

1 **Title: Reliance on polyfunctional tissue leads to a reproduction-immunity tradeoff due to**  
2 **inherent constraint**

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13

14 **Abstract:**

15 The use of one tissue for multiple purposes can result in constraints, impaired function,  
16 and tradeoffs. The insect fat body performs remarkably diverse functions including metabolic  
17 control, reproductive provisioning, and systemic immune responses. Immunity and reproduction  
18 are observed to trade off in many organisms, although the mechanistic basis for the tradeoff is  
19 generally unknown. More generally, how do polyfunctional tissues simultaneously execute  
20 multiple distinct physiological functions? Using single-nucleus sequencing, we determined the  
21 *Drosophila melanogaster* fat body executes diverse basal functions with heterogenous cellular  
22 subpopulations. However, as an emergency function, the immune response engages the entire  
23 tissue. We found that reproductively active females exhibit impaired capacity to produce new  
24 protein in response to infection, resulting in the reproduction-immunity tradeoff. We suggest that  
25 such inherent internal limitations may provide a general explanation for the wide prevalence of  
26 physiological and evolutionary tradeoffs.

27

28 **Introduction**

29 The need to balance multiple physiologically demanding and resource-intensive  
30 processes limits the ability of an organism to maximize performance in any one area. When two  
31 or more processes depend on a single tissue or resource pool, they unavoidably constrain each  
32 other, resulting in tradeoffs between the associated traits. Such tradeoffs are central to life  
33 history theory and affect the health, fitness, and evolution of all living organisms (1–3).

34   Reproduction and immunity are two traits that trade off with each other across a broad diversity  
35   of systems (4,5) but the mechanisms and physiological constraints that underlie this tradeoff are  
36   poorly understood. In *Drosophila melanogaster* females, mating results in a rapid,  
37   endocrinologically-mediated drop in resistance to bacterial infection (6). We hypothesized that  
38   this tradeoff arises due to physiological constraints of using the same tissue, the abdominal fat  
39   body, for both reproductive investment and systemic immunity, and that understanding the basis  
40   for this tradeoff could serve as a model for understanding constraints on polyfunctional tissues  
41   in general.

42           The insect fat body is a highly multifunctional tissue that is engaged in central metabolic  
43   regulation, nutrient storage, detoxification of xenobiotics, reproductive egg provisioning, and  
44   mounting of systemic immune responses (7). Thus, this single tissue performs the functions of  
45   several vertebrate organs. The fat body is remarkably dynamic. For example, a bacterial  
46   infection significantly changes the expression of several hundred genes in the fat body of  
47   *Drosophila melanogaster*, including as much as 1000-fold induction of genes encoding  
48   antimicrobial peptides and marked down-regulation of glycolytic and basal metabolic pathways  
49   (8–10). Upon mating and sperm storage, the same tissue significantly upregulates genes  
50   involved in egg provisioning as the females increase their investment egg production (7).  
51   Reproduction and immune responses are both energetically demanding (11) and a female may  
52   need to simultaneously execute these processes as well as others. Given the finite number of  
53   cells and limited capacity for transcription and translation within each cell, how does one tissue  
54   achieve so many functions at once? Is the tissue composed of specialized subpopulations of  
55   cells that are individually devoted to each function? Or do all cells of the tissue perform all  
56   functions to a limited degree? When the tissue responds to stimulus, do the identities or sizes of  
57   cellular subpopulations change, or does each cell of the tissue alter its transcriptional profile in  
58   concert? Does the simultaneous execution of multiple processes by the single tissue constrain  
59   performance of each process?

