Transcriptomic response of *Drosophila melanogaster* pupae developed in hypergravity☆

Shannon Hateley a,1, Ravikumar Hosamani b,1, Shilpa R. Bhardwaj b, Lior Pachter a,c, Sharmila Bhattacharya b,*

a Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, United States
b Space Biosciences Division, NASA Ames Research Center, Mountain View, CA 94035, United States
c Departments of Mathematics and Computer Science, University of California, Berkeley, CA 94720, United States

**A R T I C L E   I N F O**

Article history:
Received 11 May 2016
Received in revised form 12 August 2016
Accepted 8 September 2016
Available online 10 September 2016

Keywords:
Hypergravity
*Drosophila melanogaster*
Pupa
Transcriptome
Metamorphosis
RNA-Seq

**A B S T R A C T**

Altered gravity can perturb normal development and induce corresponding changes in gene expression. Understanding this relationship between the physical environment and a biological response is important for NASA's space travel goals. We use RNA-Seq and qRT-PCR techniques to profile changes in early *Drosophila melanogaster* pupae exposed to chronic hypergravity (3 g, or three times Earth's gravity). During the pupal stage, *D. melanogaster* rely upon gravitational cues for proper development. Assessing gene expression changes in the pupae under altered gravity conditions helps highlight gravity-dependent genetic pathways. A robust transcriptional response was observed in hypergravity-treated pupae compared to controls, with 1513 genes showing a significant (q < 0.05) difference in gene expression. Five major biological processes were affected: ion transport, redox homeostasis, immune response, proteolysis, and cuticle development. This outlines the underlying molecular and biological changes occurring in *Drosophila* pupae in response to hypergravity; gravity is important for many biological processes on Earth.

Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license ([http://creativecommons.org/licenses/by-nc-nd/4.0/](http://creativecommons.org/licenses/by-nc-nd/4.0/)).

1. Introduction

During the pupal stage, most holometabolous insects undergo an extensive metamorphosis from larva into winged adult. Larval structures are degenerated while adult structures are formed from the imaginal discs and histoblasts. This metamorphosis is tightly regulated transcriptionally, and is evolutionarily conserved. It also depends on Earth's gravity. For example, the orientation of the pupa is dependent upon gravitational cues for proper alignment [1] and deviation to microgravity or hypergravity environments can profoundly influence metamorphosis and alter associated gene expression [2]. Comprehensive gene expression analysis is therefore necessary to understand the underlying molecular response of developing pupae in altered gravity environments.

To date, gene expression studies on the response to altered gravity have mostly utilized microarray technology. Herranz et al. measured transcriptional response to simulated micro and hypergravity in *D. melanogaster* pupae via microarray, discovering that a significantly higher number of genes related to metamorphosis were changed in hypergravity (2 g) than in simulated microgravity [3]. Pupae exposed to even higher gravitational forces (6 g and 12 g) exhibited altered mRNA expression across multiple pathways, upsetting diverse physiological functions and behaviors [3]. In another microarray study by Herranz et al., imagos developed entirely in a diamagnetic-levitation–induced microgravity or simulated hypergravity (2 g) environment revealed changes in gene expression linked to immunity, stress, and temperature-response [4]. Other research efforts, focused on the effect of hypergravity on aging, physiological stress, and behavior [5], have shown that chronic hypergravity (3 g and 5 g for two weeks) has a beneficial effect on longevity, resistance to heat, and behavioral aging in adult *D. melanogaster*. Conversely, hypergravity in combination with cold stress can have negative impact on these same traits [6]. Life-long exposure of flies to different hypergravity (4 g, 5 g, 7.38 g) conditions can also have negative effects on longevity [7]. The wide-ranging scope of these results highlights a complex interplay between biology and gravity. Further characterization of the effects of altered gravity on an organism would benefit from the use of RNA sequencing, with its ability to address global transcriptional changes with reliable☆ Sequence data from this article have been deposited in NCBI's Gene Expression Omnibus [47] and are accessible through GEO Series accession number GSE80323 ([https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE80323](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE80323)).

☆ Corresponding author at: Principal Investigator Biomodel Performance and Behavior Laboratory, Space Biosciences Division, NASA Ames Research Center, Mail Stop – 236-5, Moffett Field, Mountain View, CA-94035, United States.

E-mail addresses: shateley@berkeley.edu (S. Hateley), ravikumar.hosamani@nasa.gov (R. Hosamani), shilpa.r.shanks@gmail.com (S.R. Bhardwaj), lpachter@math.berkeley.edu (L. Pachter), sharmila.bhattacharya@nasa.gov (S. Bhattacharya).

1 Contributed equally.
accuracy, higher sensitivity in measurement, and power to detect novel transcripts.

Here, we have characterized the effects of hypergravity on the *D. melanogaster* transcriptome via differential expression analysis of RNA-Seq data from 3 g−exposed and 1 g−control pupal samples. Our data reveal significant alterations in the expression of genes involved in ion transport, oxidative stress, immune function, proteolysis, and cuticle development in response to chronic hypergravity in *D. melanogaster*. Understanding the role of gravity in development contributes to knowledge of basic developmental biology and is a critical aspect of space biology research.

