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1 TITLE

2 # Dynamic Changes in Gene Expression Through Aging in *Drosophila melanogaster* Heads

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18 KEYWORDS

19 Gene expression; lifespan; aging; gene ontology enrichment

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22 ABSTRACT

23 Work in many systems has shown large-scale changes in gene expression during aging.
24 However, many studies employ just two, arbitrarily-chosen timepoints at which to measure
25 expression, and can only observe an increase or a decrease in expression between “young”
26 and “old” animals, failing to capture any dynamic, non-linear changes that occur throughout the
27 aging process. We used RNA sequencing to measure expression in male head tissue at 15
28 timepoints through the lifespan of an inbred *Drosophila melanogaster* strain. We detected
29 >6,000 significant, age-related genes, nearly all of which have been seen in previous fly aging
30 expression studies, and which include several known to harbor lifespan-altering mutations. We
31 grouped our gene set into 28 clusters via their temporal expression change, observing a
32 diversity of trajectories; some clusters show a linear change over time, while others show more
33 complex, non-linear patterns. Notably, re-analysis of our dataset comparing the earliest and
34 latest timepoints – mimicking a two-timepoint design – revealed fewer differentially-expressed
35 genes (around 4,500). Additionally, those genes exhibiting complex expression trajectories in
36 our multi-timepoint analysis were most impacted in this re-analysis; Their identification, and the
37 inferred change in gene expression with age, was often dependent on the timepoints chosen.
38 Informed by our trajectory-based clusters, we executed a series of gene enrichment analyses,
39 identifying enriched functions/pathways in all clusters, including the commonly seen increase in
40 stress- and immune-related gene expression with age. Finally, we developed a pair of
41 accessible shiny apps to enable exploration of our differential expression and gene enrichment
42 results.

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43 ## INTRODUCTION

44 Aging is marked by both a decline in organismal function and an increased risk for disease. As
45 complex traits, both environmental and genetic factors contribute to variation in lifespan and
46 health at old age. Identifying these factors, and their impact on life- and healthspan, enables
47 understanding of the negative effects of aging, which is increasingly important as populations
48 throughout the world age. In humans, the estimated heritability of longevity is 7-30% (Herskind
49 et al. 1996; Kaplanis et al. 2018; Mayer 1991; Ruby et al. 2018), and genome wide association
50 studies (GWAS) have been used to study the genetic basis of aging (Broer et al. 2015; Deelen
51 et al. 2011; 2014; Joshi et al. 2016; 2017; Melzer, Pilling, and Ferrucci 2020; Newman et al.
52 2010; Sebastiani et al. 2012; 2017; Pilling et al. 2016; 2017; Timmers et al. 2019; Zeng et al.
53 2016). GWAS often rely on examining sets of individuals who are >90 years old (Deelen et al.
54 2011; 2014; Newman et al. 2010; Sebastiani et al. 2012; 2017; Zeng et al. 2016), but this can
55 constrain the sample size and power of such studies (Newman and Murabito 2013; Tan et al.
56 2008). Creative approaches have enabled markedly increased sample size – for instance, Joshi
57 et al. (2016) associate offspring genotype with parental lifespan phenotype – yet human lifespan
58 GWAS have only discovered a handful of replicable associations, for instance near APOE,
59 FOXO3, and CHRNA3/5 (Broer et al. 2015; Deelen et al. 2011; Joshi et al. 2016; 2017; Pilling et
60 al. 2016; Timmers et al. 2019).

61 Outside of a deficit of power in genetic studies, studying aging directly in humans can be difficult
62 due to uncontrollable environmental variables, many of which can impact lifespan. For example,
63 calorie restriction has been shown to increase lifespan and health in model organisms (Cypser,
64 Kitzenberg, and Park 2013; Mattison et al. 2017; McCay et al. 1939; Pletcher et al. 2002), and in
65 humans a two-year calorie restriction study showed a reduction in cardiometabolic risk factors
66 including cholesterol and blood pressure (Kraus et al. 2019). However, effectively studying the
67 impact of diet on lifespan in humans is challenging due to the difficulty accurately measuring
68 calorie intake, the lack of adherence by participants, and several other concerns (F. B. Hu
69 2024). Ideally, studies investigating the genetic and molecular contributions to aging would limit
70 all other sources of variation, something that is not feasible or practical in human studies.

71 Model organisms have proven useful conduits to study aging due to their shorter lifespans, ease
72 of testing many individuals, and the ability to control both environmental and genetic variation.
73 Both genetic and environmental contributors to aging have been successfully identified in
74 vertebrate model systems (Cai, Wu, and Huang 2022; Gocmez et al. 2020; McCay et al. 1939),
75 in invertebrates such as *Drosophila melanogaster* and *Caenorhabditis elegans* (Highfill et al.
76 2017; Kenyon 2010; McCarroll et al. 2004; Pletcher et al. 2002), and in the fungi
77 *Saccharomyces cerevisiae* and *Podospora anserina* (Z. Hu et al. 2014; Philipp et al. 2013).
78 These models have helped identify and understand lifespan-associated genes and systems that
79 have relevance for human populations. For instance, FOXO3 – a member of the forkhead box
80 transcription factor O (FOXO) family – has been implicated in human aging via GWAS (Broer et
81 al. 2015; Deelen et al. 2014; Willcox et al. 2008), and studies in *C. elegans* and *D.*
82 *melanogaster* have also identified and characterized FOXO family genes that impact lifespan
83 (Alic et al. 2014; Giannakou et al. 2007; Kimura et al. 1997; Lin et al. 1997; Taormina et al.
84 2019).

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85 Cellular processes do not remain static over an individual's lifetime, and instead change, adapt,
86 and sometimes break down over time. By measuring these changes, we can understand the
87 cellular and physiological phenomena that are affected by aging. A common strategy to assess
88 such age-related changes is with the use of gene expression analyses. These analyses often
89 compare the genomewide gene expression profile between "young" and "old" individuals, and
90 identify genes that are differentially expressed with age. This approach has been successfully
91 employed in multiple systems (Bordet et al. 2021; Lu et al. 2004; X. Wang et al. 2022; Wilson et
92 al. 2015), and has resulted in expression-based hallmarks of aging (Frenk and Houseley 2018),
93 including increased expression of stress response and immunity genes (Bajgiran et al. 2021;
94 Bordet et al. 2021; Lu et al. 2004; X. Wang et al. 2022), and decreased expression of genes
95 associated with mitochondria and the electron transport chain (Bordet et al. 2021; Lu et al.
96 2004).

97 A challenge with two-timepoint, young versus old comparisons is that there is little consistency
98 in the definitions of "young" and "old", making comparisons across studies difficult. For instance,
99 various studies in *D. melanogaster* have used "young" flies sampled between 1 and 10 days old,
100 and "old" flies that were 35 to 61 days old (Bajgiran et al. 2021; Bordet et al. 2021; Carnes et al.
101 2015; Girardot et al. 2006; Landis et al. 2004). While in some cases the "old" timepoint is
102 determined by the survivorship of the aging cohort, rather than by chronological age (Doroszuk
103 et al. 2012; Highfill et al. 2017; Landis et al. 2004). Additionally, two-timepoint expression
104 analyses are limited to making binary conclusions about expression change; expression either
105 increases or decreases with age. Aging is a complex process and this simplification may often
106 fail to yield an accurate picture of age-related expression for most genes. Indeed, work in
107 several systems assaying various molecular phenotypes at multiple timepoints throughout
108 lifespan have identified many genes and gene products with non-linear trajectories (Gheorghe
109 et al. 2014; Haustead et al. 2016; Lund et al. 2002; Pletcher et al. 2002; Olecka et al. 2024;
110 Remondini et al. 2010; Schaum et al. 2020; Shen et al. 2024; Xie et al. 2022).

