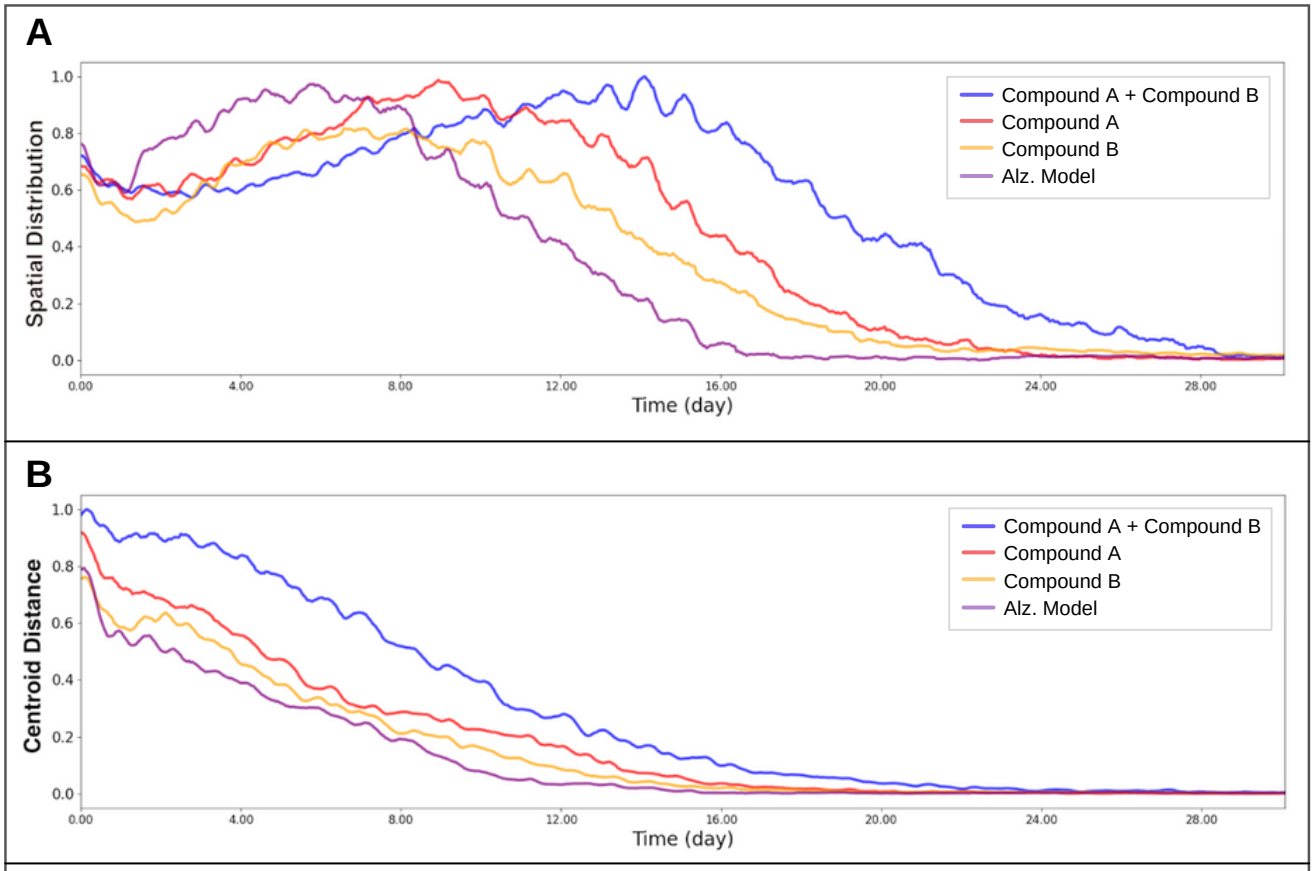

Figure 2.1. Worm Activity (Aggregate Motility) analysis over duration of lifespan.

1. Spatial Distribution. “(1-normalized mutual information)” measures changes in the spatial distribution of active worms between time points.
2. Centroid Distance. The “normalized minimum centroid distance” measures the changes in location of active worms as a group between time points.