60           To begin address these questions, we performed single-nucleus RNA sequencing  
61   (snRNAseq) on the fat bodies of *D. melanogaster* females in a replicated factorial design  
62   combining mating and bacterial infection. Mature adult female *D. melanogaster* were either  
63   mated in order to activate reproductive investment (M\_) or held as virgin to limit reproductive  
64   investment (V\_), and were either given a systemic bacterial infection with *Providencia rettgeri* to  
65   stimulate an immune response (\_I) or were held uninfected (\_U). We observed significantly  
66   lower survivorship of Mated-Infected (MI) females than Virgin-Infected (VI) females over 3 days

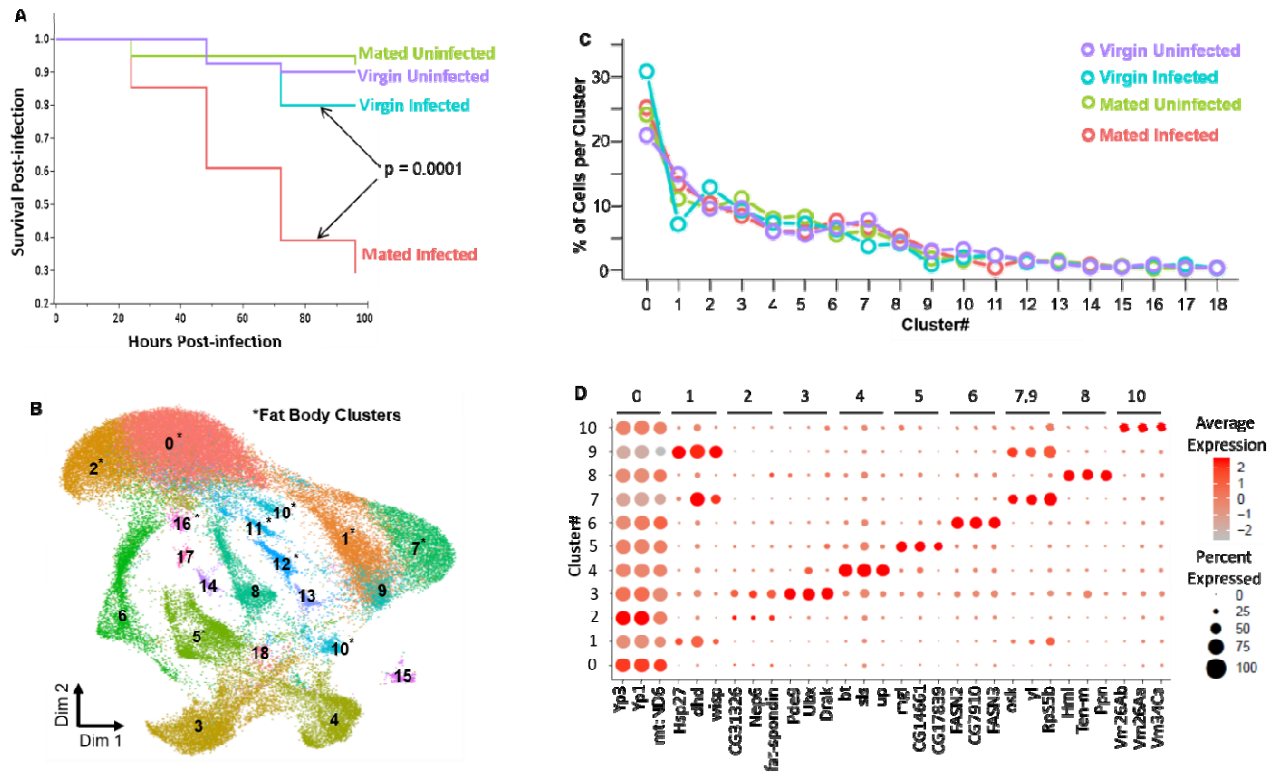
67 post-infection ( $p = 0.0001$ ; Fig. 1A), in accordance with previous observations (12,13) and  
68 demonstrating the expected tradeoff. We repeated each factorial treatment (VU, VI, MU, MI) in  
69 two independent biological replicates to generate a total of 8 samples for snRNAseq. From each  
70 sample, we dissected and pooled fat bodies from the abdomens of 40 female flies. The gut and  
71 ovaries are easily removed from the fat body tissue, but other cell types such as oenocytes,  
72 muscle cells, and hemocytes are harder to separate from fat body tissues and thus are co-  
73 isolated. We purified individual nuclei from the pooled tissues using a Dounce homogenizer  
74 followed by centrifugation onto a sucrose cushion (14). We performed snRNAseq using the 10X  
75 Genomics Chromium platform, loading at least 7000 nuclei per sample and sequencing at least  
76 16,000 reads per nucleus for a minimum of 112 million reads per sample.

