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RESEARCH ARTICLE

Drosophila transcriptomics with and without ageing

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Abstract The genomic basis of ageing still remains unknown despite being a topic of study for many years. Here, we present data from 20 experimentally evolved laboratory populations of *Drosophila melanogaster* that have undergone two different life-history selection regimes. One set of ten populations demonstrates early ageing whereas the other set of ten populations shows postponed ageing. Additionally, both types of populations consist of five long standing populations and five recently derived populations. Our primary goal was to determine which genes exhibit changes in expression levels by comparing the female transcriptome of the two population sets at two different time points. Using three different sets of increasingly restrictive criteria, we found that 2.1–15.7% (82–629 genes) of the expressed genes are associated with differential ageing between population sets. Conversely, a comparison of recently derived populations to long-standing populations reveals little to no transcriptome differentiation, suggesting that the recent selection regime has had a larger impact on the transcriptome than its more distant evolutionary history. In addition, we found very little evidence for significant enrichment for functional attributes regardless of the set of criteria used. Relative to previous ageing studies, we find little overlap with other lists of aging related genes. The disparity between our results and previously published results is likely due to the high replication used in this study coupled with our use of highly differentiated populations. Our results reinforce the notion that the use of genomic, transcriptomic, and phenotypic data to uncover the genetic basis of a complex trait like ageing can benefit from experimental designs that use highly replicated, experimentally-evolved populations.

Keywords Experimental evolution · Life history traits · Ageing · Transcriptome differentiation · *Drosophila melanogaster*

Introduction

The use of evolutionary genomics to study aging is still in its infancy (Braendle et al. 2011; Rose and Burke 2011; Hubley et al. 2016; Graves et al. 2017). Experimental evolution offers extreme phenotypic differentiation among traits in replicated populations

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(Garland and Rose 2009). Combining such populations with high-throughput omics can identify important genetic variants (Hubley et al. 2016; Bryant et al. 2017) and intermediate molecular phenotypes such as expression levels (Remolina et al. 2012; Mallard et al. 2018). This experimental and analytical framework has the potential to suggest causal genetic regions in the genome and unexpected molecular mechanisms responsible for the differentiation of ageing and characters related to it.

Despite ageing having been a subject of study for many decades, a precise understanding of its underlying physiological and molecular mechanisms remains elusive. One type of study has focused on knocking out candidate genes to examine its effect on longevity (Bray et al. 2016). Other types of studies have focused on changes at the genomic level between populations of varying ageing patterns (Remolina et al. 2012), with an extension of this approach to the analysis of transcriptome differentiation (Carlson et al. 2015; Sarup et al. 2011a, b). Many of these latter studies focus on either a single population tracked over several life stages or on different populations exposed to different treatments that result in differing ageing patterns, in order to uncover the transcriptome changes that result from altered ageing.

A more extreme kind of comparison is possible when populations of the same chronological age differ with respect to whether they are ageing at all. Burke et al. (Burke et al. 2016) have demonstrated that two sets of ten *Drosophila melanogaster* populations, called “A” and “C”, show distinctively different ageing patterns: The A populations begin demographic ageing at least 2 weeks before the C populations. These twenty populations also show extensive and consistent differences in genome-wide patterns of single-nucleotide polymorphism (SNP), transposable element (TE), and structural variant (SV) frequencies (Burke et al. 2016; Graves et al. 2017).

We sequenced the transcriptomes of the 20 A and C populations of *D. melanogaster* populations. Expression profiling was performed on whole-body females collected at both day 14 and day 21 from each cohort’s egg-stage. At these collection points, the individuals from the ten A populations had an adult age of 6 and 13 days respectively whereas those from the ten C populations had an adult age of 2 and 9 days. These time points were specifically chosen because A-type populations show demographic ageing at those times, unlike the C-type populations (Fig. 1). Given this extreme demographic contrast, we compiled three different lists of genes putatively associated with ageing in *D. melanogaster*.

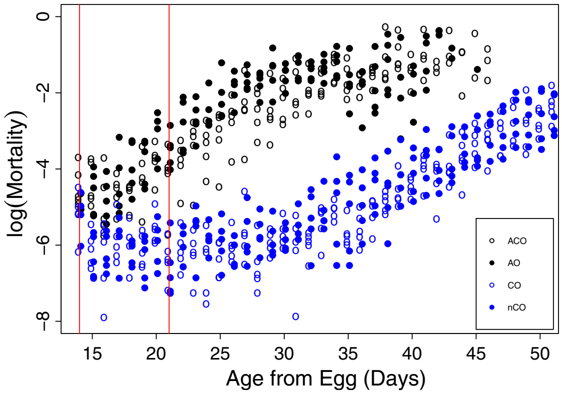

Fig. 1 Age specific log mortality plotted for the 20 populations used. Mortality graphs were plotted for both A-type (black) and C-type (blue) populations. Both types of populations consist of five long standing populations (open circles) and five recently derived populations (closed circles). The red lines show when samples were collected for RNA-sequencing. More notably, for day 21, the A-type populations are within their ageing trajectory whereas the C-type populations are still in their pre-ageing phase

Results and discussion

Evidence for transcriptional convergence within selection regimes

We employed double-stranded Illumina RNA-seq to sequence the transcriptome of whole-body females from the A and C populations to determine the magnitude and patterns of differentiation as a result of the selection regimes to which they have been exposed. Due to the recent increase in annotated noncoding RNA genes in *D. melanogaster* (Matthews et al. 2015), a fraction of which lacks poly(A) tail (Yang et al. 2011), we decided not to enrich for poly(A) mRNAs. We made this choice in order to perform an unbiased search across all transcripts. For each population type by time point combination, 10 populations were profiled.