Worm Morphology.

Again, treatment with Compound A + B yielded the largest, most robust animals, and size differences roughly correlate with movement phenotypes. The relative ranking of average worm length follows the movement and lifespan data: Compound A + B > Compound A > Compound B > Alz. Model Control (Figure 2.2 A). However, the width ranking differs in that the average widest point of Alz. Model Control is greater than that of Compound B (Figure 2.2 B).

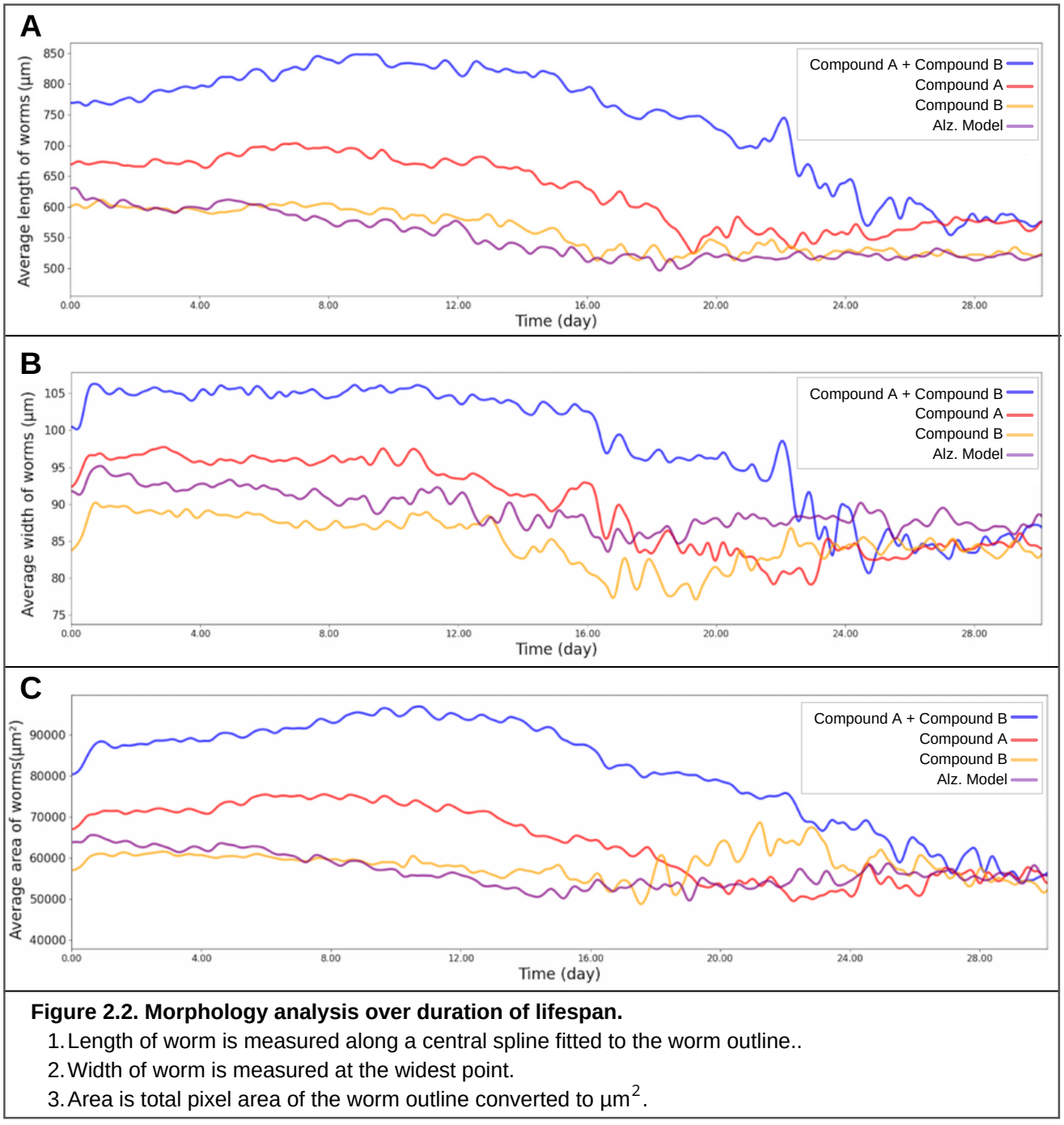

Figure 2.2. Morphology analysis over duration of lifespan.