77 We identified 19 expression clusters representing distinct cellular subpopulations (Fig.  
78 1B, Fig. S1, S2) with 90% of the nuclei present in the eleven most abundant clusters (Fig. 1C).  
79 We assigned putative functional identities to each cluster based on the significantly high  
80 expression ( $p\text{-adj} < 0.01$ ) of diagnostic marker genes. Expression of top marker genes for the  
81 first eleven clusters is shown in Fig. 1D, and a full list of key expressed genes can be found in  
82 Table S1 and is illustrated in Fig. S3. The Supplementary Online Material contains detailed  
83 descriptions of the expression patterns and inferred functions for each cluster. We found  
84 significantly high expression of marker genes that are conventionally associated with fat body in  
85 six major clusters: 0, 1, 2, 5, 7 and 10. These six clusters contain approximately 60% of all the  
86 nuclei sequenced and demonstrate that the fat body tissue is composed of heterogeneous cell  
87 subtypes. An additional 5% of nuclei map to low-abundance fat body clusters (Clusters 11, 12,  
88 13, and 16). Clusters 0 and 2 were defined by high expression of *yolk proteins 1* and 3 (Fig.  
89 1D), while cluster 1, 5, 7 and 10 respectively had high expression of *deadhead*, *megalyn*, *oskar*,  
90 and *vitelline membrane 26Ab*. All these marker genes are associated with oogenesis and egg  
91 development. Remarkably, the relative size of these clusters did not change significantly across  
92 the four treatment groups (Fig. 1C), indicating that the fat body does not respond to mating or  
93 infection by shifting the proportional representation of these specific cellular subpopulations. We  
94 infer that the remaining 35% of nuclei do not come from fat body tissue. Based on previously  
95 well-characterized cell-specific transcriptional markers, we determined that Cluster 4 is muscle  
96 (7% of sequenced nuclei), Cluster 6 is oenocytes (7%), Cluster 8 is hemocytes (5%), and  
97 Cluster 9 is uncharacterized (2%). Cluster 3 (10% of sequenced nuclei) remains  
98 uncharacterized but shows properties similar to both fat body and pericardial cells (see detailed

99 description in Supplement, Fig. S4). These tissues are physically contiguous with the fat body,  
100 and interact with and have partially overlapping functions with the fat body tissue (15,16).

101       Upon mating, *D. melanogaster* females store sperm and begin to lay fertilized eggs,  
102 which requires increased investment in oogenesis (17). We asked whether the investment in  
103 reproduction varied across the six distinct subpopulations of the fat body tissue by cluster-  
104 specific differential gene expression analysis. When comparing virgin and mated females (24  
105 hours post-mating) in the absence of infection, we found 186 differentially expressed genes  
106 across the six clusters with 145 genes significantly upregulated and 41 genes significantly  
107 downregulated (FDR <0.01; Table S2). We observed that none of the 186 genes were  
108 differentially regulated across all the six subpopulations (Fig. S5A) while 123 (66%) of these  
109 genes were differentially regulated in only one of the six subpopulations (Fig. S5A). For  
110 example, egg provisioning genes *yp1* and *yp3* were upregulated across four different clusters  
111 (Fig. S6A) while *yp2* was upregulated in only one cluster (Table S2). This indicates that the  
112 response to and investment in mating is heterogenous across fat body subpopulations. GO  
113 enrichment analysis of differentially regulated genes in each of the six subpopulations showed  
114 enrichment for diverse functions (Table S3). Upregulated genes in both Clusters 0 and 1 were  
115 enriched for one-carbon metabolism but mediated by two different mechanisms: s-adenosyl  
116 methionine (SAM; Cluster 0) and folate (Cluster 1). Cluster 1 also showed enriched upregulation  
117 of genes encoding ribosomal proteins, which were downregulated in Cluster 2. Upregulated  
118 genes in Cluster 2 showed enrichment for amino acid biosynthesis. We identified metabolic and  
119 detoxification pathways enriched in genes upregulated in Cluster 5, and upregulated genes in  
120 both Clusters 7 and 10 were related to phospholipase A1 activity. Therefore, while all six fat  
121 body subpopulations respond to mating stimulus, their heterogeneous response suggests  
122 subfunctionalization of the cellular populations.