The median number of uniquely mapped sequencing reads per population was 8.1 million, bringing the total to 332.7 million reads, which in total sequence length amounts to ∼ 21 Gb. This represents ∼ 272-

fold coverage of the fraction of the genome that has been found to produce primary transcripts (Graveley et al. 2011). Out of 17,481 gene features (from protein-coding sequences to pseudogenes to non-coding RNA genes) annotated in the R6.18 of *D. melanogaster*, 3994 were found to be reproducibly expressed at a significant level across a majority of at least one of the two population types, thereby excluding potential transcriptomic noise, and therefore were considered suitable for downstream analyses ("Materials and methods" section).

After data normalization relative to the total transcript output, we performed a principal component analysis on the transcript level of each of the expressed genes. This led to a clear segregation of the samples by time point and selection regime (Fig. S2). Due to sequencing batch discrepancies at day 21, we employed a linear mixed-effects model to account for the effect of sequencing batch.

We then calculated the pairwise correlation coefficients between the expression levels of the 20 populations assayed. To study the relationships among all 20 populations studied, we used Pheatmap (Kolde 2015), a clustering program, that uses these correlation coefficients and generates a dendrogram. The resulting dendrogram perfectly separates the A- and C-population types (Fig. S3). It is remotely conceivable that these populations are grouped in the expected manner due to chance. To address whether or not this result occurred due to chance, we shuffled the expression values within each gene and recreated 1000 simulated dendrograms. We found that none of the simulated dendrograms showed the same topology as our experimental result ($P < 0.001$). This is a particularly remarkable result, because the populations within each of the A and C types feature two subsets that have only recently experienced parallel selection regimes. This result provides strong evidence of genome-wide transcriptional convergence of the two subtypes of populations within each selection regime, which is precisely in alignment with the convergence they exhibit for both life history traits and genome-wide variation (Burke et al. 2016; Graves et al. 2017).

Transcriptional differentiation between population types

At a 5% false discovery rate (FDR) per gene, we found 906 genes differentially expressed in the A and C populations: 277 at day 14 only, 366 at day 21 only, and 263 at both time points (Fig. 2). 24.6% (i.e. 133) of the differentially expressed genes at 14 days and 23.5% (i.e. 148) of the differentially expressed genes at 21 days were found to differ by 2-fold or more, respectively (Fig. 3a, b; Table S2). The highest fraction of differentially expressed genes, across all genes analyzed, is seen on day 21 (16.2% of expressed genes vs. 14.7% on day 14). 24.9% (i.e. 225) of the genes that are significantly differentiated at one of the two time points are not significantly differentiated at the other time point. Of the 540 differentially expressed genes found at day 14, 229 (42.4%) genes were significantly overexpressed in the A-type populations in relation to the C-type populations whereas 311 genes showed the opposite pattern. At day 21, the proportion of significantly overexpressed genes in the A-type versus the C-type population is very similar, with 282 (44.8%) genes significantly overexpressed out of 629.

Gene ontology (GO), KEGG pathway, and chromosomal enrichment analyses found no biologically interpretable patterns of differentiation at either time point. Notably, when reviewing the gene-feature types among the differentially expressed genes, we found numerous non-coding RNA genes (ncRNA). Specifically, we found significant differentiation for 402 and 482 protein-coding genes for day 14 and day 21, respectively. There were 136 and 246 ncRNA genes that were significantly differentiated for those same time points, respectively. One annotated pseudogene (CR18275) appeared also as differentially expressed at each time point, and one tRNA gene was also found differentially expressed on day 14. In light of the numerous ncRNA genes found to be differentially expressed, and the fact that there is little known about them at the functional level, we repeated the GO analyses without the inclusion of non-protein coding genes. However, this more selective analysis provided no evidence for significant enrichment with respect to any biologically interpretable pattern.

Transcriptional differentiation due to ageing

There are numerous ways of curating these data to obtain a list of genes differentially expressed due to ageing. We adopted three different approaches: I, II, and III. The relationships among these lists are highlighted in Fig. 4.