111 Here we set out to robustly measure expression trajectories throughout the adult lifespan of an
112 inbred *D. melanogaster* strain, focusing on head tissue from males. Flies were aged for 59 days,
113 and sampled for RNAseq at 15 timepoints. We recorded the number of deaths in our aging
114 cohort daily, allowing us to calculate a survival-based "physiological age" in addition to the
115 "chronological age" for each time point, and endeavored to sample roughly evenly through both
116 metrics. Subsequently, using multiple analyses we identified genes whose expression changed
117 with aging, and clustered differentially expressed genes based on their expression trajectories.
118 With the idea that genes with similar expression trajectories may share similar functional roles
119 (e.g. Zhang et al. 2004), we did a series of enrichment analyses for each of our clusters,
120 identifying numerous enriched pathways. To better understand the value of a multi-timepoint
121 expression study, we subsequently re-analyzed our data in a two-timepoint, young versus old
122 analysis framework. Finally, we compared the results of our work with a series of prior
123 expression-based *D. melanogaster* aging studies.

124 MATERIALS AND METHODS

125 **Fly rearing, maintenance, and aging:** We employed a single inbred *D. melanogaster* strain,
126 A4, which is one of the founders of the *Drosophila* Synthetic Population Resource (E. G. King et

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127 al. 2012; Chakraborty et al. 2018), a panel of recombinant strains enabling the genetic
128 characterization of complex traits. Following several generations of expansion, we generated
129 200 replicate vials of the A4 strain, clearing adults to maintain roughly even egg/larval densities
130 over vials. In the following generation we harvested 0-2 day old A4 males over CO_2 anesthesia,
131 collecting 132 vials of 40 males (for subsequent collection of aged animals for RNAseq), and 5
132 vials of 10 males (for collection of 3 day old flies for RNAseq). The aging cohort was maintained
133 in vials, flies were tipped to new vials every 2-3 days without anesthesia, and periodically the
134 entire cohort was anesthetized and re-arrayed into groups of 40 animals in vials. Dead animals
135 were counted daily. Supplementary Table A provides further description of how the aging cohort
136 was treated.

137 Flies were raised and maintained in standard narrow fly vials (Fisher Scientific, AS515)
138 containing ~10-ml of a cornmeal-yeast-molasses media (see Supplementary Text A), and kept
139 in an incubator under the following environmental conditions: 25°C, 50% relative humidity, and a
140 12:12 Light:Dark cycle.

141 **Fly/tissue sampling:** Multiple groups of flies were sampled from the population through the
142 aging process, with animals for the first – Day 3 – timepoint coming from those vials initially
143 holding 10 flies (see above). Flies were sampled without anesthesia via manual aspiration, and
144 sampled flies were always given at least 24 hours between CO_2 anesthesia and sampling. See
145 Supplementary Table A for details on when flies were sampled.

146 On collection, groups of 10 male flies were moved into screw-top tubes, flash frozen in liquid
147 nitrogen, and kept at –80°C. Subsequently – after the entire cohort had died and flies from all
148 timepoints had been sampled – tubes were removed from the freezer into aluminum dry bath
149 blocks held on dry ice. We then went through the tubes one by one, subjected each to liquid
150 nitrogen, briefly vortexed to separate heads/bodies, poured the body parts into the flat surface
151 of an aluminum dry bath block placed on dry ice, and used a paint brush to manually collect
152 heads into a fresh screw-top tube. These destination tubes were pre-filled with 4-6 glass beads
153 (BioSpec Products, 11079127) that had been previously washed in bleach and thoroughly
154 rinsed with distilled water.

155 **RNA isolation, sequencing library preparation, and sequencing:** RNA was isolated using
156 the Zymo Direct-zol MicroPrep kit (Zymo, R2062), largely following the manufacturer's protocol
157 (see Supplementary Text B). Samples were isolated over 6 batches, replicate samples from a
158 given timepoint were isolated in different batches, and RNA quantity was measured using a
159 NanoDrop ND-1000.

160 Forty-eight RNA samples were used to generate mRNA sequencing libraries; 14 timepoints had
161 3 replicates each, while one (Day 59, the final timepoint) had 6 replicates. We used 200-500ng
162 of total RNA from these samples to initiate half-reaction volume mRNA sequencing library
163 construction (Illumina TruSeq stranded HT kit using dual indexing), generating libraries across 2
164 batches of 24 samples each. Libraries were quantified using a Qubit fluorometer, and a subset
165 of 8 libraries from each batch were run on an Agilent TapeStation. Each of these libraries
166 showed a single library peak, no evidence of adapter dimers, and estimated average fragment
167 sizes of 277-294bp. See Supplementary Table B for details on RNA isolation / library
168 preparation batching and quantification. Given the relatively uniform fragment sizes, equal
169 quantities of all 48 libraries were pooled together. The final 48-plex pool had an average
170 fragment size of 289bp, and was run over two Illumina NextSeq550 PE75 flowcells, yielding a

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171 total of over 635 million read pairs, with an average of 13.2 million per sample (range = 9.0 –
172 17.4 million).

173 **Quantifying expression level and identifying expression changes during aging**: Reads
174 from each of the 48 samples were processed using Salmon (Patro et al. 2017), employing
175 release BDGP6.32 of the *Drosophila melanogaster* transcriptome/annotation from Ensembl.
176 Salmon quantifications were then summarized to gene level using R/tximeta (Love et al. 2020).
177 To identify genes whose expression significantly changed with aging (adjusted p-value < 0.05)
178 we used R/DESeq2 (Love, Huber, and Anders 2014), executing three different analyses. First,
179 we identified genes associated with chronological age (the "Day analysis") by treating the age of
180 the flies in each RNAseq sample as a continuous variable. Second, we identified genes
181 associated with physiological age (the "Survival analysis") by using the fraction of dead animals
182 in the entire cohort at the point flies were sampled for RNAseq as a continuous variable
183 (Supplementary Table C). Third, we identified genes showing expression variation through
184 aging by considering the 15 sampling points as levels of a categorical variable (the "Sampling
185 Point analysis"). This final analysis sought to identify genes with expression patterns not easily
186 captured by the two continuous variables (day and survival).

187 **Clustering genes by their expression trajectories:** Our three differential expression analyses
188 (see above) collectively identified 6,142 unique genes, and we sought to cluster these genes
189 into groups based on their expression trajectories through aging. To focus on expression
190 trajectories, and avoid confounding with varying expression levels, we standardized the
191 expression counts of each gene via z-scores. Briefly, for each gene we calculated the mean
192 expression across replicates for each sampling point, along with the overall mean expression
193 across all sampling points and replicates. We then subtracted the overall mean from the mean
194 of each sampling point and divided by the overall standard deviation.

195 The calculated z-scores for the 6,142 genes were used to create a dissimilarity matrix using the
196 Pearson correlation method in R/factoextra (Kassambara and Mundt 2020). We used this
197 dissimilarity matrix for hierarchical clustering of our identified genes, and then "cut" the resulting
198 dendrogram into a designated number of gene groups/clusters. This was done using the hclust
199 and cutree functions from the R/stats package (R Core Team 2021).

200 There are a variety of ways to cut a gene dissimilarity matrix into clusters, and variation in the
201 approach and parameters will yield different numbers and sizes of clusters. Our goal was to
202 examine whether clusters of genes with similar expression trajectories were enriched for
203 particular properties (e.g., gene ontology terms). To facilitate this, we sought to avoid clusters
204 with either very small or very large numbers of genes, so targeted clusters with between 50 and
205 500 genes. After exploring several methods, we grouped our 6,142 differentially expressed
206 genes into 28 clusters (see Supplementary Table D and Supplementary Figure 3 for more
207 information).

208 **Summarizing and classifying cluster expression trajectories:** For each of the 28 clusters
209 we created a representative expression curve by smoothing the mean z-score from all genes in
210 the cluster for each sampling point (Figure 3). The smoothing was executed using
211 geom_smooth from R/ggplot2 (Wickham 2016) and spline modeling with rcs from R/rms (Harrell
212 Jr 2023). The resulting 28 curves show a diversity of trajectories, with some being generally
213 linear, while others show a more complex pattern. To classify the cluster trajectories, we ran a
214 linear regression between the mean z-scores and the age of the sampled flies. A trajectory was

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215 designated as “Linear” if the *p*-value was less than 0.002 (0.05/28), or “Complex” otherwise.
216 (Repeating this analysis using survival, or the numbered sampling points, 1-15, yields the same
217 designations). Linear trajectories were further designated as “Up” (gene expression increases
218 with aging) or “Down” (gene expression decreases with aging) based on the sign of the linear
219 regression coefficient. To simplify subsequent discussion, clusters are named with these
220 classifications (i.e., Complex, LinearUp or LinearDown), and given numeric codes based on the
221 cluster position within the dendrogram (bottom to top in Figure 2 and left to right in
222 Supplementary Figure 4). See Supplementary Table E for details on the trajectory-designating
223 linear regression analyses.