1. Length of worm is measured along a central spline fitted to the worm outline..
2. Width of worm is measured at the widest point.
3. Area is total pixel area of the worm outline converted to μm^2.

Average Circularity.

The Average Circularity Assay indicates worm heath by describing how closely the shape and posture of the worms is enclosed within a circle. Healthy, active worms maintain an elongated, albeit sinusoidal posture. As unhealthy and aged worms lose muscle function they increasingly adopt a curled, bunched, or folded state in addition to a stout and wrinkled morphology. Hence, the shape of unhealthy worms is more readily enclosed by a perfect circle--their circularity is closer to 1.

In agreement with the other healthspan metrics, the Alz. Model Control has the highest circularity and Compound A + B has the lowest. The worms' circularity follows a pattern of being inversely correlated with the other healthspan metrics: Alz. Model Control > Compound B > Compound A > Compound A + B (Figure 3.3).

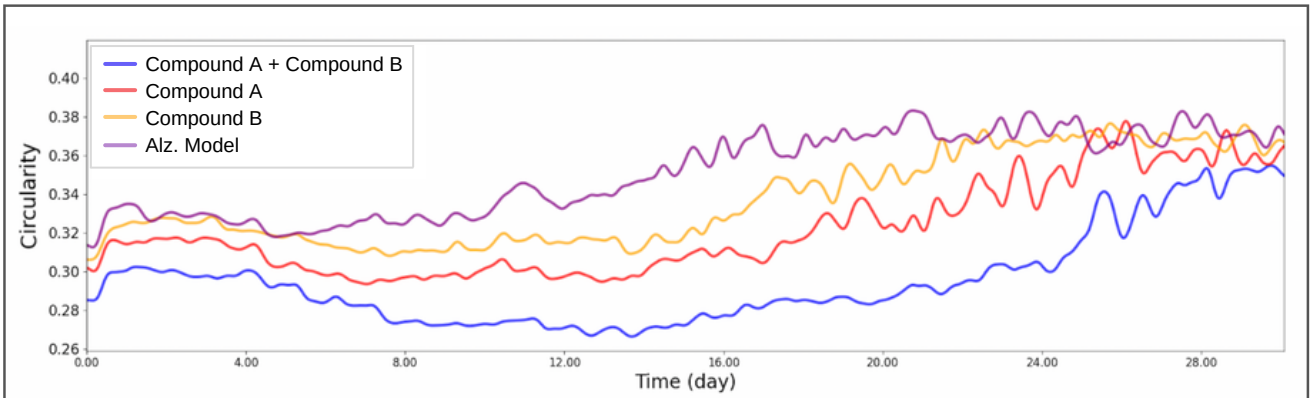

Figure 2.3. Average Circularity. Measures how close the worm's shape is to a perfect circle, with a perfect circle having circularity of 1.

Question 3: What genes are up and down regulated when animals are treated with the formulation?

To determine the mechanism of action of the lifespan extension, animals were exposed to compounds or vehicle alone at 48 hours after hatching (~L4 stage, -1 day to adulthood) and were harvested on Days 3 and 10 of adulthood. RNA was extracted and assessed for quality. All RNA had a quality index of greater than 9.5 (out of a possible 10). Libraries were prepared in a strand-specific manner and were sequenced on the Illumina platform obtaining paired-end sequence of 150 bp per end. Differential gene expression was performed with EdgeR using false likelihood ratio tests based on fitting linear models. The likelihood ratios were used to determine the p-values which were subsequently corrected for using the BH false discovery rate

(fdr) method. Differential expressed genes were defined as genes with fdr-corrected p-value of 0.05 or lower, as well as a change in expression of at least 2-fold in a given between-group comparison.