2. Results and discussion

2.1. Hypergravity accelerates time to pupal development

To see if gravity-induced gene expression changed in proportion to increase in gravitational force, in addition to 3 g samples, we also allowed *D. melanogaster* to develop at 5 g and 8 g for evaluation using qRT-PCR. Pupae that developed at 3 g from egg to stage P6 [8] did not show any change in developmental phenotype. However, as we increased gravitational force from 3 g to 5 g, pupal development accelerated significantly, as evidenced by a higher number of advanced stage pupae and increased eclosion rate compared to 3 g (Table 1). Next, we increased g-force to 8 g and looked for developmental defects. At 8 g, egg laying and hatching were minimal. While at 1 g and 3 g around 150 pupae developed, at 9 g only 16 were counted. Among the 16, 9 developed into 3rd instar larvae and died before pupation, and only 7 attained pupal stage. The 8 g environment was detrimental to normal development. The low count of pupae and adults at 8 g (Table 1) illustrate that the force exceeded the viability threshold. Those that did manage to pupate developed at a much faster rate than at 5 g. Previous studies have shown that heat stress can accelerate growth in *Drosophila* [9]. To ascertain whether this phenotype was due to a change in gravity or due to heat stress, we fixed an iButton to the rotating arm inside the centrifuge to monitor the temperature profile through the course of the experiment. The iButton recorded the temperature every 15 min until the end of the experiment. The temperature ranged from 22.5 to 24.5 °C inside the centrifuge, indicating no significant change in both the 3 g and 5 g environments. These data rule out the possibility of heat stress influencing the developmental process in pupae. Thus, chronic hypergravity significantly influenced pupal development.

A clear change in developmental phenotype was evident as we increased the gravitational acceleration from 5 g to 8 g. However, we were specifically interested in understanding the transcriptional response to 3 g for two reasons. First, during space flight, astronauts are not only exposed to microgravity but also experience around 3 g during launch and 3 g or more during reentry. More importantly, chronic hypergravity models have been exploited to complement and predict microgravity-associated changes [3]. Since 3 g exerts enough force for chronic hypergravity exposure without decreasing overall viability, we sought to elucidate 3 g-induced transcriptional changes in *D. melanogaster* pupae in our study.

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>1 g</th>
<th>3 g</th>
<th>5 g</th>
<th>8 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early pupae</td>
<td>151</td>
<td>149</td>
<td>142</td>
<td>0</td>
</tr>
<tr>
<td>Dark pupae</td>
<td>2</td>
<td>3</td>
<td>16</td>
<td>7</td>
</tr>
<tr>
<td>Adult eclosion</td>
<td>1</td>
<td>1</td>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

Pupal development was monitored and samples were counted immediately post-centrifugation. While there was no change in pupal development at 1 g and 3 g, at 5 g we observed some acceleration in pupal development. At 8 g there was a profound effect on development and survival as was evident from the low pupal counts.

2.2. Hypergravity induces consistent transcriptional response

To discern the transcriptional response to hypergravity, we performed differential expression analysis on control and 3 g−exposed pupae. First, poly (A+) libraries from four control and four 3 g samples were pooled and sequenced on one lane of the Illumina HiSeq 2000, yielding a total of 214.5 million Casava quality-filtered reads [10]. After trimming, reads were aligned to the *D. melanogaster* reference transcriptome using Bowtie 2 [11] and transcript abundances estimated using eXpress [12]. Principal component analysis (PCA) revealed the gene expression data are well clustered between different biological replicates of both control and hypergravity-exposed samples, suggesting hypergravity is the greatest variable factor across samples (Fig. 1a). Due to technical constraints (see Section 4.2 for details), pupae could not be sexed before sample preparation. To check for possible sex bias of pupae and ensure that sex was not a confounding factor in our analyses, we compared Y chromosome and sex-specific gene expression across samples. Y chromosome transcripts confirm that males were present in each sample, with expression patterns uncorrelated to PCA components 1 and 2 (Fig. 1b). Comparison to the sex-specific transcripts in identically-staged pupae as detailed by Lebo et al. [13] show only 19 of the same genes to be significantly differentially expressed, of which there is no sex-specific bias per sample or between conditions (Fig. 1c,d). Differential expression analysis with DESeq2 [14] revealed 1513 genes were significantly (q < 0.05) altered in response to chronic hypergravity, of which 704 were upregulated and 809 were downregulated in 3 g samples compared to control (Supplemental Table S1). Gene ontology analysis was performed using the PANTHER classification system [15]. Among the 1513 genes differentially expressed, as many as 475 genes have no defined information on their molecular functions or biological processes. This study focuses upon the remaining 1038 genes. Based upon the hierarchical relation of gene ontology molecular and biological associations [16], we divided the 1038 genes into 13 functional classifications (Fig. 2). Using PANTHER statistical overrepresentation and enrichment tests along with these functional classifications, we determined that the 3 g treatment predominantly affected five major processes: ATP synthesis/redox, ion channels/transport, defense/immunity, cuticle development, and proteolysis. Overrepresentation tests reported enrichment of ion channel/transport genes up to 5.3 times (padj = 5.70E-05), cuticular genes up to 3.0 times (padj = 1.68E-05), and redox genes up to 6.0 times (padj = 4.37E-04) their expected levels compared to control genes. Translation related genes were also overrepresented up to 2.2 times (padj = 7.95E-06), but with minimal fold change in expression. The enrichment tests found defense and proteolysis genes to be enriched (padj = 2.27E-03 and 3.08E-08, respectively), and redox genes to be under-enriched (padj = 6.59E-03). (For results of statistical tests, see Supplemental Tables S2–S5.) The major affected processes are discussed in detail in Section 2.4.