224 **Cluster-specific enrichment analyses:** To understand whether genes with similar expression
225 trajectories share similar properties/functions, we used PANGEA (Version 1.1 beta December
226 2022) (Y. Hu et al. 2023), an online gene set enrichment tool that can perform Gene Ontology
227 (GO) analysis, identify enrichment of particular gene groups or pathways, and – importantly for
228 our needs – can execute analyses on multiple gene lists simultaneously. For each cluster we
229 ran 6 separate enrichment analyses, examining 3 *Drosophila* GO Subsets (SLIM2 GO BP –
230 biological process, SLIM2 GO CC – cellular component, SLIM2 GO MF – molecular function), 2
231 collections of gene groups (DRSC GLAD and FlyBase), and the REACTOME pathway set. For
232 each analysis we used a custom background set of 13,303 genes that included only those with
233 at least one mapped read in our dataset. We identified terms significantly enriched in each
234 cluster using a Benjamini Hochberg false discovery rate (FDR) of 0.05. Each of the PANGEA
235 tables are available as Supplementary Tables F-K and our enrichment analysis code is
236 available at https://github.com/Hanson19/RNAseq-Aging.

237 **Comparing trajectory-based, multi-timepoint analysis results to analyses contrasting**
238 **groups of young and old animals:** Our design differs from some previous examinations of
239 age-related expression in that we generated expression data from many points through the
240 aging process. To examine what might be gained from our approach, we re-analyzed our data
241 after dropping the bulk of the timepoints. The samples from Day 3 and Day 6 (N=6) collectively
242 made up our “young” sample, while our “old” sample came from the last collection day, Day 59
243 (N=6). Using R/DESeq2 (Love, Huber, and Anders 2014) we identified genes whose expression
244 changed significantly between these age groups, and determined if gene expression increased
245 or decreased over time. We subsequently repeated this analysis, comparing the Day 3+6
246 “young” timepoint against every sequential pair of older timepoints (e.g., we compared Day 3+6
247 to Day 10+14, Day 3+6 to Day 14+17, and so on).

248 **Comparison with previous *Drosophila* aging genomewide expression studies**: We
249 compared our set of 6,142 multi-timepoint significant genes with those identified in 8 previously
250 published, two-timepoint aging expression studies (Supplementary Table M). These papers vary
251 in the strains/populations employed, the sex of the animals targeted, the tissue that was
252 employed, and the actual timepoints during aging that were sampled. We validated all gene IDs
253 using FlyBase (FB2024_01) (Jenkins et al. 2022), identified genes shared between our study
254 and these previous works, and compared the expression change reported in the previous
255 studies (up or down in expression with age) with the expression trajectories these genes were
256 grouped into in our study (LinearUp, LinearDown, Complex; Supplementary Figure 14).

257 **Shiny apps to enable data exploration:** Our analyses generated a considerable amount of
258 data, and to make our results more accessible, we developed two interactive apps using
259 R/shiny (version 1.8.0) (Chang et al. 2023). The Gene and Cluster app allows users to look up

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260 specific genes, receive information about the whether the gene was identified in our analysis,
261 and if so in which cluster it was found, and what its expression trajectory is over time. The
262 Cluster Enrichment app allows users to select specific clusters, or sets of clusters, and identify
263 any enriched terms. For more information on how to run and use these apps see
264 Supplementary Text C and D.

265 **Data availability:** The A4 strain is available on request from the corresponding author. Raw
266 FASTQ sequencing data is available from the NCBI SRA under BioProject accession number
267 PRJNA1194574. All summary data and results are presented in supplementary files (available
268 at WILL_INSERT_G3_FIGSHARE_SITE_URL_HERE), and all analysis code is available via
269 GitHub (https://github.com/Hanson19/RNAseq-Aging).

270 RESULTS AND DISCUSSION

271 **Over 6000 genes show expression change during aging:** We aged a cohort of 5,330
272 *Drosophila melanogaster* males from a single inbred strain, recorded the number of fly deaths
273 each day, and collected several replicates of 10 flies at 15 timepoints throughout the adult
274 lifespan. Samples were collected every ~4 days between day 3 (99.9% flies alive) and day 59
275 (1.92% alive) to roughly evenly sample flies throughout lifespan (see Kaplan-Meier survivorship
276 curve in Supplementary Figure 1 and detail on the timing of the sampling in Supplementary
277 Table C). RNA was isolated from the heads of collected flies, converted into RNAseq libraries
278 and sequenced, and subsequently reads were assembled to the transcriptome to quantify gene
279 expression.

280 Three separate analyses were used to identify genes with significant expression changes
281 through aging. For two of the analyses, we associated gene expression with a continuous
282 variable, defined as either the chronological age of the flies on the day samples were collected
283 (Day analysis), or as the survivorship of the aging cohort upon sampling (Survival analysis). In
284 the third analysis, we associated expression with a categorical variable with 15 levels,
285 representing the sampling points when flies were collected for RNAseq (Sampling Point
286 analysis). This third analysis has the potential to identify genes missed by the pair of continuous
287 variable analyses, since expression variation of some genes may not follow a simple,
288 continuous temporal pattern.

289 The three analyses collectively identified 6,142 unique genes that were differentially expressed
290 through lifespan (Figure 1), representing a little under half of the genes with detectable
291 expression in our study (N=13,303). The analyses individually identified 5,449 (Day), 5,264
292 (Survival), and 4,449 (Sampling Point) genes. Around 60% of the genes (3,706) were identified
293 by all three analyses, and an additional 28% (1,432) were identified by both continuous variable
294 analyses, Day and Survival. The large overlap of genes identified in both the Day and Survival
295 analyses – well over 90% of the genes identified in each analysis are shared among the two – is
296 expected since age in days and survivorship are strongly correlated ($r = 0.98$, $p < 10^{-9}$;
297 Supplementary Figure 2). The Sampling Point analysis identified the most genes unique to one
298 analysis – 567 (9% of the total number of unique genes identified) – demonstrating its utility in
299 capturing genes not easily found by either continuous variable analysis.

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300 **A diversity of gene expression trajectories through aging:** Genes with similar functions, or
301 that function in the same pathways, might be expected to share similar expression trends over
302 time (e.g. Eisen et al. 1998; Zhang et al. 2004). To enable investigation of this, we normalized
303 the expression trajectories of all 6,142 identified genes via *z*-scores and clustered them into 28
304 groups (Figure 2, Supplementary Figure 4). Based on the results of linear regressions of cluster-
305 specific expression patterns (Figure 3) against time in Days, each cluster was assigned a
306 trajectory designation of LinearUp or LinearDown (the mean cluster expression is significantly
307 associated with time, and either goes up or down over time, respectively), or Complex (there is
308 no significant association with time after correcting for multiple testing). See "Materials and
309 Methods" for further details on this process and Supplementary Table E for statistical
310 information. Over 80% of genes fall into the 6 LinearDown (2,596) and 8 LinearUp (2,536)
311 clusters, with the remaining 1,010 genes split among 14 Complex clusters (Figure 3). In general,
312 the Complex clusters harbor considerably fewer genes than the more linear clusters.

313 Clearly the LinearDown and LinearUp clusters do not show perfect linear patterns of expression;
314 For instance, genes in LinearDown-1 (Figure 3) show a slight increase in expression in mid-life,
315 before decreasing in expression towards late life. However, the curves for the linear clusters
316 typically appear more linear than those of the Complex clusters (compare LinearUp-8 to
317 Complex-1), and primarily show monotonic increases/decreases in expression over time (see
318 LinearUp-7). Furthermore, among the Complex class of clusters we see a great deal of variation
319 in expression trajectory; Many are decidedly non-linear, and show wave-like patterns (e.g.,
320 Complex-5), or are curved with the highest expression in mid-life (e.g., Complex-10). However,
321 some Complex cluster are somewhat linear and exhibit patterns not dissimilar to those of
322 LinearUp/Down clusters (e.g., compare Complex-6 with LinearUp-4). We recognize that our
323 "linear" clusters are not perfectly linear, and do vary in their trajectories over time, and that our
324 "complex" clusters exhibit a wide spectrum of trajectories. However, to simplify presentation, we
325 elected to employ a straightforward trajectory-based naming scheme for the clusters we identify
326 (i.e., LinearUp, LinearDown, Complex).