The data presented in this report is of two types. The volcano plot (Figure 3) and Heat map (Figure 4) provide a single gene resolution of expression changes caused by the compounds as the worm ages. The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis (Table 3, Figure 5) provides insight into which pathways and/or cellular systems might be involved.

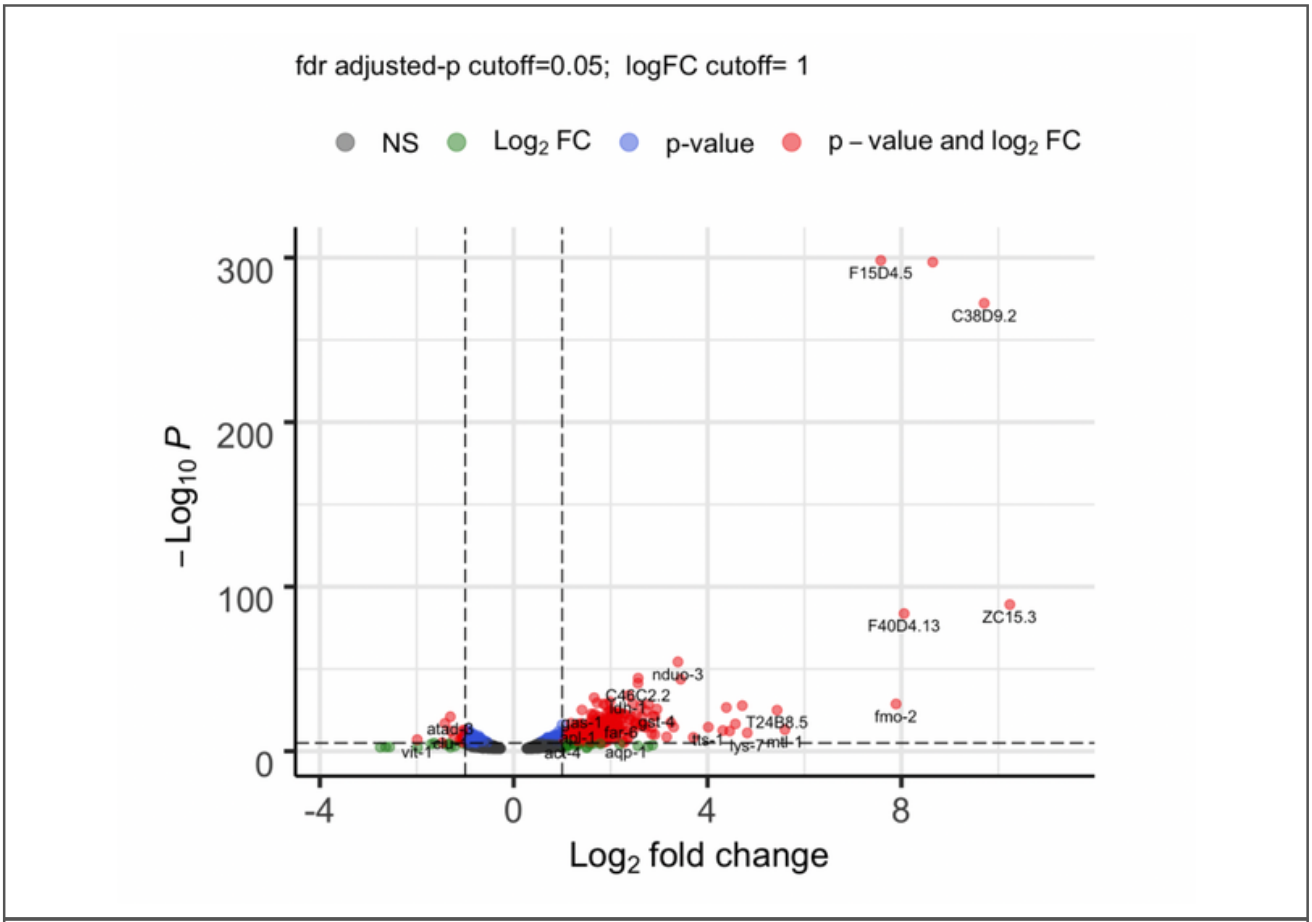

Figure 3.1. Volcano plot showing log fold change of gene expression against its p-value. Positive values mean that the gene is more expressed in Treatment, and negative values means the gene is more expressed in Control. The farther