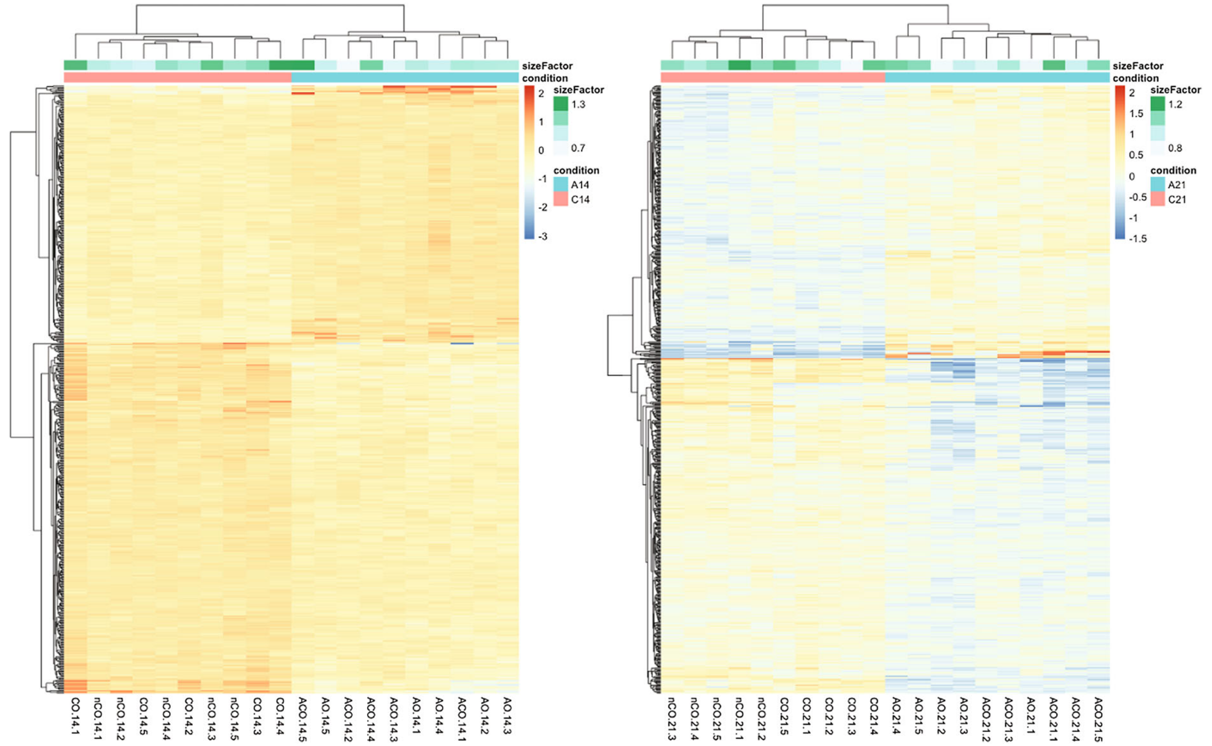

Fig. 2 Clustering of differentially expressed genes in the female transcriptome of *D. melanogaster* between two populations types. Heatmaps for the two time points assayed: 14 days (left), 492 genes; 21 days (right), 603 genes. Heatmaps were generated using hierarchical clustering of gene normalized mean expression levels. Ward's minimum variance was used as a distance metric. Gene clustering simultaneously results in a perfect grouping of populations based on the type of selection regime to which they were exposed. Blue, underexpression; red, overexpression. The identity of each population is shown at the bottom of each chart

List for approach I

First, we simply compiled the genes that were differentially expressed at day 21 between the A-type and C-type populations. In this case, we purposefully ignored day 14 because at day 14 both A-type and C-type populations were transferred from vials to cages shortly before being collected for sequencing, potentially influencing expression levels for some of the transcribed genes. In addition, day 21 is not affected by the females from the C-type populations not being fully sexually mature. More importantly, the degree of mortality-rate differentiation between A and C population types at day 21 is substantially higher than at day 14 (Fig. 1).

We found 629 differentially expressed genes between A-type and C-type populations at day 21. Of these genes, 282 were significantly more expressed in the A-type populations while 347 were more expressed in the C-type populations (Fig. 2b). Searching for biologically interpretable patterns within this list found no significant pattern. This result did not change after omitting 146 ncRNA genes and 1 pseudogene.

List for approach II

Next, we adopted a more stringent approach and analyzed only genes that were differentially expressed at both day 14 and day 21 between the A-type and C-type populations. The rationale behind this approach was that, at both time points, the A-type populations are ageing whereas the C-type populations have yet to age (Fig. 1). Essentially, we were interested in those genes that exhibit sustained interpopulation differences across 7 days. We found 263 differentially expressed genes between A-type and C-type populations at both day 14 and day 21. Of them, 94 genes exhibit significantly higher expression in the A-type populations whereas 169 do so in the

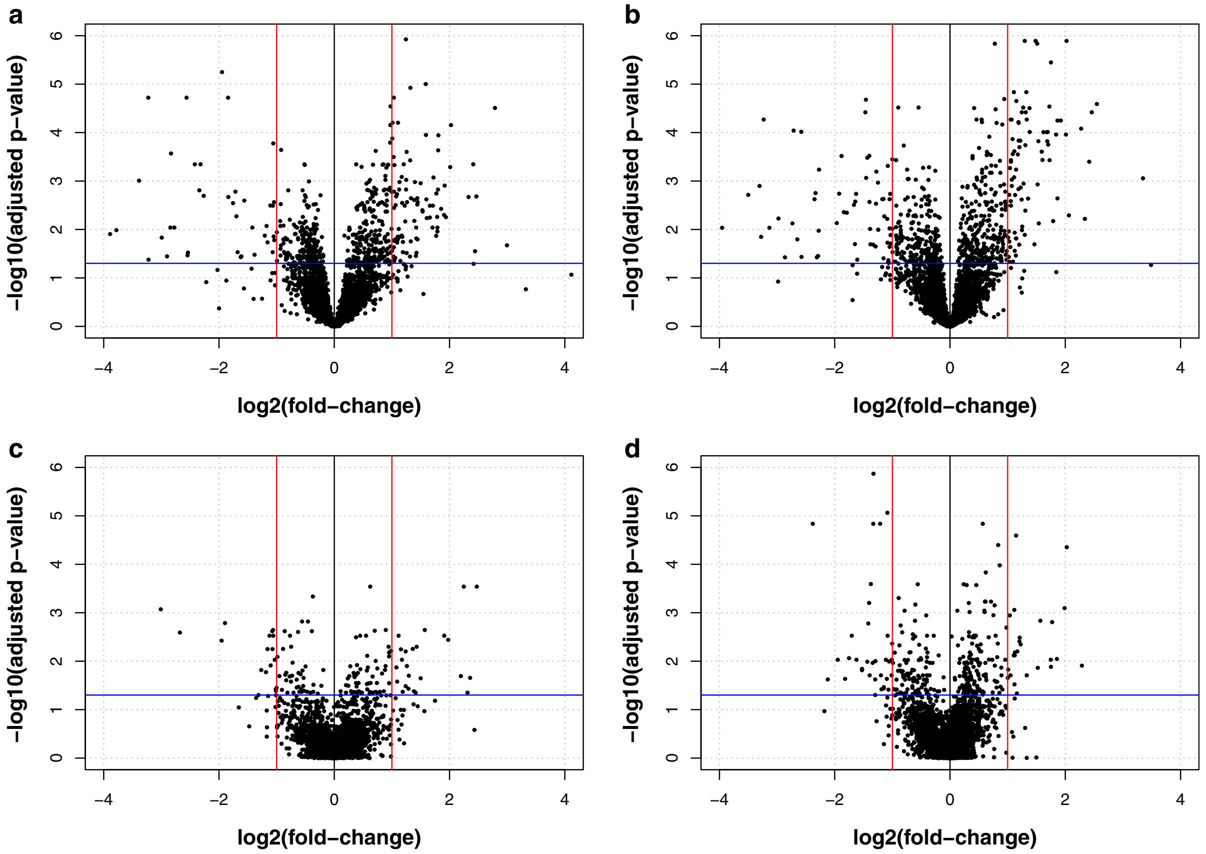

Fig. 3 Differences in mRNA abundance in the female transcriptome between two population types and two time points. Volcano plots for day 14 and day 21 (**a**, **b**) time points show the differences in expression between the populations types A and C. Similar plots for A-type and C-type (**c**, **d**) populations showing differences in expression between the two time points. *x* axis, difference in normalized $\log_2$-transformed expression difference in mRNA abundance between day-21 and day-14; *y* axis, significance of the differences as $-\log_{10}(P_{\mathrm{adj}}$ value). Statistically significant differences were determined using a linear mixed effect model. Blue dotted line, $P_{\mathrm{adj}} = 0.05$