327 **Genes with complex expression trajectories are often identified via the sampling point**
328 **analysis:** We examined the relationship between the statistical analyses a gene was identified
329 in (Day, Survival, Sampling Point), the cluster in which it resides, and the trajectory it was
330 assigned (LinearUp, LinearDown, Complex). More than 75% of the genes identified solely in the
331 continuous variable analyses (i.e., Day only, Survival only, or both) reside in the LinearUp/Down
332 clusters, whereas ~43% of those genes found in Complex clusters were uniquely identified in
333 the Sampling Point analysis (Supplementary Figure 5). This result does not appear to be driven
334 by specific clusters (Supplementary Figure 6). This again implies that the Sampling Point
335 analysis has the potential to identify genes whose age-related changes in expression are
336 difficult to capture with linear analyses based on chronological time or survivorship.

337 **Identification of known aging-relevant genes:** Our analyses identified many genes previously
338 associated with aging in *Drosophila*. We identified 107/176 genes associated with the Gene
339 Ontology (GO) term "determination of adult lifespan" (GO:0008340), with at least one such gene
340 being present in 24 of the 28 clusters. For instance, we identified *I'm not dead yet* (Complex-6,
341 FBgn0036816), a transporter of Krebs cycle intermediates, which when mutated increases
342 lifespan via a mechanism resembling the effect of caloric restriction (Rogina et al. 2000). We

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343 found insulin signaling genes, including *chico* (LinearUp-8, FBgn0024248) and *Insulin-like*
344 *peptide 2* (Complex-11, FBgn0036046), for which loss-of-function mutations increase lifespan
345 (Clancy et al. 2001; Grönke et al. 2010). We also identified the heat shock proteins *Hsp22*
346 (LinearUp-6, FBgn0001223), *Hsp26* (LinearUp-8, FBgn0001225), and *Hsp68* (LinearUp-5,
347 FBgn0001230) which when overexpressed can increase lifespan (Morrow et al. 2004; H.-D.
348 Wang, Kazemi-Esfarjani, and Benzer 2004; M. C. Wang, Bohmann, and Jasper 2003).

349 **Exploring the biological functions of expression trajectory clusters:** To understand if
350 genes with similar expression trajectories share similar functions, we executed a series of
351 enrichment analyses using the software PANGEA (Y. Hu et al. 2023), identifying enriched GO
352 terms, gene groups and pathways within each of our 28 clusters. GO terms derive from the
353 *Drosophila* GO subsets available within PANGEA (terms are indicated below by the "GO" stem),
354 the gene groups are from DRSC GLAD ("GLAD") and FlyBase ("FBgg"), and pathways come
355 from Reactome information ("R-DME"). In total we identified 732 unique enriched terms, 595 of
356 which are specific to just one of our expression trajectory designations (i.e., LinearUp,
357 LinearDown, Complex), suggesting our three designations represent largely distinct sets of
358 biological processes. Furthermore, most of the unique enrichment terms are found in just a
359 single cluster, with only 67 terms being shared by multiple clusters. (The PANGEA output tables
360 are available in Supplementary Tables F-K).

361 *Confirming common aging-related gene expression patterns:*

362 In *Drosophila* aging expression studies it is commonly observed that both general stress
363 response genes and immunity genes increase in expression with age (Bordet et al. 2021;
364 Carlson et al. 2015; Girardot et al. 2006; Highfill et al. 2017; Landis et al. 2004; Pletcher et al.
365 2002; Zane et al. 2023), and we recapitulate this finding. Five of our clusters are enriched for
366 genes that respond to stress (GO:0006950), all of which are designated LinearUp (LinearUp-2,
367 3, 5, 7, 8; Figure 3). Previous studies have seen heat shock proteins increase in expression with
368 age (V. King and Tower 1999; Landis et al. 2004; Manière et al. 2014), and we see these genes
369 (FBgg0000501) enriched in two LinearUp clusters (LinearUp-5, 7). Of the 5 clusters that are
370 enriched for stress response genes, 3 are more specifically enriched for immune response
371 genes (GO: 0006955; LinearUp-2, 5, 8). LinearUp-5 and 8 are enriched for antimicrobial
372 peptides (FBgg0001101), including *Attacin-A* (LinearUp-8, FBgn0012042), *Listericin* (LinearUp-
373 8, FBgn0033593), and *Drosocin* (LinearUp-5, FBgn0013088) which are commonly found to
374 increase in expression with age in *Drosophila* expression studies (Bajgiran et al. 2021; Bordet et
375 al. 2021; Carnes et al. 2015; Highfill et al. 2017; Lai et al. 2007; Landis et al. 2004; Zane et al.
376 2023).

377 A decrease in cognitive ability with advanced age has been reported in *D. melanogaster*
378 (Tamura et al. 2003; Haddadi et al. 2014; Pacifico et al. 2018), mice (Lamberty and Gower
379 1990; Kubanis, Gobbel, and Zornetzer 1981; Healy et al. 2024), and rats (Rowe et al. 1998;
380 Sagheddu et al. 2024). Similarly, age-related neurodegeneration is commonly observed, with
381 decreased neurogenesis with aging being reported in both mice (Maslov et al. 2004) and rats
382 (Kuhn, Dickinson-Anson, and Gage 1996), and synaptic deterioration seen in the motor neurons
383 of aged *Caenorhabditis elegans* (J. Liu et al. 2013). Three of our clusters – LinearDown-3, 4 and
384 6 – are enriched for genes that are found in synapses (GO:0045202), are involved in synapse

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385 organization (GO:0050808), as well as those associated with cognition (GO:0050890) and
386 nervous system development (GO:0007399). LinearDown-4 and 6 are also enriched for genes
387 encoding voltage-gated potassium and sodium channel subunits (FBgg0000506,
388 FBgg0000595). Notably, LinearDown-6 harbors *Atpα* (FBgn0002921), a gene encoding a
389 subunit of the NA+/K+ exchanging ATPase pump, and which has been shown to affect lifespan
390 (Palladino et al. 2003).

391 A decrease in the expression of genes involved with the electron transport chain (ETC) is a
392 common finding in samples of aged individuals (Bordet et al. 2021; Girardot et al. 2006; Highfill
393 et al. 2017; Landis et al. 2004; Pletcher et al. 2002; Zane et al. 2023). Such a decrease could
394 lead to reduced ATP production, disruption of the NAD+/NADH ratio, and cellular senescence
395 (Lenaz et al. 1997; Miwa et al. 2014; 2022). Our study supports a decrease in ETC-related gene
396 expression; Genes in Complex-12 and LinearDown-6 show an overall decrease in expression
397 across lifespan (Figure 3), and these clusters are adjacent to each other in the dendrogram
398 resulting from hierarchical clustering (Figure 2 and Supplementary Figure 4). These two clusters
399 are enriched for genes encoding mitochondrial ETC complex I and V (FBgg001836,
400 FBgg0001849). LinearDown-6 is also enriched for genes that are part of mitochondrial complex
401 III and IV (FBgg0001850, FBgg0001847). A series of ETC-related genes shown to influence
402 lifespan are also present in these clusters; These include *ND-20* (Complex-12, FBgn0030718)
403 and *ND-SGDH* (LinearDown-6, FBgn0011455) (Copeland et al. 2009), *levy* (LinearDown-6,
404 FBgn0034877) (W. Liu et al. 2007), and *ATPsynD* (LinearDown-6, FBgn0016120) (Sun et al.
405 2014). Additionally, LinearDown-6 harbors *stress-sensitive B* (FBgn0003360), which is involved
406 in the transport of ADP and ATP in and out of the mitochondrial matrix, and when mutated
407 shortens lifespan (Celotto et al. 2006; Reynolds 2018).