the dot is from the origin point on the x-axis, the greater the expression fold-change. Genes represented in green show a larger than 2-fold change in expression but are not deemed statistically significant due to replicate variability or low read count. Genes represented by red dots show greater than 2-fold change and expression that is statistically significant. The higher on the y-axis, the greater the calculated level of significance.

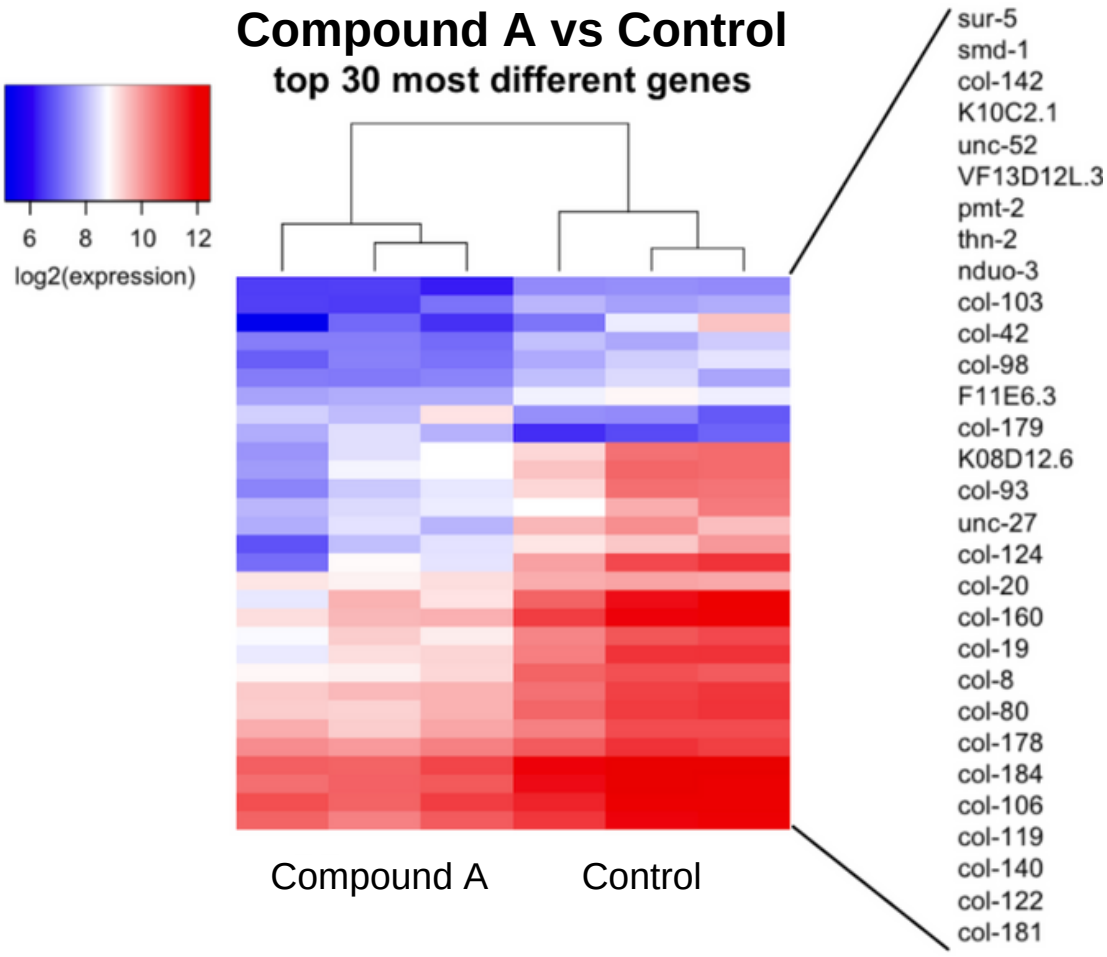

Figure 4. Heat Map comparing gene expression for the top 30 most significant differentially regulated genes between conditions and replicates.