C-type populations (Fig. 3a, b). Again, no biologically interpretable patterns were found, which did not change upon omitting 73 ncRNA genes.

List for approach III

Lastly, we focused on genes that were differentially expressed in the aging populations (A-type), but not in the non-aging populations (C-type). More specifically, we were interested in the genes that were differentially expressed in the A-type populations between day 14 and day 21 but showed statistically similar expression in the C-type populations between day 14 and day 21. The A-type populations are clearly ageing between day 14 and day 21 (Fig. 1) and therefore identifying genes that are differentially expressed between these two time points would capture potential ageing related genes. As the C-type populations are not ageing during this same time frame, the cross examination of the two sets of genes should eliminate any genes that are differentially expressed due to any random environmental artifacts at day 14. We ultimately found 82 genes that fit these criteria. Of these 82 genes, 38 had higher expression in the day 14 A-type populations and 44 had higher expression in the day 21 A-type populations (Fig. 3c, d). Further, the search for biologically interpretable patterns among these 82 genes revealed an enrichment for genes associated with the ABC transporter-like pathway (GO:0005524, GO:0016887: $P_{\mathrm{adj}} = 0.021$). ABC transporter proteins are part of the ATP-Binding Cassette (ABC) superfamily and are widely used in the hydrolysis of ATP to energize many biological processes. These transporters are key for the import and export of many

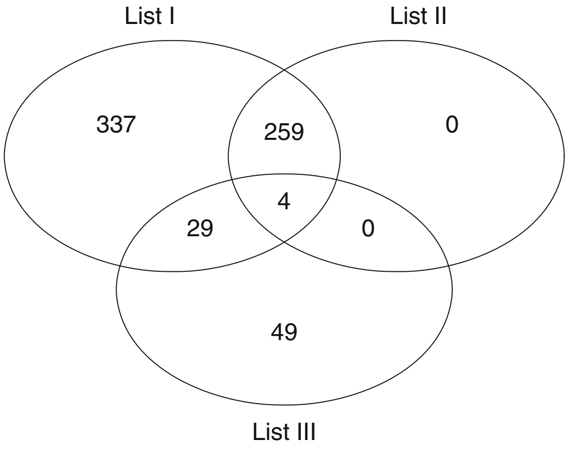

Fig. 4 Venn diagram showing the overlap between the three different lists of ageing related genes. The three gene lists were curated using different outlooks on the same data set

substrates, in particular toxins (Saurin et al. 1999). Similar to the previous analyses, there were numerous ncRNA genes within this list. Specifically, we found 61 protein coding genes, 19 ncRNA genes and 2 tRNA genes. Removing the 19 ncRNA genes due to their poor functional annotation led to the detection of no additional patterns.

The gene lists obtained upon applying these three sets of criteria not only differ in the total number of genes included, but also in the degree of overlap (Fig. 4). List *II* is essentially a more restricted version of list I, as the transcriptome differentiation in day 14 is incorporated. List III differs substantially from list I and list II, with 49 (59.8%) of the genes being specific to it.

Genomic comparison

We compared our three lists of ageing-related genes with that previously identified from genomic analysis (Graves et al. 2017), in order to determine what fraction of the differentially expressed genes between population types harbored significantly differentiated SVs, TE insertions, and SNPs. For SVs specifically, we were interested in in duplications and deletion events that could modify expression levels (Cardoso-Moreira et al. 2016). Next, we checked for local TE insertions that may also affect gene expression (Cridland et al. 2015). Lastly for SNPs, we were interested in finding differentiation in gene regions that can accommodate *cis*-regulatory motifs, i.e. the 5′ and 3′ UTRs and the promoter. The latter was taken to reside within 1 kb upstream of the transcriptional start site of the gene. To this purpose, we examined both coding and non-coding gene regions, including exons, introns, and the aforementioned untranslated regions, as well as the upstream region for each of the differentially expressed genes.

We identified 12, 8, and 2, in lists I, II, and III respectively, differentially expressed genes that harbor differentiated SVs (Table S2). In contrast, only 3, 2, and 0, differentially expressed genes between the A-type and C-type populations contain differentiated TE. Further, we identified 48, 26, and 4 differentially expressed genes (Table 1) that harbored differentiated SNPs in lists I, II, and III respectively. Of these genes, 66.7% (32), 69.2% (18), and 75% (3), respectively, had such SNPs within exonic or intronic regions. More importantly, 60.4% (29), 65.4% (17), and 50% (2) of the genes, respectively, contained SNPs in at least one gene region where *cis*-regulatory motifs reside, potentially contributing to the detected differences in mRNA abundance. The remaining differentially expressed genes either have no differentiated SNPs or the SNPs are located in gene regions usually devoid of *cis*-regulatory motifs. Differences in gene expression for this latter gene set should be primarily influenced by trans-regulatory effects.