408 *A distinction between cytosolic and mitochondrial ribosomal gene expression responses:*

409 A decrease in the expression of ribosomal proteins and ribosomal biogenesis genes with age
410 has been documented in a number of yeast studies (Choi et al. 2018; Janssens et al. 2015;
411 Kamei et al. 2014; Philipp et al. 2013; Yiu et al. 2008), with three of these showing a reduction in
412 cytosolic ribosome (GO: 0022626) gene expression over time (Choi et al. 2018; Philipp et al.
413 2013; Yiu et al. 2008). One fly study we identified (Doroszuk et al. 2012) observed a similar
414 response, with enrichment of ribosome-related ontology terms in genes showing a reduction in
415 expression with age in a given strain, including genes involved in ribosome biogenesis
416 (GO:0042254) and mitochondrial ribosome (GO:0005761).

417 In the analyses of our dataset, we saw enrichment of ribosomal-related ontology terms in six
418 clusters. Five of these clusters show a general increase in gene expression with age (LinearUp-
419 1, 2, 4, 6, Complex-6; see Figure 3), and include genes associated with the terms ribosome
420 biogenesis (GO:0042254), rRNA processing (R-DME-72312), cytoplasmic ribosomal proteins
421 (FBgg0000141), and structural constituent of ribosomes (GO:0003735). The sixth (LinearDown-
422 6) shows reduced expression with age, and shows enrichment of mitochondrial ribosomal
423 proteins (FBgg0000059). Thus, in an apparent contrast with prior results, it appears that in our
424 data many ribosome-associated genes increase in expression with age, while only
425 mitochondrial ribosomal protein genes decrease with advanced age.

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426 To examine this further we extracted from our complete set of 6,142 differentially-expressed
427 genes all those affiliated with any ribosome-related term (N=270, See Supplementary Table L
428 for list of terms). We plotted their age-related expression, separating out mitochondrial
429 ribosomal proteins (N=36, FBgg0000059), and can clearly see that while all mitochondrial
430 ribosomal protein genes go down in expression with age, nearly all other ribosomal genes
431 (220/234) increase in expression with age (Supplementary Figure 8). Furthermore, just
432 contrasting cytoplasmic (N=80, FBgg0000141) and mitochondrial (N=36, FBgg0000059)
433 ribosomal proteins, we see the former all go up, and the latter all go down in expression with
434 age (Supplementary Figure 7).

435 A *D. melanogaster* brain-specific, single-cell RNAseq study (Davie et al. 2018) appears to
436 support our finding that – outside of mitochondrial ribosomes – ribosomal genes increase in
437 expression with age. First, Davie et al. report that the cytosolic small ribosomal subunit (GO:
438 0022627) gene *stubarista* (FBgn0003517) is statistically upregulated during aging; We also
439 identified this gene in our LinearUp-5 cluster. Second, while Davie et al. see a reduction in the
440 overall levels of RNA and transcription through aging – a result seen previously (Tahoe,
441 Mokhtarzadeh, and Curtsinger 2004), and which we also observed (Supplementary Table B and
442 Supplementary Figure 9) – on average, ribosomal protein genes show a lower decline in
443 expression than other genes (see Fig. 5C in Davie et al. 2018). In a bulk RNAseq analysis
444 framework this result would be expected to translate to a relative increase in the expression of
445 ribosomal proteins over time. Nonetheless, that we see a result that contrasts with some of the
446 prior work on gene expression changes through aging is intriguing and worthy of future
447 examination.

448 *Various metabolic processes are enriched in some Complex clusters:*

449 Many of our Complex clusters have small gene counts, and relatively few enriched terms.
450 However, 8/14 Complex clusters (2, 3, 4, 6, 7, 9, 10 and 12) are enriched for genes associated
451 with metabolism (R-DME-1430728 and GLAD:24593). While this term is broad, when we focus
452 on individual clusters more specific metabolic functions are evident. As described earlier,
453 Complex-12 is enriched for genes involved with the ETC. Complex-2, 6, and 10 are specifically
454 enriched for genes involved in the pentose phosphate pathway (R-DME-71336), a glucose
455 catabolism pathway that produces NADPH and ribose sugars for nucleotide synthesis (Stincone
456 et al. 2015). Notably, Complex-2 harbors *Pgd* (FBgn0004654) and *G6pd* (FBgn0004057) which
457 are the two reducing enzymes involved in the pentose phosphate pathway (Gvozdev et al.
458 1976; Geer, Bowman, and Simmons 1974). Complex-3 is uniquely enriched for genes involved
459 in galactose catabolism (R-DME-70370) and glycogen synthesis (R-DME-3322077), one of
460 which – *Agbe* (FBgn0053138) – is involved in lifespan (Paik et al. 2012). That all of these
461 metabolic pathways/genes emerged from our Complex clusters suggests that nonlinear
462 changes in metabolic activity occur throughout lifespan, as has been suggested in a large, multi-
463 omic study in humans (Shen et al. 2024).

464 *Enrichment for protein folding, modification, and transport in the LinearUp-7 cluster:*

465 LinearUp-7 – which shows only limited change in expression for the first third of life, followed by
466 increasing expression until end of life – is enriched for multiple terms involved with proper

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467 protein folding and modification, and transporting proteins from the endoplasmic reticulum to the
468 golgi apparatus. We see enrichment for chaperones and co-chaperones (FBgg0001643), and
469 heatshock proteins (see above), which can bind onto unfolded proteins (GO:0051082) and help
470 correctly fold them (GO:0006457). Additionally, LinearUp-7 is enriched for genes involved in
471 post-translation protein modification pathways (R-DME-597592). LinearUp-7 is the only cluster
472 to be enriched for genes associated with both the endoplasmic reticulum (GO:0005783) and the
473 golgi apparatus (GO:0005794). It includes genes that are involved in transport between these
474 two organelles, and is enriched for both coat protein complex I (FBgg0000087) and II
475 (FBgg0000116) genes, and genes involved in ER-to-Golgi anterograde transport (R-DME-
476 199977), and Golgi-to-ER retrograde transport (R-DME-8856688). These enrichment patterns
477 further demonstrate that genes with similar roles can have very similar temporal expression
478 patterns.

479 **Multi-timepoint trajectory-based datasets offer more detail than two-timepoint studies:**
480 Often aging expression studies compare “young” and “old” samples, and we sought to re-
481 analyze our data in this framework to discover what is gained from a multi-timepoint approach.
482 Our “young” timepoint combined all the Day 3 and Day 6 samples (to achieve a sample size of
483 6), and our “old” timepoint used the six Day 59 samples. Contrasting these two sets of samples
484 yielded 4,533 differentially-expressed genes. Of the 6,142 genes identified in our multi-timepoint
485 analysis, 4,347 (~71%) were re-identified in this two-timepoint analysis. The reduction in the
486 number of genes is likely a combination of the switch in analytical approach, and a simple loss
487 of power (since we have gone from 48 to 12 samples). Considering the assigned expression
488 trajectories from our multi-timepoint analysis, the two-timepoint analysis recovered 77.5% of the
489 LinearDown cluster genes, 72% of the LinearUp genes, but only 50.5% of the Complex genes
490 (Figure 4). As anticipated, genes that do not exhibit a straightforward, monotonic
491 increase/decrease in expression through lifespan are much less likely to be identified when
492 sampling is restricted to very young and very old animals.

493 A challenge with a two-timepoint analysis is that the only conclusion one can draw about the
494 expression change is that it goes up or down with age. For those genes present in
495 LinearUp/LinearDown clusters that were replicated in the two-timepoint analysis, the inferred
496 direction of the expression change matched expectations 98-99% of the time (Figure 4), and
497 this trend is consistent across the different LinearUp/LinearDown clusters (90%-100% matched)
498 (Supplementary Figure 10). However, for the 50.5% of Complex genes that were re-identified in
499 the two-timepoint analysis, 56% of them showed an increase in expression in old samples, and
500 44% showed a decrease (Figure 4). Examining the two-timepoint expression change direction
501 calls across Complex clusters reveals cluster-to-cluster variation (see Supplementary Figure
502 10), and in most cases, re-identified genes within a given Complex cluster exhibit the same
503 direction of expression change in the two-timepoint analysis (see Supplementary Figure 10).
504 This likely reflects the particular, cluster-specific nature of each Complex expression trajectory,
505 and what the precise expression levels were at the start and end of our experiment. For
506 instance, Complex-7 shows a general increase in expression over time (Figure 3), and all genes
507 re-identified in the two-timepoint analysis are marked as increasing in expression
508 (Supplementary Figure 10).