Question 4: What mechanisms of action and genetic pathways are impacted when animals are treated with compound/ingredient A?

Technical background on KEGG pathway analysis

KEGG (Kyoto Encyclopedia of Genes and Genomes) is a collection of databases used for inferring biological function from genomic data. Orthologous genes across species are linked to specific molecular functions through KEGG Orthology Identifiers or KO-numbers. KO numbers

are linked to cellular- and organism-level functions within the PATHWAY and MODULE databases. The PATHWAY database contains curated maps of well-established cellular pathways including several related to longevity. Mapping genes from RNA-seq datasets to KEGG PATHWAYS is a standard and widely-used method of interpreting transcriptomic data.

Technical summary of KEGG pathway analysis

We performed a longevity-focused KEGG analysis by mapping differentially-expressed genes to the KEGG pathways and examining linkages between pathways related to longevity. For each condition, the differentially-expressed genes were filtered on a P-value cutoff and then assigned a score based on the fold change weighted by the log of the P-value. Color mapping based on the score indicated the degree of enrichment for specific pathways. Starting with the established Longevity Regulating Pathway, we then examined the intersections with supporting pathways to expand the pool of evidence of whether a pathway is impacted by the treatment.

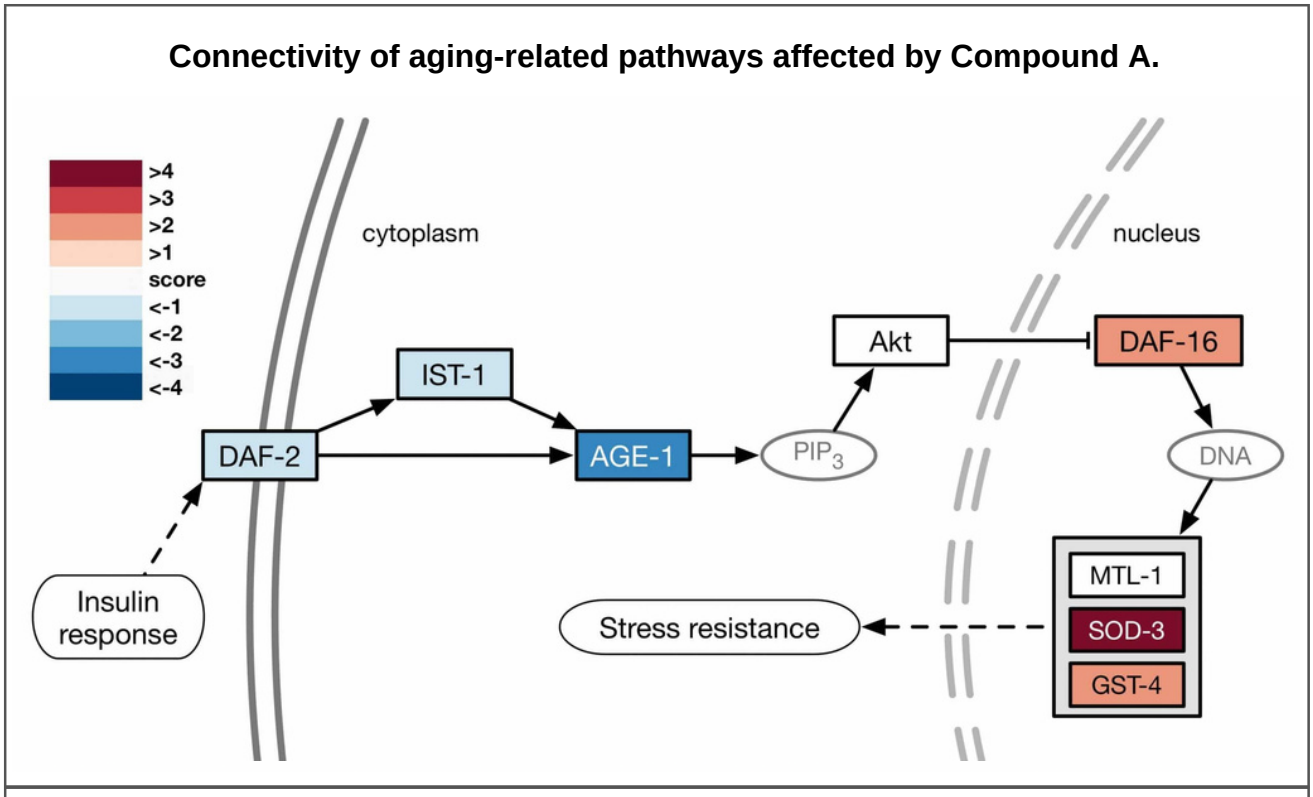

Figure 5. Summary of KEGG pathway mapping to recognized aging-related pathways for Day 10 Compound A treatment. Colored score increments indicate degree of up-(red) or down-(blue) regulation weighted by the log of the P-value. Solid lines indicate direct pathway connections and dashed lines indicate indirect or multi-step connections. Where lists of differentially-expressed genes have been condensed into a module, the number of genes is indicated.

Table 3. Top KEGG pathways enriched with differentially-expressed genes from Compound A treatment.

Pathway	Genes
Day 10 "Aged" Adults	
cel00190 Oxidative phosphorylation	15 genes