123



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125

126 **Fig. 1. Single-nucleus sequencing of *Drosophila* fat body tissue**

127 (A) Cox proportional hazard analysis showed that the mated *Drosophila melanogaster* females  
 128 have significantly lower survival than virgin females ( $n = 40$ ;  $p = 0.0001$ ) after infection with the  
 129 Gram-negative bacterium *Providencia rettgeri*. Survival of uninfected virgin and mated females  
 130 was not different over four days. (B) Combined Uniform Manifold Approximation and Projection  
 131 (UMAP) of 56,000 nuclei from two replicates each of Virgin Uninfected, Virgin Infected, Mated  
 132 Uninfected, and Mated Infected colored by their treatment identity. Clusters 0,1,2,5,7, and 10  
 133 marked with asterisk (\*) represent subpopulations of the fat body tissue. (C) Percentage  
 134 distribution of nuclei from four treatments (Virgin Uninfected, Virgin Infected, Mated Uninfected,  
 135 and Mated Infected) across 19 clusters. All clusters are present in constant proportion across all  
 136 four treatments. (D) Dot Plot showing expression of marker genes per cluster for top eleven  
 137 clusters. Average expression of a marker gene in a cluster is represented by gradient of the  
 138 colored dot and dot size represents the cell percentage per cluster expressing the marker.

139

140 The fat body mounts an intense and rapid immune response to bacterial infection (9,18)  
 141 so we asked whether the whole tissue is engaged in that response or whether it maps to a  
 142 restricted set of subpopulations. The answer, interestingly, is both. All clusters showed  
 143 significant upregulation of immune response genes in both mated and virgin females, including