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509 In our initial analysis we chose Days 3+6 and Day 59 to represent our "young" and "old"
510 samples. However, studies have used flies of quite different ages to represent young and old
511 animals (Bajgiran et al. 2021; Bordet et al. 2021; Carnes et al. 2015; Girardot et al. 2006; Landis
512 et al. 2004). To understand the impact of varying the "old" timepoint, we repeated the two-
513 timepoint analysis several times. In each case, we fixed the young timepoint using our Days 3+6
514 data, and derived the old timepoint from two sequential sampling days (e.g., Days 10+14, Days
515 14+17, Days 17+23, and so on) such that each analysis compared two sets of 6 samples. We
516 then compared the differentially-expressed genes that emerged from these analyses, along with
517 the inferred expression change, with our initial Day 3+6 versus Day 59 analysis. As might be
518 expected, using old sampling points that occur earlier in life results in fewer significant
519 differentially expressed genes (Supplementary Figure 11); there has simply been less time for
520 change. As the old timepoint moves later in life there is increasingly greater overlap with the
521 Day 3+6 versus Day 59 analysis, and 97-99% of the shared genes show the same change in
522 expression (Supplementary Figure 11). Nonetheless, every alternative analysis with a different
523 old timepoint identified genes we initially identified in our multi-timepoint trajectory analysis, but
524 that were missed in our initial two-timepoint analysis (Supplementary Figure 11). Particularly in
525 those analyses using old timepoints that occur earlier in life (up to Day 36, a little over halfway
526 through the lifespan of our cohort), such genes are often associated with our multi-timepoint
527 Complex trajectory clusters (Supplementary Figure 12). Between 9-17% of Complex genes
528 found using the earlier alternative old timepoints, were not found in our initial Day3+6 versus
529 Day 59 test. It is likely that the observed change in the expression of these genes over time is
530 highly dependent on the exact timepoints chosen. In general, our analyses clearly demonstrate
531 that the age when individuals are sampled impacts the genes identified.

532 **Comparison with other aging expression studies in flies:** We compared the outcome of our
533 multi-timepoint analysis with 9 datasets from 8 previously published expression analyses in *D.*
534 *melanogaster* (Bajgiran et al. 2021; Bordet et al. 2021; Carnes et al. 2015; Girardot et al. 2006;
535 Highfill, Reeves, and Macdonald 2016; Lai et al. 2007; Landis et al. 2004; Zane et al. 2023).
536 These were all two-timepoint studies that varied in the sex of the flies, the target tissue, and the
537 sampling points employed (Supplementary Table M). Around 95% of the genes resulting from
538 our multi-timepoint analysis were identified in at least 1 of these datasets, and the number of
539 previous studies that identified a given gene was not clearly associated with the trajectory
540 designation (LinearUp, LinearDown, Complex) we assigned (Supplementary Figure 13). The
541 high rate of gene re-identification across studies is notable given the diversity of the study
542 designs in terms of fly sex, the tissue targeted, the sampling points used (Supplementary Table
543 M), and the likely many differences in the precise rearing/maintenance conditions employed.
544 That the same sets of genes are regularly identified suggests some similarity across
545 genotype/sex/tissue in the age-related expression profile (Izgi et al. 2022).

546 Similar to the comparisons between multi- and two-timepoint analyses within our own dataset
547 (above), when we look at genes in our LinearUp/LinearDown clusters that were identified in
548 prior studies, they broadly show the direction of expression change we would predict
549 (Supplementary Figure 14). However, as might also be expected, the fraction of such genes
550 showing the predicted expression change in a prior study is often lower than it is in our within-
551 study methodological comparison (compare Figure 4 with Supplementary Figure 14). The

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552 highest fraction of LinearUp/LinearDown genes showing the expected expression change in a
553 prior study – 95-98% – is with Highfill et al. (2016; Supplementary Figure 14), a study published
554 by our group that used the same tissue type, employed a very similar fly maintenance
555 environment, but targeted females rather than males. The other prior studies we examined
556 varied more in their design, and these differences likely contribute to shared genes more often
557 showing mis-matched expression changes (see Supplementary Figure 14).

558 **Benefits of a multi-timepoint, trajectory-based transcriptomics approach to exploring**
559 **dynamic biological processes:** It is increasingly clear that a complete view of the gene
560 regulatory changes underlying a range of dynamic processes – from the response to infection
561 (Schlamp et al. 2021) to cellular differentiation (Strober et al. 2019) to aging (Pletcher et al.
562 2002; Shen et al. 2024; Gheorghe et al. 2014) – requires a timecourse experimental design,
563 interrogating samples taken throughout the process of interest. Here, we showed that a two-
564 timepoint, young versus old analysis can successfully identify, and correctly infer the direction of
565 expression change of many genes that have largely linear expression trajectories through aging.
566 But we also showed that the set of genes identified depends on the pair of timepoints chosen.
567 Furthermore, we showed that by limiting the sampling to just two timepoints, we would have
568 failed to identify almost half of the genes with more complex, non-linear expression trajectories,
569 and would not have captured the nature of the trajectories for genes that were identified. Even
570 for those genes in the clusters we consider to be "linear" there is variation in rate of expression
571 change with time, which can only be captured with a multi-timepoint approach (Gheorghe et al.
572 2014; Haustead et al. 2016; Lu et al. 2004; Schaum et al. 2020; Shen et al. 2024).

573 Another major benefit of being able to consider the trajectory of gene expression is that it
574 enables the identification of genes with similar longitudinal expression patterns, and allows
575 assessment of whether genes with similar patterns have similar functional/molecular properties.
576 Our enrichment analyses support the contention that genes with similar expression trajectories
577 share similar functions. Due to the multiple points we sampled throughout the aging process,
578 around 70% of all significant, enriched gene ontology terms we identified were unique to a
579 single cluster. This specificity allowed us to more precisely characterize the expression patterns
580 of various sets of genes with related functional roles, rather than only – with a two-timepoint
581 framework – being able to state that particular groups of genes are up- or down-regulated with
582 age. Better understanding of the dynamic molecular changes underling aging will facilitate a
583 deeper understanding of the cellular and physiological changes that occur as organisms age.

584 **Caveats:** We recognize that the scope of our results may be somewhat limited due to the use of
585 only males from one inbred strain, and the use of a single tissue type. Additionally, while our
586 target tissue – the fly head – is enriched for brain/neuronal cells, since it also includes a mixture
587 of other cells types, we lack true tissue specificity. This said, we were able to demonstrate some
588 consistency over studies in the genes and expression patterns identified, despite these studies
589 varying in multiple ways, making our results a useful resource for future aging investigations.

590 **Accessible data exploration via interactive shiny apps:** Our expression, gene enrichment,
591 and comparative analyses generated a significant amount of data. Above we have only
592 discussed a subset of our observations. To make the results more accessible – in addition to
593 sharing our raw data, summary data, and analytical code – we have developed two interactive

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594 R/shiny apps that enable individuals to explore our gene identification, clustering, and cluster
595 enrichment results. The apps allow users to look up specific genes, examine their expression
596 trajectory through time in our cohort of flies, the cluster they belong to, as well as to explore
597 enriched terms within and across clusters. See the Materials and Methods, along with
598 Supplementary Texts C and D for more information on the development of the apps, and how to
599 access and use them.