Table 1 *Cis*-regulatory nature of population-differentiating-SNPs in ageing-related differentially expressed genes

	Cis-regulatory motifs				Non-*cis*-regulatory motifs			
	Promoter	5′UTR	3′UTR	Combined*	Exons	Introns	Combined*	Total*
List I	20	9	7	29	18	19	32	48
List II	13	4	4	17	12	14	18	26
List III	2	0	0	2	2	2	3	4

*Non-redundant among different gene regions

Comparison of ageing genes to previous lists of ageing-related genes

We compared our lists of candidate ageing genes to others obtained in similar studies. First, we considered the gene list at the repository for *D. melanogaster* at GenAge (de Magalhaes 2014). Of the 193 genes present in the GenAge database, only four were also part of List I. Lists II and III showed no overlap with the GenAge list. This lack of overlap may be due to the fact that GenAge specifically focuses on genes that have an ortholog associated with ageing in humans. In addition, GenAge focuses on genes that are only ageing-specific, and omits genes that may play roles in other biological processes.

Subsequently, and more in the context of transcriptomic changes associated with ageing, we compared our lists to that from a previously published expression profiling study in *D. melanogaster* (Carlson et al. 2015). That study found 1581 genes differentially expressed across 11 time points and 79 days, compared to a control sample consisting of 2-day old (after eclosion) females. We identified 51, 20, and 5 genes from their list that were also present in our I, II and III lists, respectively. Interestingly, this is lower than what we found by randomly selecting 1581 genes from the reference genome and compare it to our ageing lists. After 1000 simulations, we found that the probability of having at least 51, 20, or 5 overlapping genes from lists I, II, and III respectively was 0.824, 0.825, and 0.890. Thus random selections of genes have more overlap with our results than the genes identified by Carlson et al. (Carlson et al. 2015). Conversely, if we were to take a random sample from the reference genome using the length of our three lists individually and compared it to their list of 1581 genes, we found the probability of having at least the same number of overlapping genes as observed was 0.445, 0.574, and 0.759 for lists I, II, and III respectively. A major difference between our study and that of Carlson et al. (Carlson et al. 2015) is how a gene was ultimately considered to be associated with ageing. The gene list in Carlson et al. (Carlson et al. 2015) was obtained by studying transcriptional changes in one large population over time, always relative to the first time point. By contrast, the lists generated in this study derive from comparing an ageing set of populations and a non-ageing set of populations under three different sets of assumptions. An additional difference is that the females collected in the study of Carlson et al. (Carlson et al. 2015) were exposed to males for only 24 h before the beginning of the assay, whereas our samples contain females that were exposed to males the entire time up until their sampling point.

Power of replication

We evaluated how our level of replication might have impacted our inferences. The power to detect differentially expressed genes is negligible with only four or fewer replicates, which holds regardless of the set of criteria used to deem a gene as ageing-related (Fig. 5). Therefore, it is surprising that studies with this low level of replication find even more differentially expressed genes (Carlson et al. 2015; Hsu et al. 2019). Combining multiple inbred or isofemale lines is commonplace in *Drosophila* studies, but they might in fact contribute to the large number of differentially expressed genes documented. Increased linkage disequilibrium, which is a typical consequence of combining multiple lines, and having insufficient replication are likely to result in unforeseen effects in gene expression. Therefore, our results strongly suggest that, given the phenotypic plasticity of expression levels (Scheiner et al. 2012; Dayan et al. 2015), using properly maintained evolved populations that derived from an outbred population plus high replication levels are crucial to reduce both type I errors and type II errors when generating a portrait of differences in gene expression.

Conclusions

Having two clearly defined sets of *D. melanogaster* populations has enabled us to dissect what is ageing-related in the transcriptome. Specifically, one set of ten populations (A-type) is demographically ageing between day 14 and day 21 and the other set of ten populations (C-type) is not demographically ageing (Fig. 1). This extreme contrast of ageing vs non-ageing populations is particularly useful, because we can assay them at the same chronological ages, day 14 and day 21. In addition, these populations are not exposed to any metabolic arrest or other manipulation [e.g. nematodes, (Ayyadevara et al. 2008)]; their differences are genetic. Lastly, these two sets of

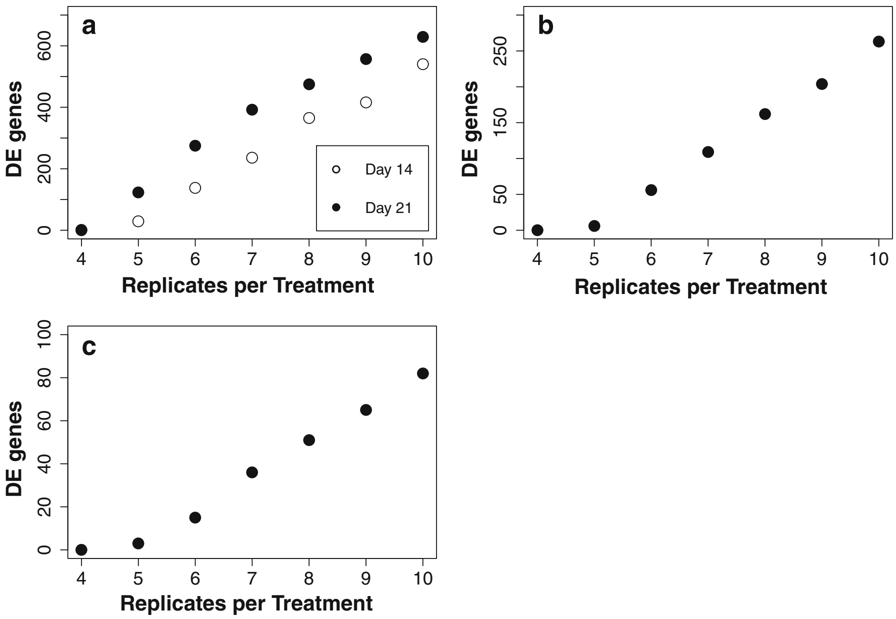

Fig. 5 Differentially expressed genes versus number of replicates in each treatment. **a** Number of differentially expressed genes determined between the A-type and C-type for both day 14 and day 21 depending on the number of replicates included in the analysis. **b** Number of differentially expressed genes between A-type and C-type populations sustained at both day 14 and day 21. **c** Number of differentially expressed genes deemed ageing-related between ageing populations and non-ageing populations

populations are closely related, despite the stark difference in their ageing patterns between day 14 and day 21.