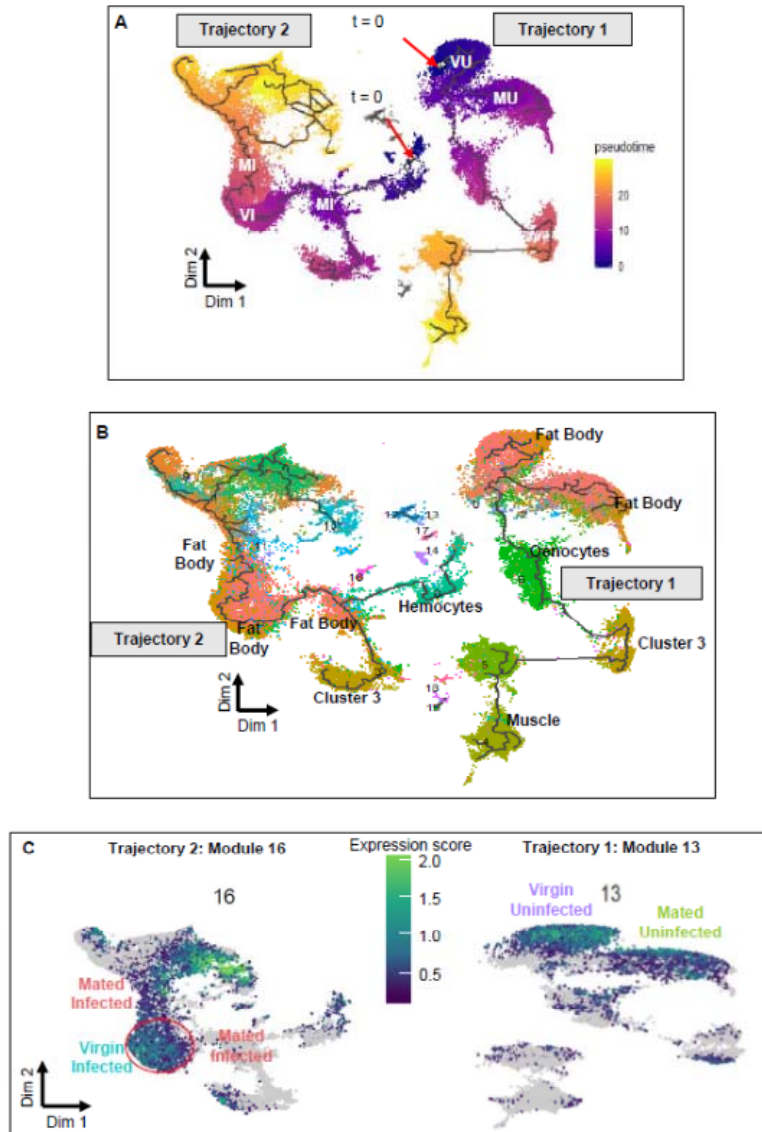
144 genes that encode secreted antimicrobial peptides (Figs.S6B, S6C). However, the precise  
145 expression patterns were heterogeneous after infection, with particular combinations of immune  
146 genes induced most strongly in different subsets of clusters. Across the six major fat body  
147 subpopulations, 47 genes were induced by infection in both virgin and mated females. However,  
148 twice as many genes showed significant induction after infection in virgin females than in mated  
149 females (124 versus 63, FDR <0.01; Table S4, S5), indicating a negative impact of mating on  
150 the transcriptional response to infection. We found three genes (*attacin A*, *CG42807*, and  
151 *CG14322*) to be upregulated across all six fat body subpopulations in virgins (Table S4, Fig.  
152 S5B) while no genes were upregulated across every subpopulation in mated females (Table S5,  
153 Fig. S5C). Around 11% genes were upregulated in 4 or more of the six subpopulations in virgins  
154 compared to 6% in mated females. To understand the functional heterogeneity of the genes  
155 expressed in each cluster, we performed cluster-specific GO enrichment analysis of the genes  
156 that are differently expressed after infection in mated and virgin females separately (Table S6).  
157 Protein processing and secretion was a significantly enriched function of upregulated genes in  
158 Clusters 0 and 2 in both virgin and mated flies. Cluster 2 in virgin infected females also showed  
159 enrichment of genes for phagosome formation (Table S6). Downregulation of ribosome  
160 constituents was observed in Cluster 1 of mated flies and Cluster 2 of virgin females. We  
161 observed that 54% of differentially expressed genes in virgins and 65% of differentially  
162 expressed genes in mated were differentially regulated in only one of the six subpopulations  
163 (Figs.S5B, S5C). These data reveal heterogeneity in infection response across the fat body and  
164 demonstrate that the tissue-wide transcriptional response to infection is dampened by mating,  
165 probably contributing to the tradeoff between reproduction and immunity.

166 Most of the mating- and infection- induced transcriptional changes were heavily driven  
167 by clusters 0, 1, and 2 (Table S7), representing ~70% of all the nuclei from the six fat body  
168 subpopulations. We hypothesized that the involvement of such a large majority of fat body cells  
169 in resource-intensive physiological functions might constrain resource allocation, which could be  
170 reflected in coordinated regulation of gene expression networks or modules. To identify these  
171 modules, we constructed pseudotime trajectories from all the four treatments with Monocle (19–  
172 21), representing the transition of cells between differential functional states in response to  
173 mating or infection. An initial analysis revealed that the infected and uninfected fat body cells  
174 resolved into two completely disjointed trajectories defined by infection status. Trajectory 1  
175 contained a majority of nuclei from VU and MU treatments while Trajectory 2 contained a  
176 majority of nuclei from VI and MI (Fig. 2A, Fig. S7). This suggests that fat body cells rapidly and