2.3. Extrapolation with qRT-PCR

We selected 18 differentially expressed genes representing the diverse biological functions for further study using quantitative RT-PCR. Comparison of RNA fold-change and pattern of gene expression between RNA-Seq data and quantitative RT-PCR is highly consistent across biological replicates (Fig. 3) with Spearman correlation = 0.885, P = 1.075E-06.

After confirming our RNA-Seq results from the 1 g and 3 g conditions, we examined whether there was any relationship between changes in gene expression and changing gravitational force by performing qRT-PCR on pupae exposed to increasing gravity levels (3 g, 5 g and 8 g). There was no clear dose-responsive pattern observed in gene expression of these genes at the higher g−levels (see Section 4.5 for methods and Supplemental Table S10 for qRT-PCR data). This is consistent with the fact that higher g−loads seem to induce additional
stressful effects on the flies including affecting development at 5 g and loss of viability at 8 g (Table 1).

2.4. Major processes involved in hypergravity response

2.4.1. Hypergravity alters expression of genes encoding mitochondrial electron transport, oxidative phosphorylation, and redox status

Of the 1038 differentially expressed genes, 17.5% (182 transcripts) were related to mitochondrial electron carrier activity, TCA cycle, oxidative phosphorylation, and redox, with 140 downregulated and 42 upregulated in the hypergravity environment (Table 2); (See Supplemental Fig. S1 for oxidoreductase gene network). Notably, genes related to all four electron transport chain (ETC) enzymes (Complexes I, II, III, & IV) and hydrogen-exporting ATP Synthase (Complex V) were highly downregulated. These robust changes may indicate impaired mitochondrial function and ATP synthesis in pupae subjected to chronic hypergravity. Anomalous ETC enzyme activity can cause electron leakage and free radical generation in mitochondria, leading to the state of oxidative stress [17]. Decrease in the expression of these important genes in pupae developing in a hypergravity environment might adversely impact the process of metamorphosis.

Genes in the cytochrome P450 family (terminal oxidase enzymes in the electron transfer chain) were significantly elevated in hypergravity, suggesting a change in redox homeostasis. Increased expression of cytochrome P450 genes here could partially explain the induction of oxidative stress in hypergravity-exposed pupae due to impaired mitochondrial function and ATP synthesis. Some studies suggest that mild hypergravity exposure is beneficial to the stress response of adult flies, although no correlation with antioxidant defense, particularly SOD and catalase activity, was found [18]. Conversely, several reports have indicated that altered gravity (hypergravity/microgravity) induces oxidative stress in multiple organisms [19]. A recent study with endothelial cells found a significant decrease in expression of genes related to oxidative phosphorylation in microgravity [20]. Similarly in rodents, spaceflight-induced impairments to mitochondrial function, redox status, and associated oxidative stress were reported [21]. In the same study, post-flight-induced lipid peroxidation and diminished antioxidant defense were attributed to the stress associated with reentry into the Earth’s gravity, including a brief hypergravity exposure.
2.4.2. Hypergravity affects ion channels/transmembrane transporters

Metamorphosis in pupae involves the dramatic rearrangement of cells and changes in cell adhesion. To sense and respond to an external physical force that may affect the precision of these rearrangements, cells can activate mechanosensors. Ion channels and transmembrane transport proteins can transduce mechanical force into biochemical cell signaling pathways [22]. In our study, GO classification revealed that expression of several ion channel and transmembrane transport protein genes were altered in response to hypergravity. Among the 158 genes (15% of the total) in this category, 94 genes were down-regulated and 64 upregulated. Nearly half (73 of 158) are related to Na\(^+\) (26), Ca\(^{2+}\) (34), and K\(^+\) (13) ion channels (shown in Figs. 4a, b, and c respectively). See Supplemental Fig. S2 for differentially expressed genes involved in transport not related to Na\(^+\), K\(^+\), or Ca\(^{2+}\). The majority of the transcripts related to Na\(^+\) and K\(^+\) ion channels (16 of 26 Na\(^+\) and 10 of 13 K\(^+\)) were decreased in expression in hypergravity compared to control. Na\(^+\) and K\(^+\) ion channels ensure optimum action potential across the membranes of excitable cells such as neurons and myocytes [23]. A decrease in the expression of these channels in response to hypergravity could impair the action potential across the cell membrane, ultimately affecting neurotransmission and muscle contraction in pupae. We also observed that most genes related to Ca\(^{2+}\) ion binding and Ca\(^{2+}\)-dependent ion channel activity were down-regulated (26 of 34 Ca\(^{2+}\)-related genes) whereas only 8 were upregulated (Prestin, aralar1, Trpml, Rgk1, aay, Rph, Fim, and Cib2) in response to hypergravity (Fig. 4b).