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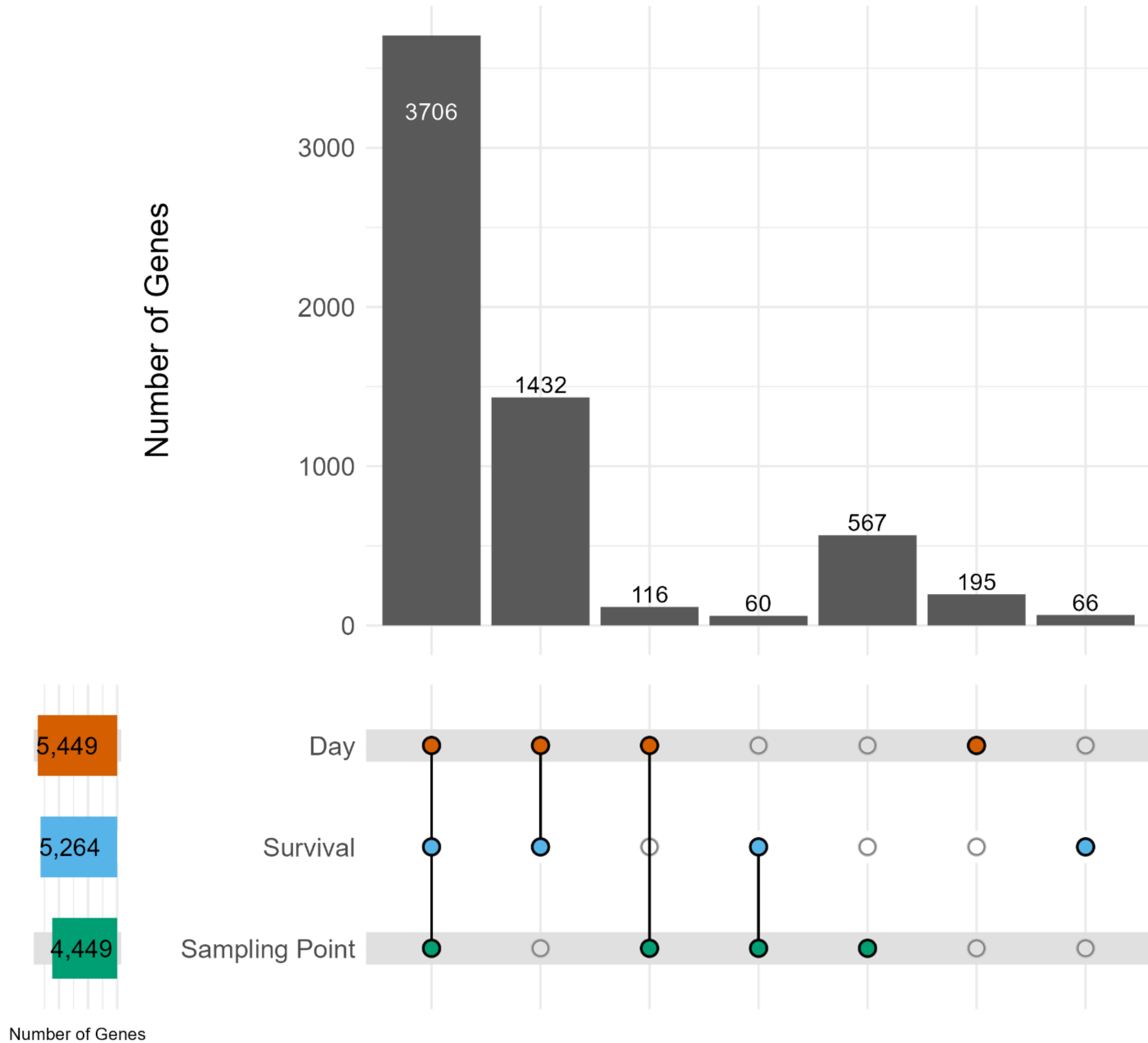

1032
1033 **Figure 1: Identification of 6,142 genes with age-related gene expression.** We identified
1034 genes whose expression was significantly associated with Day of life (N=5,449), with Survival
1035 (N=5,264), and with Sampling point (N=4,449). The upper bar chart shows the number of
1036 significant genes identified, with colored circles below showing which analysis the genes were
1037 identified in (3706 genes were identified in all three analyses, 1432 genes were identified in both
1038 the Day and Survival analyses, and so on).

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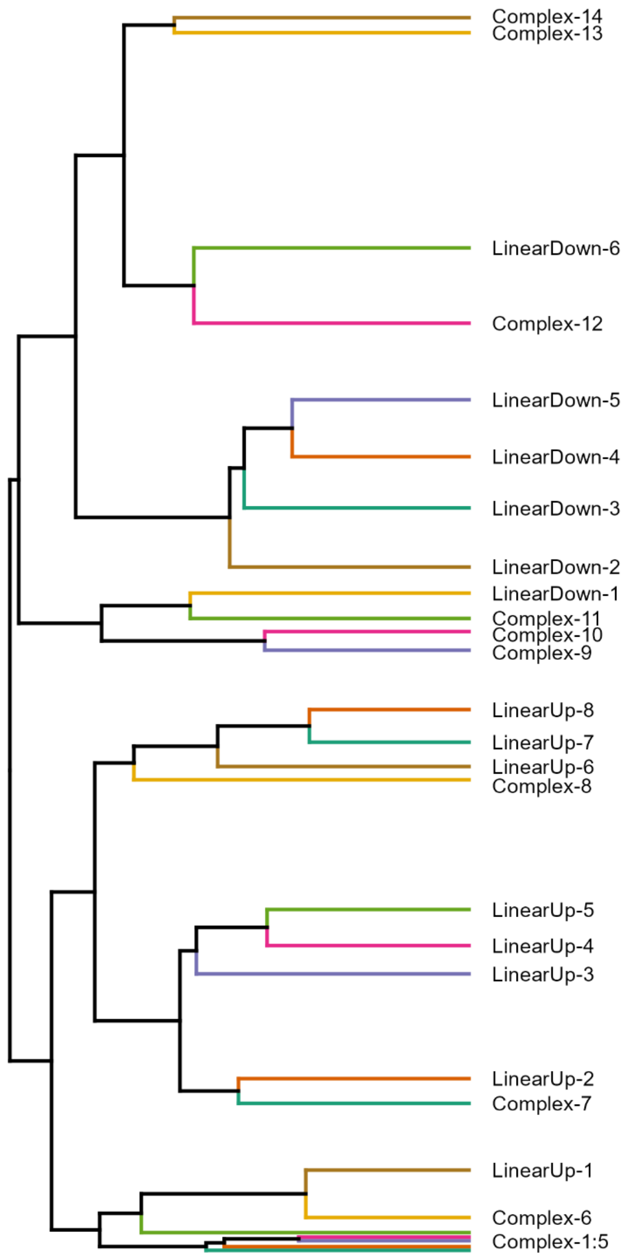

1040 **Figure 2: Clustering all 6,142**
1041 **age-related genes into 28**
1042 **clusters via their expression**
1043 **trajectories through lifespan.**
1044 A simplified dendrogram
1045 representing the hierarchical
1046 clustering of our gene
1047 expression trajectory data.
1048 Each horizontal colored line
1049 represents all those genes in
1050 each of our 28 clusters; The
1051 closer clusters are to each
1052 other on the plot, the more
1053 similar their expression
1054 trajectories (see Figure 3).
1055 Each cluster is named based
1056 on their expression trajectory
1057 (LinearUp, LinearDown,
1058 Complex). Complex-1 to
1059 Complex-5 clusters are not
1060 individually labeled since they
1061 are very close together in the
1062 plot. Supplementary Figure 4
1063 shows the full dendrogram
1064 highlighting the relationships
1065 among all 6,142 genes.