Furthermore, these two sets of ten populations have been extensively characterized for genomic differentiation (Graves et al. 2017), developmental differentiation (Burke et al. 2016), and physiological differentiation (Rose et al. 2004) (Kezos et al. in prep.). Developmentally, the two sets of ten populations show SNP, TE and SV differentiation. Phenotypically, the 10 A-type populations develop from egg to pupation and pupation to eclosion significantly faster than the 10 C-type populations. Physiologically, the A-type populations have a shorter time to starvation than the C-type populations and the same is true for desiccation. Considering the stark difference between the two sets of populations at the genomic and phenotypic levels, it is not surprisingly that we find here a substantial amount of transcriptomic differentiation.

Unlike the statistical results testing for differentiation between A-type and C-type populations, within A-type comparisons of populations and within C-type comparisons show little to no differentiation. That is, the transcriptomic results are parallel to those previously found for within-type convergence with respect to life-history phenotypes (Burke et al. 2016) and genomics (Graves et al. 2017). As before, this convergence has arisen despite two very different selection histories underlying the differentiation of members of the A and C sets of populations. Specifically, the A group is made up of five populations that share long-standing A-type selection (the ACO), as well as five *other* populations that have undergone A-type selection for substantially fewer generations (the AO). Likewise, the C group is made up of five populations that share relatively long-standing C-type selection (the CO), as well as five other populations that have undergone C-type selection for substantially fewer generations (the nCO). The newly derived populations are much more closely-related phylogenetically than the longer-standing populations, with just 327 generations separating them versus 1171 generations separating the longer-standing populations. When comparing the newly derived populations with each other and with their selection regime counter parts (AO with ACO; nCO with CO), we find that the newly-derived populations show no evidence of less differentiation, suggesting that recent selection regime has a larger impact on the transcriptome than evolutionary history, as we have previously found both phenotypically and genomically (Burke et al. 2016; Graves et al. 2017). We believe this notion only stands true for populations that maintain moderate census size to combat the effects of inbreeding. With inbred populations, we would expect evolutionary history to have a larger impact on the transcriptome than recent selection regime (Sarup et al. 2011a, b).

When reviewing the nature of each differentiated gene, there was a high proportion of non-coding RNA genes that were differentially expressed. These genes are currently poorly understood. But due to their abundance among systematically differentiated transcripts in our data, our findings support the hypothesis that they can play an important role in gene regulation (Deveson et al. 2017). Another striking result was that only 4–6%, depending on which criteria used, of the differentially expressed genes between the A-type and O-type populations harbored SVs, TE insertions, or SNPs in *cis*-regulatory regions, suggesting that much of the transcriptome differentiation between population types stems primarily from trans-regulatory effects.

Next, when comparing our lists of ageing-related genes to that of previous ageing related work (Carlson et al. 2015; Hsu et al. 2019), we find very little overlap with previous findings. In addition, we find little to no enrichment for biological processes delimited in GO with our ageing-related lists of differentiated transcripts. These two results together suggest that our lists are novel relative to the current state of ageing transcriptomics in *D. melanogaster*.

Here we have presented two-time point comparisons using ten replicates for each set of populations. But it is clear from Fig. 5 that even at ten replicates we have not reached saturation for detection of differentially expressed genes, suggesting that still more replication would allow better detection of transcripts that differentiate with respect to ageing versus non-ageing. In addition to increased replication, more time point sampling should improve our understanding of how the transcriptome is affected by ageing. Lastly, integrating genomic analysis with still more powerful transcriptomics should further improve our understanding of how differentially expressed transcripts are regulated.

Currently, we have only sequenced the transcriptomes of our A-type and C-type populations, because of their clear difference in ageing. Adding additional types of populations to transcriptomic analysis should foster the parsing of transcriptomic differentiation with respect to whether or not any particular transcript difference is involved in the differentiation of ageing versus other types of phenotypic differentiation. In previous phenotypic and genomic work, we have used an intermediate set of ten populations, the B-type, in addition to the current sets (Burke et al. 2016; Graves et al. 2017; Mueller et al. 2018). The B-type populations have a generation length of 14 days, which falls between that of the A-type and C-type populations. The B-type populations would be therefore a prime candidate to add to bolster our ability to parse transcriptomic differentiation.

Although having a full suite of genomic, transcriptomic, and phenotypic data is powerful in itself, parsing all three bodies of data together is challenging. Due to the inherent complexity of ageing, complexity should be maintained and not sacrificed when analyzing all these data together. Modeling techniques commonly called “AI”, but more properly defined as statistical learning (Hastie et al. 2009), allow us to address this challenge of parsing complexity. Currently, the Fused Lasso Additive Model or FLAM (Petersen et al. 2016; Mueller et al. 2018) shows promise in making sense of large data sets, such as those of genomic data. Ultimately, we propose that combining the omics of highly replicated experimentally evolved populations with statistical learning tools could prove promising for uncovering the foundations of any complex trait, those of ageing included.

Materials and methods

Experimental populations

The populations used here were experimentally evolved over numerous generations (Rose et al. 2004; Burke et al. 2016; Graves et al. 2017). These populations were subject to two selection regimes which differed in length of their discrete generations. Each selection regime was applied to two sets of five populations, each with known distinct evolutionary histories (Fig. S1). The ACO and AO sets are both A-type, whereas the CO and nCO populations are both C-type. Although the individuals of the two population-types differ in body size, there is no evidence of major allometric differences.