Mechanical loading such as hypergravity can activate mechanotransduction pathways. Several reports suggest roles of mechanoreceptors in sensing gravitational acceleration [24]. In mammals, mechanical stimuli play a vital role in bone remodeling, especially bone cell differentiation, proliferation, and maturation, which are extremely sensitive to mechanical cues [25]. Similarly, mechanotransduction pathways regulate root gravity-sensing cells and calcium homeostasis in plants [26]. However, it is not yet clear exactly how altered gravity manipulates transport proteins and ion channel activity. One possibility is that interaction between integral...
response to hypergravity, suggesting a direct impact on innate immune regulation, affecting metamorphosis in pupae, but also modulates in-hypervarability. Thus, it is likely that hypergravity not only alters hormone-induced genes with dual roles in both metamorphosis and defense response against bacterium were significantly increased, suggesting a role of ecdysone hormone in regulating innate humoral immune response. Juvenile hormone (JH) and 20-hydroxy-ecdysone (20E) play critical roles in metamorphosis, and evidence suggests that both JH and 20E modulate innate immune response as well [32]. Related to this, when we increased hypergravity to 8g, we discovered not only accelerated pupal development but also a significant reduction of adult eclosion. This phenotype may partially be attributed to robust changes in the expression of ecdysone-induced genes in response to hypergravity. Thus, it is likely that hypergravity not only alters hormonal regulation, affecting metamorphosis in pupae, but also modulates innate immune response. We also found increased mRNA transcripts related to ion channels and transport could be part of the mechanotransduction-biological membranes. Thus, changes in mRNA transcripts related to ion and functional properties of alamethicin, a synthetic polypeptide that forms voltage-dependent ion pores in artificial and biological membranes [27]. These simplified membrane studies indicate detection of changes in gravitational acceleration might be an intrinsic property of biological membranes. Thus, changes in mRNA transcripts related to ion channels and transport could be part of the mechanotransduction-mediated manipulation causing metamorphosing cells in pupae to actively adapt to the change in gravitational acceleration.

2.4.3. Hypergravity elicits immune response in pupae

It is widely recognized that altered gravitational force has a profound influence on immune function. Spaceflight and ground-based studies validate this in different organisms [28–31]. In the present study, one major category of differentially expressed genes related to immune pathways. 5% of differentially expressed genes were immune-related genes that were highly upregulated in response to the chronic hypergravity environment (Fig. 5). The expression of several ecdysone-induced genes with dual roles in both metamorphosis and defense response against bacterium were significantly increased, suggesting a role of ecdysone hormone in regulating innate humoral immune response. Juvenile hormone (JH) and 20-hydroxy-ecdysone (20E) play critical roles in metamorphosis, and evidence suggests that both JH and 20E modulate innate immune response as well [32]. Related to this, when we increased hypergravity to 8g, we discovered not only accelerated pupal development but also a significant reduction of adult eclosion. This phenotype may partially be attributed to robust changes in the expression of ecdysone-induced genes in response to hypergravity. Thus, it is likely that hypergravity not only alters hormonal regulation, affecting metamorphosis in pupae, but also modulates innate immune response. We also found increased mRNA transcripts related to ion channels and transport could be part of the mechanotransduction-biological membranes. Thus, changes in mRNA transcripts related to ion and functional properties of alamethicin, a synthetic polypeptide that forms voltage-dependent ion pores in artificial and biological membranes [27]. These simplified membrane studies indicate detection of changes in gravitational acceleration might be an intrinsic property of biological membranes. Thus, changes in mRNA transcripts related to ion channels and transport could be part of the mechanotransduction-mediated manipulation causing metamorphosing cells in pupae to actively adapt to the change in gravitational acceleration.