1066

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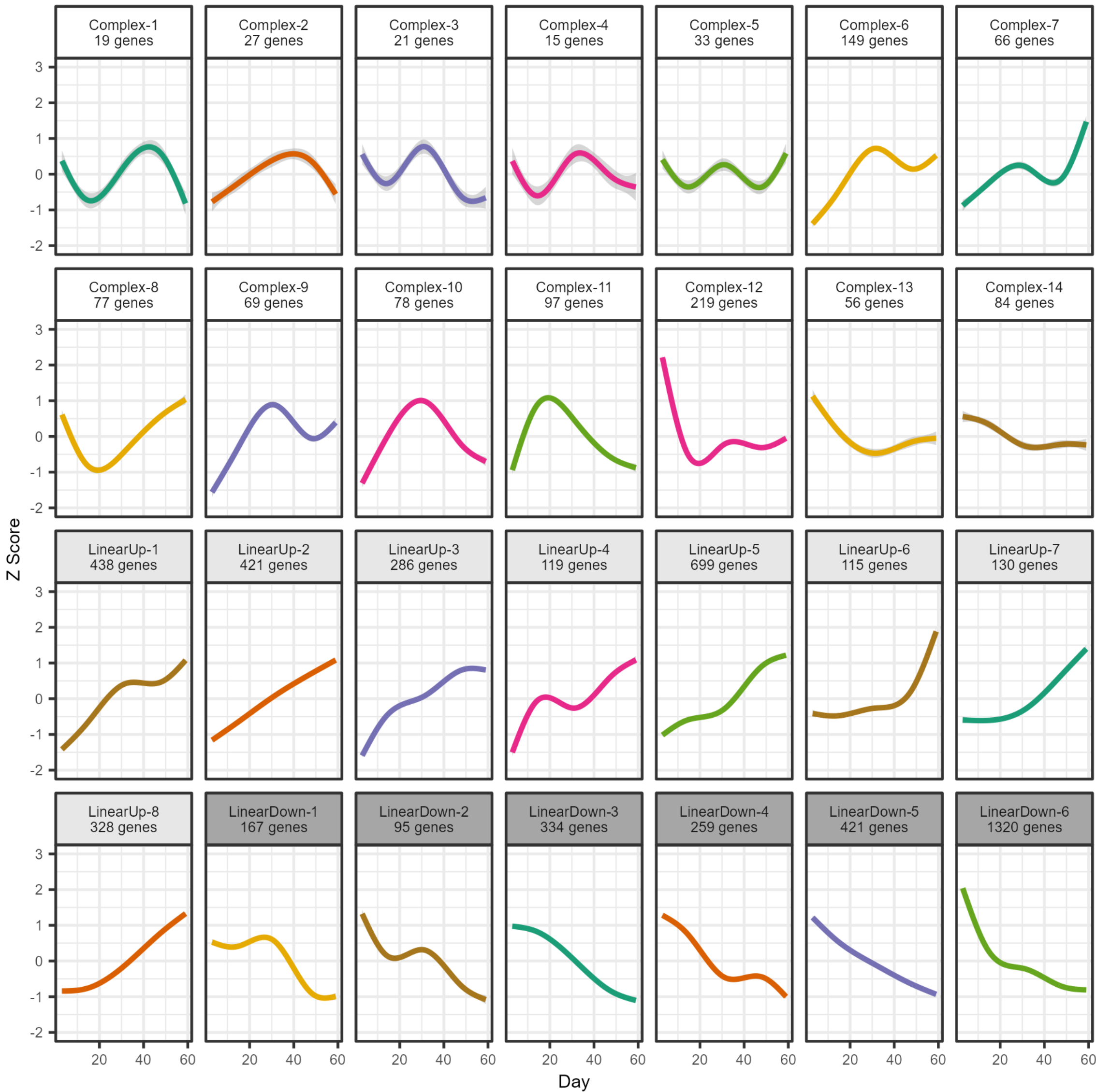

1067
1068 **Figure 3: Representative expression trajectories for all 28 clusters.** Within a cluster we
1069 calculated the average *z*-score over genes for each timepoint, and present a smoothed curve
1070 through those points highlighting the cluster-specific changes in gene expression over time. We
1071 determined whether each cluster-specific set of mean *z*-scores was statistically associated with
1072 age, and used this information to designate each cluster as LinearUp or LinearDown (we found
1073 a significant association, and expression either increases or decreases over time), or as
1074 Complex (there was no significant association between expression and age). This led to 14
1075 Complex, 8 LinearUp, and 6 LinearDown clusters.

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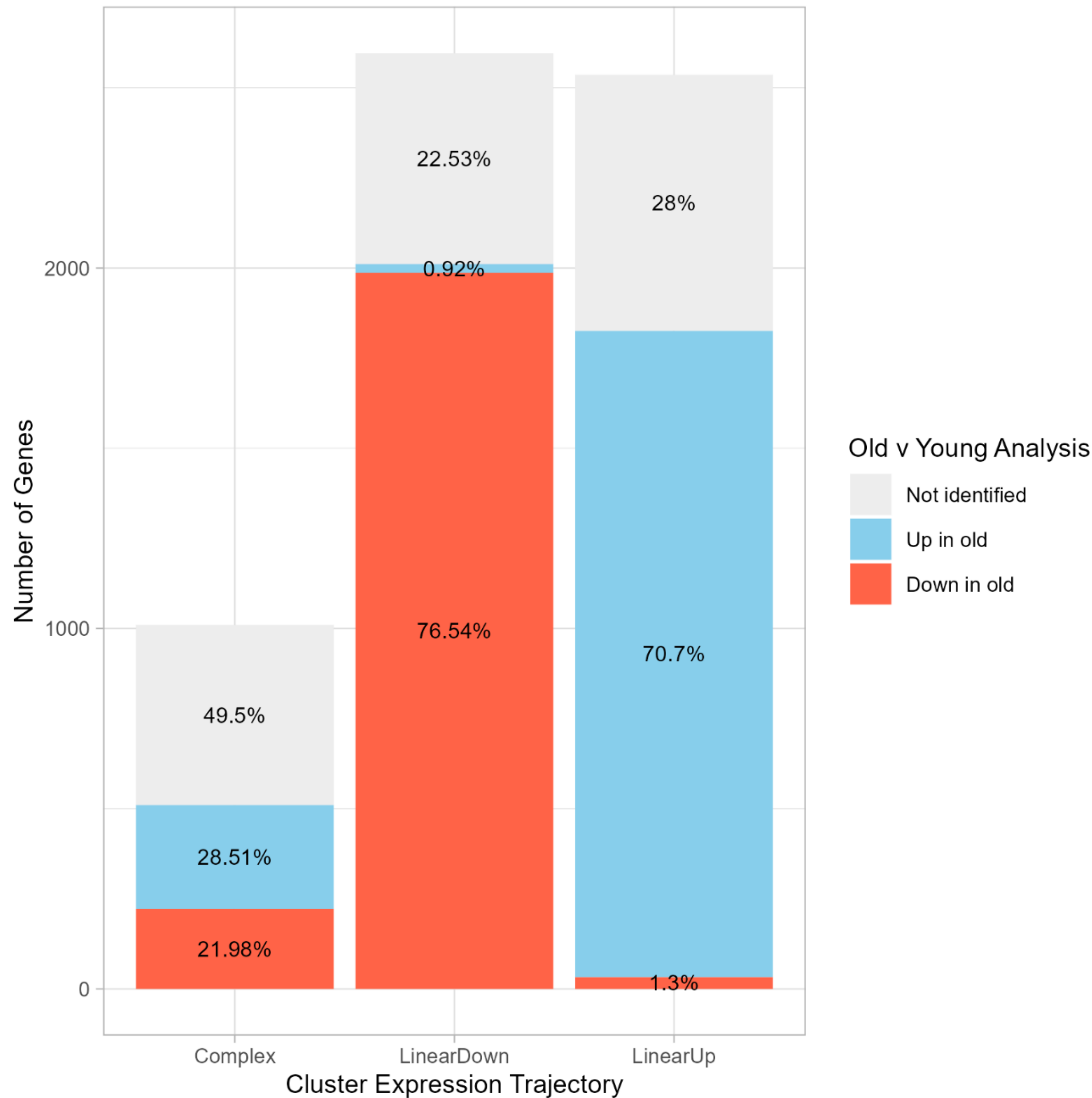

1076
1077 **Figure 4: Differences between a multi-timepoint and a two-timepoint analysis:** Our 3
1078 trajectory-based analyses revealed > 6,000 differentially-expressed genes that were grouped
1079 into 3 trajectories (Complex, LinearDown, LinearUp). We compared these results to a re-
1080 analysis of a subset of the same data, where we directly contrasted expression between young
1081 (Day 3+6) and old (Day 59) samples. Each vertical bar depicts the fraction (in the figure) and
1082 the number (*y*-axis) of genes in each of our expression trajectories that are absent in the young
1083 versus old test (gray), are significantly up-regulated in old animals (blue) or are significantly
1084 down-regulated in old animals (red). Most genes with linear trajectories are re-identified, and
1085 ~99% of those show the expected direction of change with age. However, only ~50% of the
1086 Complex trajectory genes are re-identified in the two-timepoint analysis, and these are split
1087 between those that appear to increase or to decrease in expression with age.