Fly husbandry

Each population is maintained over generations at a census size of ~ 2000 individuals in order to reduce the effects of genetic drift on genetic variation. Flies are kept in 8-dram plastic vials during development and placed in Plexiglass cages on day 10 (A-type) and day 14 (C-type). For the A-type treatment, once the flies are placed in cages, they are given a 24 h oviposition window on fresh food. For the C-type treatment, the flies are left in cages until day 26, when they are given 48 h to oviposit on fresh food. All populations are fed with fresh medium made with cooked bananas, corn syrup, yeast, barley malt, and agar (Rose et al. 2004). Fresh food is supplemented with 5% live yeast paste to enhance oviposition duration egg-laying. All populations are kept at about 23 °C and exposed to a 24-h light cycle.

RNA preparation and sequencing

For each population and time point combination, 150 whole-body females from the same cohort were submerged in TRIzol, snap-frozen in liquid nitrogen, and stored at − 80C until extraction. Total RNA was purified using the RNeasy Mini Kit (Qiagen). RNA concentration, purity, and integrity were estimated using a NanoDrop 8000 Spectrophotometer and the RNA 6000 Nano Chip Kit (Agilent Technologies) with an Agilent 2100 Bioanalyzer. Ribodepleted, strand-specific paired-end libraries were prepared using the Ribo-Zero Gold Set A and the TruSeq Total RNA Library Prep kits from Illumina. Libraries were multiplexed and sequenced from both ends for 75 cycles over four lanes on an Illumina HiSeq2500 at the University California Irvine Genomics High Throughput Facility.

Gene expression analysis

Quality checks of each RNA paired-end sequencing output were performed with FastQC v0.11.5 (Andrews 2010). Subsequent sequence processing with Trimmomatic v0.35 (Bolger et al. 2014) included removal of adapter sequences, trimming of 3′ nucleotide calls with Phred score lower than 30, and filtering out of sequencing reads with a final length lower than 36 bp or overall Phred score lower than 30. Paired-end reads were mapped to the *D. melanogaster* Release v6.18 reference genome assembly (dos Santos et al. 2015) using STAR (Dobin et al. 2013)—under default settings except for an adjustment to avoid the detection of novel junctions—as this tool shows enhanced accuracy mapping rates in relation to other alignment tools (Baruzzo et al. 2017). The average alignment rate for the 40 sequencing outputs was 89.5% (minimum = 73.3%, maximum = 97.8%). Alignment post-processing was performed with SAMtools v0.1.19 (Li et al. 2009). Read counting per gene and population was done using HTSeq v0.6.1p1 (Anders et al. 2013) at default settings. Genome coverage was estimated using the genomeCoverageBed utility from BEDTools v2.25.0 (Cridland et al. 2015). Summary statistics and NCBI SRA accession numbers are provided in Table S1.

For each sample, per gene read counts were normalized using the default DESeq2 settings (Love et al. 2014). Genes showing normalized count values > 4 in at least eight out of ten populations, within at least one of the treatment types, were included in downstream analyses. To see any relationships between populations, we conducted a principal component analysis with the normalized count data using prcomp and ggplot2 (Wickham 2016). To accommodate any block effect associated with different rounds of extraction and sequencing, the normalized count data for reproducibly expressed genes were analyzed using a linear mixed effects model (R Development Core Team 2016). In each population we have a measure of gene expression, e_{ijk}, from selection treatment-i (i = 1 (A) or 2(C)), block-j (j = 1 (ACO and CO) or 2 (AO and nCO)), and population-k (k = 1,..,20). We can then model expression with the mixed linear effects function,

$$e_{ijk} = \mu + \delta_i\alpha_i + b_j + \varepsilon_k,$$

where $\delta_i = 0$, if $i = 1$ and 1 otherwise, b and ε are assumed to be independent random variables with a normal distribution with zero mean and variances σ_1^2 and σ_2^2 respectively. Significant treatment effects are determined by testing whether α_2 is significantly different from zero.

Statistical significance for differential expression of any given gene was set at a 5% FDR for ~ 4000 tests, i.e. the number of expressed genes that passed filtering (Benjamini and Hochberg 1995).

Searches for biological patterns across GO and KEGG terms, and other genome features such as chromosome distribution, were performed using DAVID 6.8 (da Huang et al. 2009). The Benjamini–Hochberg correction for multiple tests was applied given that we were performing 50-600 tests, i.e. the number of differentially expressed genes inputted into DAVID, across different gene lists.

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References

Anders S, McCarthy DJ, Chen Y, Okoniewski M, Smyth GK, Huber W, Robinson MD (2013) Count-based differential expression analysis of RNA sequencing data using R and Bioconductor. Nat Protoc 8(9):1765–1786

Andrews S (2010) FastQC: a quality control tool for high throughput sequence data. Babraham Institute. Available at http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Ayyadevara S, Alla R, Thaden JJ, Shmookler Reis RJ (2008) Remarkable longevity and stress resistance of nematode PI3 K-null mutants. Aging Cell 7(1):13–22

Baruzzo G, Hayer KE, Kim EJ, Di Camillo B, FitzGerald GA, Grant GR (2017) Simulation-based comprehensive benchmarking of RNA-seq aligners. Nat Methods 14(2):135–139

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate—a practical and powerful approach to multiple testing. J R Stat Soc Ser B 57(1):289–300

Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30(15):2114–2120

Braendle C, Heyland A, Flatt T (2011) Integrating mechanistic and evolutionary analysis of life history variation. In: Flatt T, Heyland A (eds) Mechanisms of life history evolution: the genetics and physiology of life history traits and trade-offs. Oxford University Press, New York, pp 1–10

Bray NL, Pimentel H, Melsted P, Pachter L (2016) Erratum: near-optimal probabilistic RNA-seq quantification. Nat Biotechnol 34(8):888

Bryant DM, Johnson K, DiTommaso T, Tickle T, Couger MB, Payzin-Dogru D, Lee TJ, Leigh ND, Kuo T-H, Davis FG et al (2017) A tissue-mapped axolotl de novo transcriptome enables identification of limb regeneration factors. Cell Rep 18(3):762–776