Table 2

<table>
<thead>
<tr>
<th>Upregulated genes</th>
<th>Log2 FC³</th>
<th>P-value²</th>
<th>Downregulated genes</th>
<th>Log2 FC³</th>
<th>P-value²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ix</td>
<td>5.608</td>
<td>2.36E-43</td>
<td>Cyp904A1</td>
<td>2.544</td>
<td>4.01E-49</td>
</tr>
<tr>
<td>CG2070</td>
<td>4.045</td>
<td>2.38E-13</td>
<td>Cyp6v1</td>
<td>1.955</td>
<td>0.000484</td>
</tr>
<tr>
<td>CG11878</td>
<td>3.600</td>
<td>4.10E-17</td>
<td>CG4085</td>
<td>1.953</td>
<td>9.02E-07</td>
</tr>
<tr>
<td>Eo</td>
<td>3.432</td>
<td>1.66E-21</td>
<td>CG34172</td>
<td>1.953</td>
<td>6.32E-09</td>
</tr>
<tr>
<td>Cyp304A1</td>
<td>2.502</td>
<td>5.77E-32</td>
<td>CG8757</td>
<td>1.618</td>
<td>0.000672</td>
</tr>
<tr>
<td>CG11535</td>
<td>2.423</td>
<td>2.01E-22</td>
<td>CG8630</td>
<td>1.587</td>
<td>7.04E-05</td>
</tr>
<tr>
<td>Hbp2</td>
<td>2.167</td>
<td>3.30E-05</td>
<td>CG5955</td>
<td>1.332</td>
<td>1.00E-06</td>
</tr>
<tr>
<td>CG10131</td>
<td>1.957</td>
<td>6.90E-07</td>
<td>CG5955</td>
<td>1.332</td>
<td>1.00E-06</td>
</tr>
<tr>
<td>CG5914</td>
<td>1.831</td>
<td>2.06E-05</td>
<td>CG9150</td>
<td>1.273</td>
<td>0.002439</td>
</tr>
<tr>
<td>ImpL3</td>
<td>1.662</td>
<td>0.000119</td>
<td>Cyp4p1</td>
<td>1.371</td>
<td>5.97E-05</td>
</tr>
<tr>
<td>Cyp6a8</td>
<td>1.661</td>
<td>0.000583</td>
<td>l(2)91.289</td>
<td>1.365</td>
<td>8.60E-05</td>
</tr>
<tr>
<td>CG12268</td>
<td>1.637</td>
<td>0.000711</td>
<td>Cyp4g15</td>
<td>1.163</td>
<td>2.88E-05</td>
</tr>
<tr>
<td>mex1</td>
<td>1.595</td>
<td>3.24E-05</td>
<td>l(1)G0156</td>
<td>1.055</td>
<td>5.95E-05</td>
</tr>
<tr>
<td>CG3518</td>
<td>1.590</td>
<td>0.001960</td>
<td>Cyp6a9</td>
<td>1.206</td>
<td>0.004563</td>
</tr>
<tr>
<td>CG9747</td>
<td>1.582</td>
<td>0.001417</td>
<td>Cyp4f9</td>
<td>1.201</td>
<td>5.24E-06</td>
</tr>
<tr>
<td>Gilt2</td>
<td>1.566</td>
<td>0.000100</td>
<td>Cyp4g15</td>
<td>1.163</td>
<td>2.88E-05</td>
</tr>
<tr>
<td>Spat</td>
<td>1.562</td>
<td>0.000268</td>
<td>l(1)1G0156</td>
<td>1.055</td>
<td>5.95E-05</td>
</tr>
<tr>
<td>CG10211</td>
<td>1.552</td>
<td>0.001267</td>
<td>CG5028</td>
<td>1.041</td>
<td>6.08E-05</td>
</tr>
<tr>
<td>CG5512</td>
<td>1.538</td>
<td>0.000275</td>
<td>CG311a1</td>
<td>1.027</td>
<td>-4.10E37</td>
</tr>
</tbody>
</table>

Top 20 upregulated and downregulated genes in pupae that developed in hypergravity compared to 1g.

³ Upregulated and downregulated genes are indicated as positive and negative fold change respectively.

² P-value adjusted for FDR = 0.05.
function. In the absence of microbial infection, increased expression of antimicrobial peptides indicates an immune boost in response to hypergravity. Additional studies support our finding that hypergravity boosts immune function. Hypergravity-exposed flies (wild type and immune-compromised) exhibited increased survival against fungal infection [33]. In addition, in the absence of microbial infection, Lee et al. have shown that mechanical loading and stress activate immune responses in rat and human cell lines [34]. In contrast, microgravity exerts the opposite response by compromising immune function. For instance, adult flies returned from space showed increased vulnerability to microbial infection [31,33]. Similarly, previous studies from our group also show altered expression of antimicrobial peptides in response to space flight in both larvae and adults [31]. Hypergravity may have an advantage in terms of host immune defense response to combat against microbial infection.

2.4.4. Hypergravity alters genes encoding chitin metabolism and cuticle development

During development D. melanogaster produce five separate cuticles: three larval cuticles (first, second, and third instar), a pupal cuticle, and an adult cuticle [35]. Pupal cuticle is well understood as it is easy to synthesize in vitro by mass-culturing imaginal discs under defined hormonal conditions. The pupal cuticle proteins are synthesized in two phases by the imaginal disc epithelium [35]. Prior to pupation, sets of low–molecular weight proteins are synthesized and deposited. Post pupation, high–molecular weight proteins are synthesized and incorporated into the pupal cuticle. In the present study, 22 genes (12 upregulated, 10 downregulated) encoding chitin metabolic processes and 43 chitin-based cuticle development genes were altered in response to hypergravity (Fig. 6). Chitin plays a major role in structural integrity by stabilizing the layered organization of the cuticle [36]. Changes in the expression of these genes suggest altered chitin binding and metabolism. This data corroborates well with previous findings by Herranz et al., that genes related to insect cuticle proteins were significantly altered during metamorphosis, with most being downregulated [37]. In insects, cuticle serves a function similar to that of the skeletomuscular system in mammals. However, unlike the mammalian model, not many studies have been carried out in insects to understand the effect of altered gravity on cuticle development and chitin biosynthesis. One study, carried out on the space shuttle Challenger in 1985, found a pronounced decline in larvae hatched from the embryonic cuticle, as well as alterations in larval head, anterior, and thoracic cuticle segments [38]. Our lab’s research from Space Shuttle Discovery (STS-121, 2006) data revealed that defects in the pupal cuticle correspond with decreased eclosion rate. Many of the flies attempting eclosion rather died half-eclosed from the pupal case, suggesting that a structural change in the pupal case during spaceflight prevented the flies from properly eclosing (unpublished observations). The significant defects in the embryonic, larval, and pupal cuticle echoes the skeletomuscular system of mammals: like skeletomuscular tissues, insect cuticle is highly vulnerable to microgravity/spaceflight effects. In the present study, overrepresentation of cuticular genes due to hypergravity lends support to the phenotypes observed in the spaceflight experiments and suggests candidate genes affecting cuticle development in the pupae exposed to microgravity.