cel04140 Autophagy	• *let-363*; Target of rapamycin homolog • *daf-15*; Raptor_N domain-containing protein • *pek-1*; Eukaryotic translation initiation factor 2-alpha kinase pek-1 • *atg-2*; Autophagy-related protein 2 • *cpl-1*; Cathepsin L-like • *epg-7*; ATG11 domain-containing protein • *ragc-1*; RAs-related GTP binding protein C homolog • *unc-51*; Serine/threonine-protein kinase unc-51
cel04150 mTOR Signaling Pathway	• *let-363*; Target of rapamycin homolog • *daf-15*; Raptor_N domain-containing protein • *rict-1*; RICTOR_V domain-containing protein • *T08A11.1*; DEP domain-containing protein • *ragc-1*; RAs-related GTP binding protein C homolog • *Y32H12A.8*; WD_Repeats domain-containing protein • *daf-2*; Insulin-like receptor subunit beta • *unc-51*; Serine/threonine-protein kinase unc-51
cel04212 Longevity regulating pathway - worm	• *let-363*; Target of rapamycin homolog • *sod-2*; Superoxide dismutase [Mn] 1, mitochondrial • *gst-7*; Probable glutathione S-transferase 7 • *fat-6*; Delta(9)-fatty-acid desaturase fat-6 • *daf-2*; Insulin-like receptor subunit beta • *unc-51*; Serine/threonine-protein kinase unc-51
cel04010 MAPK signaling pathway	• *mtk-1*; Protein kinase domain-containing protein • *fln-2*; FiLamiN (actin binding protein) homolog • *nsy-1*; Mitogen-activated protein kinase kinase kinase nsy-1 • *mnk-1*; MAP kinase-interacting serine/threonine-protein kinase mnk-1 • *pxf-1*; Rap guanine nucleotide exchange factor • *ced-2*; Cell death abnormality protein 2 • *daf-2*; Insulin-like receptor subunit beta

About InVivo Biosystems

InVivo Biosystems provides essential services to help pharmaceutical, nutraceutical, biotechnology companies and academic research institutions around the globe accelerate their research and drug development efforts.

An expert in CRISPR genome editing, InVivo Biosystems creates custom genome edited C. elegans and zebrafish models to enable aging and other disease studies. In addition, InVivo Biosystems provides in-vivo analytical services to produce data and insights for companies that need to make go/no-go decisions quickly in early-stage development of new compounds.

Contact us to start a conversation about how our services can support your innovation.

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