Burke MK, Barter TT, Cabral LG, Kezos JN, Phillips MA, Rutledge GA, Phung KH, Chen RH, Nguyen HD, Mueller LD et al (2016) Rapid divergence and convergence of life-history in experimentally evolved *Drosophila melanogaster*. Evolution 70(9):2085–2098

Cardoso-Moreira M, Arguello JR, Gottipati S, Harshman LG, Grenier JK, Clark AG (2016) Evidence for the fixation of gene duplications by positive selection in *Drosophila*. Genome Res 26(6):787–798

Carlson KA, Gardner K, Pashaj A, Carlson DJ, Yu F, Eudy JD, Zhang C, Harshman LG (2015) Genome-wide gene expression in relation to age in large laboratory cohorts of *Drosophila melanogaster*. Genet Res Int 2015:835624

Cridland JM, Thornton KR, Long AD (2015) Gene expression variation in *Drosophila melanogaster* due to rare transposable element insertion alleles of large effect. Genetics 199(1):85–93

da Huang W, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4(1):44–57

Dayan DI, Crawford DL, Oleksiak MF (2015) Phenotypic plasticity in gene expression contributes to divergence of locally adapted populations of *Fundulus heteroclitus*. Mol Ecol 24(13):3345–3359

de Magalhaes JP (2014) Why genes extending lifespan in model organisms have not been consistently associated with human longevity and what it means to translation research. Cell Cycle 13(17):2671–2673

Development Core Team R (2016) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna

Deveson IW, Hardwick SA, Mercer TR, Mattick JS (2017) The dimensions, dynamics, and relevance of the mammalian noncoding transcriptome. Trends Genet 33(7):464–478

Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR (2013) STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29(1):15–21

dos Santos G, Schroeder AJ, Goodman JL, Strelets VB, Crosby MA, Thurmond J, Emmert DB, Gelbart WM, FlyBase C (2015) FlyBase: introduction of the *Drosophila melanogaster* release 6 reference genome assembly and large-scale migration of genome annotations. Nucleic Acids Res 43(Database issue):D690–D697

Garland T, Rose MR (2009) Experimental evolution. University of California Press, Berkeley, CA

Graveley BR, Brooks AN, Carlson JW, Duff MO, Landolin JM, Yang L, Artieri CG, van Baren MJ, Boley N, Booth BW et al (2011) The developmental transcriptome of *Drosophila melanogaster*. Nature 471(7339):473–479

Graves JL Jr, Hertweck KL, Phillips MA, Han MV, Cabral LG, Barter TT, Greer LF, Burke MK, Mueller LD, Rose MR (2017) Genomics of parallel experimental evolution in *Drosophila*. Mol Biol Evol 34(4):831–842

Hastie T, Tibshirani R, Friedman JH (2009) The elements of statistical learning: data mining, inference, and prediction. Springer, New York

Hsu SK, Jaksic AM, Nolte V, Barghi N, Mallard F, Otte KA, Schlotterer C (2019) A 24 h age difference causes twice as much gene expression divergence as 100 generations of adaptation to a novel environment. Genes 10(2):89

Hubley R, Finn RD, Clements J, Eddy SR, Jones TA, Bao W, Smit AF, Wheeler TJ (2016) The Dfam database of repetitive DNA families. Nucleic Acids Res 44(D1):D81–D89

Kolde R (2015) pheatmap: Pretty Heatmaps. R package version 1.0.8

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, Genome Project Data Processing S (2009) The sequence alignment/map format and SAMtools. Bioinformatics 25(16):2078–2079

Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15(12):550

Mallard F, Nolte V, Tobler R, Kapun M, Schlotterer C (2018) A simple genetic basis of adaptation to a novel thermal environment results in complex metabolic rewiring in *Drosophila*. Genome Biol 19(1):119

Matthews BB, dos Santos G, Crosby MA, Emmert DB, St Pierre SE, Gramates LS, Zhou PL, Schroeder AJ, Falls K, Strelets V et al (2015) Gene model annotations for *Drosophila melanogaster*: impact of high-throughput data. G3 5(8):1721–1736

Mueller LD, Phillips MA, Barter TT, Greenspan ZS, Rose MR (2018) Genome-wide mapping of gene-phenotype relationships in experimentally evolved populations. Mol Biol Evol 35(8):2085–2095

Petersen A, Witten D, Simon N (2016) Fused lasso additive model. J Comput Graph Stat 25(4):1005–1025

Remolina SC, Chang PL, Leips J, Nuzhdin SV, Hughes KA (2012) Genomic basis of aging and life-history evolution in *Drosophila melanogaster*. Evolution 66(11):3390–3403

Rose MR (1984) Laboratory evolution of postponed senescence in *Drosophila melanogaster*. Evolution 38(5):1004–1010

Rose MR, Burke MK (2011) Genomic croesus: experimental evolutionary genetics of *Drosophila* aging. Exp Geront 46(5):397–403

Rose MR, Passananti HB, Matos M (2004) Methuselah flies. World Scientific Publishing, Singapore

Sarup P, Sørensen JG, Kristensen TN, Hoffmann AA, Loeschcke V, Paige KN, Sørensen P (2011a) Candidate genes detected in transcriptome studies are strongly dependent on genetic background. PLoS ONE 6(1):e15644

Sarup P, Sørensen P, Loeshcke V (2011b) Flies selected for longevity retain a young gene expression profile. Age 33(1):69–80

Saurin W, Hofnung M, Dassa E (1999) Getting in or out: early segregation between importers and exporters in the evolution of ATP-binding cassette (ABC) transporters. J Mol Evol 48(1):22–41

Scheiner SM, Barfield M, Holt RD (2012) The genetics of phenotypic plasticity. XI. Joint evolution of plasticity and dispersal rate. Ecol Evol 2(8):2027–2039

Wickham H (2016) ggplot2: elegant graphics for data analysis. Springer, New York

Yang L, Duff MO, Graveley BR, Carmichael GG, Chen LL (2011) Genomewide characterization of non-polyadenylated RNAs. Genome Biol 12(2):R16