2.4.5. Proteolysis altered in hypergravity

During metamorphosis, active restructuring of organelles occurs in the early pupa. A perfect balance between protein degradation and synthesis must occur during this period [39]. Endopeptidases catalyze protein degradation, freeing amino acids that in turn can be recycled for protein biosynthesis. Changes in proteolytic gene expression (65 upregulated and 24 down-regulated) in hypergravity may indicate an imbalance in protein biosynthesis.
2.5. Additional transcriptional response in hypergravity

59 genes encoding mitochondrial ribosomal proteins were significantly downregulated. 37 large subunit (mRpL) and 22 small subunit (mRpS) ribosomal protein encoding genes were decreased in their expression in pupae developed at 3 g. Mitoribosomes are located in the mitochondrial matrix and synthesize proteins involved in oxidative phosphorylation [40]. The expression of genes coding for mitochondrial electron carrier activity, oxidative phosphorylation, and redox homeostasis were the largest group of genes altered in this study. Decrease in the expression of these genes could cause a cell-autonomous defect in protein biosynthesis in hypergravity.

Nine genes with isomerase activity related to cuticle pigmentation and melanin biosynthetic processes were altered in the hypergravity environment in pupae. Genes related to carbohydrate, lipid metabolic processes, protein glycosylation, glycolipid biosynthetic processes, catecholamine metabolism, acetyl-CoA biosynthetic processes, xenobiotic metabolism, ketone body catabolic processes and oligosaccharide biosynthetic processes were also altered in pupae developed in the hypergravity environment.

2.6. Hypergravity versus pro-oxidant–induced transcriptional response

It has been reported that hypergravity exposure is associated with oxidative stress [20]. We sought to discern which gene expression changes in our study were specific to hypergravity and which overlapped with other oxidative stress responses triggered by common pro-oxidants. We compared our data with Girardot et al., 2004, who studied transcriptional responses to common pro-oxidants such as paraquat and hydrogen peroxide [41]. While the Girardot et al. study used adult flies and our study deals with early pupae, oxidative stress response is conserved at a molecular level across the developmental stages and across species [42,43]. With this caveat, we compared our data to Girardot’s results. Genes that were affected by hypergravity and encode mitochondrial electron transport chain, redox, and oxidative phosphorylation proteins significantly overlapped with those induced by paraquat and hydrogen peroxide exposure. Chronic hypergravity might be inducing reactive oxygen species (ROS)–mediated oxidative stress in pupae similarly to pro-oxidants in adults. In contrast, genes encoding ion channel and transport proteins, cuticle development, and immune response overlapped the least with previously published data. This preliminary comparison of transcriptomic data suggests hypergravity may induce oxidative stress comparable to many of these pro-oxidants, but that also it has specific effects on mechanotransduction, cuticle development, and immune response.

To assure that these specific effects are unique to hypergravity, comparison of our data to environmental stress-induced transcriptional responses reported by Brown et al. [44] show that, while hypergravity response is slightly correlated to other stressors (Spearman between 0.27 for heat shock and −0.033 5 mM paraquat), the main responses elicited are particular to hypergravity (Supplemental Fig. S3). 782 of our genes were altered under stress conditions in Brown et al. including broad upregulation of cytochrome P450s across treatments (Supplemental Table S6). Correlation between our samples and the study’s homogenous response to environmental stressors’ could be affected by the latter’s use of adult flies versus our use of pupae.
3. Conclusions

We performed RNA-Seq on pupae in hypergravity to determine the most important factors in transcriptional response to abnormal gravity. We found 1513 genes differentially expressed in response to hypergravity, with 1038 of the genes falling into 13 functional categories. Using qRT-PCR, we were unable to find a linear relationship between transcript levels and magnitude of g force possibly because higher g-levels caused non-specific stress and decreased viability. However, we did find that increased gravitational force increases the rate of development. As the expression profile for hypergravity deviates from other known stress expression profiles, we conclude that this rate increase is a gravity-specific response. Further studies will inform this observation.

In planning this study, we expected to see changes in specific developmental pathways in D. melanogaster pupae since we had previously observed an increase in pupal lethality after spaceflight (Bhattacharya, unpublished data). We found a wide array of biological processes altered in response to hypergravity exposure. A possible explanation for this diverse transcriptomic response lies in the evolutionary adaptation of an organism like Drosophila melanogaster to Earth’s gravity. Organisms are constantly sensing and adapting to Earth’s different environmental cues. Specific genes therefore have evolved in concordance with a constant gravitational force of 1 g. Orienting themselves with respect to Earth’s gravity is an essential act of most multicellular organisms including plants, arthropods, and chordates. It is to be expected therefore that changes in gravity elicit responses in multiple pathways of an organism. As a result, the transcriptome in its entirety may respond quite diversely to cope with this change in gravitational force of three times its normal level. Thus, we see hypergravity affecting a broad range of biological processes in Drosophila melanogaster pupae.

4. Materials and methods

4.1. Drosophila culture and maintenance

Canton-S wild type flies were cultivated at 25 °C and 50% relative humidity on standard fly food (torula yeast 25.6 g; dextrose 103.2 g; cornmeal 48.8 g; agar as a solidifying agent 7.44 g; and tegosopt as an antifungal 18 mL/L H2O) with 12 h light–dark cycles. To generate synchronized 2 to 3 day old F1 flies, adult flies were allowed to lay for 48 h in a bottle, then parent flies were discarded, and the bottles with eggs were allowed to grow into adult flies at 25 °C. From the F1 generation, 2 to 3 day old flies were used for the chronic hypergravity studies using standard laboratory fly vials.

4.2. Hypergravity exposure

We define our hypergravity condition as 3 times the Earth’s gravitational acceleration (3 g). Hypergravity was mimicked using the standard centrifugation method (using a modified BECKMAN GS-6R centrifuge designed to attain low g levels - 97 RPM is equal to 3 g). For chronic hypergravity exposure, 20 female and 20 male synchronized 2 to 3 day old flies were allowed to lay eggs while being maintained in the 3 g environment. Centrifugation was briefly stopped to discard the parent flies after 24 h of egg laying, and then immediately continued until the eggs developed into P6-staged pupae. P6-stage of pupae typically lasts between 25 and 43 h after pupation initiation (white puparium stage). We ascertained this stage by isolating pupae at 40 h after pupation, which is well within the P-6 stage. The development of green malpigian tubules is the hallmark of this stage, and we confirmed this by dissecting representative pupae. Similarly, 1 g controls were maintained in the dark, with the same angle of vial orientation so as to mimic the hypergravity-exposed flies. It is not possible to sex pupae at stage P6, as the differentiating male sex combs do not darken and become visible until stage P12 [45]. We considered sexing at the larval stage by looking for the enlarged male gonad and then separating the sexes into separate vials to develop, but we were concerned that the time taken to do this would interrupt the prolonged 3G-exposure environment we were creating. Therefore we used downstream statistical methods to confirm that our gene expression results correlated with hypergravity treatment and not to the sex of the organism, and is described in Section 2.2.

4.3. Total RNA isolation and RNA-Seq library preparation

Post-hypergravity exposure, P6-staged early pupae were collected. Using Qiagen RNeasy mini kit, total RNA was extracted from four pooled pupae for both conditions (3 g and 1 g), and in addition four such independent biological replicates were processed to ensure statistical reliability. Total RNA for these 8 samples (4 from each condition) were treated with DNase (tURboDNase) and prepared for sequencing (Illumina mRNA TruSeq vol 2 kit) as per the manufacturer’s protocol. Size distribution and concentration of DNA fragments were measured using chip electrophoresis (Agilent Bioanalyzer) and fluorometer (Qubit 2.0). Indexed libraries were pooled (8 samples total) and sequenced on one flowcell lane (Illumina HiSeq 2000) to obtain 100-base paired-end reads.

4.4. Transcriptome analysis

Raw sequenced reads were filtered to remove reads not passing Illumina’s Casava quality score, and then trimmed (Trimmmomatic) [10] to remove adapters and low-quality reads. Trimmed reads were aligned to the Drosophila melanogaster reference transcriptome build R6.02 (Bowtie 2, default settings) [Supplemental Table S7]. Aligned reads were subsequently quantified (eXpress). The transcript TPMs were merged to gene level and scaled up (×100) for downstream analysis. Differential expression analysis was performed (DE-Seq2) to measure fold change and significance between 1 g and 3 g conditions. Genes with q-values ≤0.05 were considered significantly differentially expressed. Pathway analysis was performed to determine significant affected processes (PANTHER). Using the Gene Ontology hierarchical structure, where specialized ‘child’ terms are nested within broader ‘parent’ terms, we grouped terms that had the same molecular functions and biological processes. PANTHER statistical overrepresentation tests for biological processes and molecular functions were performed with the control samples’ genes as reference. PANTHER statistical enrichment tests were performed for biological processes and molecular functions using default settings. All statistical tests were corrected for multiple testing using the Bonferroni method. Results of these tests are detailed in Supplemental Tables S2–S5. Gene network figures were generated using GeneMANIA [46]. These networks provide information on genetic interactions, physical interactions, coexpression, colocalization, and shared protein domains between genes. Software versions and settings in Supplemental Table S8.

4.5. qRT-PCR

Based on GO analysis, we selected 18 genes representing various pathways for validation by qRT-PCR. Relative gene expression levels were quantified (7500 Real Time PCR instrument from Applied Biosystems, and SYBR green PCR kit, QuantiTect, Qiagen). Primers were designed (Primer–Blast, NCBI). Real-time cyclers conditions were; 95 °C for 15 min for initial activation step, and 40 cycles of 94 °C for 15 s, 50 °C for 30 s, and 72 °C for 30 s for denaturation, annealing, and extension respectively. Data was normalized to the reference gene alpha tubulin 84B. The comparative cycle threshold (CT) method was used for data analysis. Primers used for target genes listed in Supplemental Table S9. Results for qRT-PCR tests on samples exposed to 3 g, 5 g and 8 g are provided in Supplemental Table S10. Exposure to 3 g, 5 g and 8 g hypergravity levels were conducted as described previously.
in Section 4.2 and corresponded to 97 rpm, 161 rpm and 258 rpm set-
tings respectively on the modified BECKMAN GS-6R centrifuge.

Declarations

Availability of data and material

The datasets supporting the conclusions of this article have been de-
posited in NCBI’s Gene Expression Omnibus [47] and are accessible
through GEO Series accession number GSE80323 (https://www.ncbi.
nlm.nih.gov/geo/query/acc.cgi?acc=GSE80323). Other supporting
material is available within the article and its supplemental files.
Supplementary data to this article can be found online at http://dx.

Competing interests

Authors state that they have no competing interests.

Authors’ contributions

SH, RH, and SB wrote the manuscript text. RH and SRB carried out
the experiments. SH performed the sequencing library preparation. SH
performed bioinformatic analysis including QC, mapping, expression es-
timates, and differential expression analysis; pathway analysis was per-
formed jointly by SH and RH. RH and SRB performed qRT-PCR and
responding analysis. SB and LP supervised the research and edited
the final manuscript. All authors read and approved the final manuscript.

Funding and acknowledgements

This work was funded by NASA grants to SB (NNX15AB42G and
NNX13AN38G). RH was supported by a NASA Post-Doctoral Program
(NPP) Fellowship. SH was supported by the NSF Graduate Research Fel-
nership. SH was supported by the NSF Graduate Research Fel-

References

or/10.1146/annurev.ento.08.100163.000523.

1016/S0891-9527(00)00317-8.


[4] E. Le Bourg, Combined effects of two mild stresses (cold and hypergravity) on lon-


or/10.1016/S0261-5317(02)00938-3.

[10] A. Roberts, L. Pachter, Streaming fragment assignment for real-time analysis of se-


[12] A. Roberts, L. Pachter, Streaming fragment assignment for real-time analysis of se-

1016/S0891-9527(00)00317-8.

[14] E. Le Bourg, Combined effects of two mild stresses (cold and hypergravity) on lon-


[17] É. Le Bourg, Combined effects of two mild stresses (cold and hypergravity) on lon-


[19] E. Le Bourg, Combined effects of two mild stresses (cold and hypergravity) on lon-

[20] M. Goldermann, W. Hanke, Ion channel are sensitive to gravity changes, Micrograv-

or/10.1016/S0261-5317(02)00938-3.

[22] Z. Cheng, G. Bao, N. Wang, Cell mechanics: mechanical response, cell adhesion, and
defences? A study of superoxide dismutase and catalase in Drosophila melanogaster


[25] Y.S. Kolesnikov, S.V. Kretovin, N. Wang, Cell mechanics: mechanical response, cell adhesion, and
defences? A study of superoxide dismutase and catalase in Drosophila melanogaster


[27] M. Goldermann, W. Hanke, Ion channel are sensitive to gravity changes, Micrograv-

[28] M. Goldermann, W. Hanke, Ion channel are sensitive to gravity changes, Micrograv-

[29] M. Goldermann, W. Hanke, Ion channel are sensitive to gravity changes, Micrograv-

[30] M. Goldermann, W. Hanke, Ion channel are sensitive to gravity changes, Micrograv-

[31] M. Goldermann, W. Hanke, Ion channel are sensitive to gravity changes, Micrograv-

[32] M. Goldermann, W. Hanke, Ion channel are sensitive to gravity changes, Micrograv-


[34] M. Goldermann, W. Hanke, Ion channel are sensitive to gravity changes, Micrograv-

[35] M. Goldermann, W. Hanke, Ion channel are sensitive to gravity changes, Micrograv-


[37] K. Taylor, K. Kleinhesselink, M.D. George, R. Morgan, T. Smallwood, A.S. Hammonds,

[38] M. Goldermann, W. Hanke, Ion channel are sensitive to gravity changes, Micrograv-

[39] M. Goldermann, W. Hanke, Ion channel are sensitive to gravity changes, Micrograv-

[40] M. Goldermann, W. Hanke, Ion channel are sensitive to gravity changes, Micrograv-

[41] M. Goldermann, W. Hanke, Ion channel are sensitive to gravity changes, Micrograv-

[42] M. Goldermann, W. Hanke, Ion channel are sensitive to gravity changes, Micrograv-

[43] M. Goldermann, W. Hanke, Ion channel are sensitive to gravity changes, Micrograv-

[44] M. Goldermann, W. Hanke, Ion channel are sensitive to gravity changes, Micrograv-

[45] M. Goldermann, W. Hanke, Ion channel are sensitive to gravity changes, Micrograv-