177 dramatically change expression profile upon infection with no intermediate states visible at the  
178 6-hour post-infection sampling time point. Only fat body cells (inferred using Seurat-based  
179 cluster analysis) were present in both of these trajectories (Fig. 2B). Other co-isolated cell types  
180 were present in only one of the two trajectories; indicating that they are not strongly  
181 transcriptionally responsive to infection. Using Louvain clustering in the two trajectories, we  
182 identified several modules of co-regulated genes that were enriched for specific functional  
183 ontologies. In Trajectory 1, we identified a module (Module 13, Fig. 2C, Table S8) with low  
184 aggregate expression score that was enriched in ribosome biogenesis (Fig. S8) in mated  
185 uninfected nuclei (MU) relative to virgin uninfected nuclei (VU), including CAP-dependent  
186 translation initiation factors. Surprisingly, the same set of genes (Module 16, Fig. 2C, Table S9)  
187 with the addition of one gene (*O-fucosyltransferase 2*) had a low aggregate expression score in  
188 mated infected (MI) nuclei contrasted to virgin infected nuclei (VI) in Trajectory 2 (Module 16).  
189 This suggests that protein synthesis might be reduced in the fat body after mating due to  
190 suppressed ribosome biogenesis. Furthermore, a subset of MI nuclei showed high expression of  
191 a module enriched in protein folding and degradation (Fig. S9), including genes involved in ER  
192 stress and unfolded protein response (UPR; Table S10). Electron microscopy confirmed dilated  
193 ER membranes in MI fat bodies (Fig. 3), indicative of ER stress (22). Since alleviation of ER  
194 stress is often attained via suppression of ribosome biogenesis to limit protein synthesis in the  
195 cell (22,23), a key factor underlying the observed reproduction-immunity tradeoff could be  
196 reduced capacity to produce immune-related proteins in mated females due to reduced protein  
197 synthesis.

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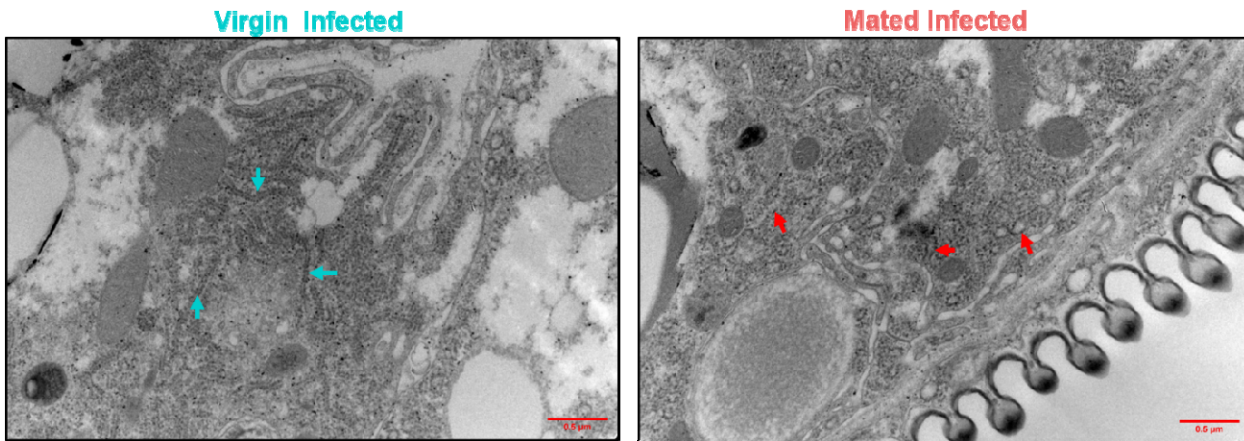
201 **Fig. 2 Pseudotime analysis showing differentially expressed gene modules**

202 (A) Pseudotemporal ordering of nuclei along the two trajectories calculated from trajectory-specific  
203  $t = 0$  points. Nuclei from four treatments (Virgin Uninfected (VU), Virgin Infected (VI),  
204 Mated Uninfected (MU), and Mated Infected (MI)) separate at different pseudo-time scales. The  
205 trajectories from infected nuclei are completely disjoint from the trajectories of uninfected nuclei,  
206 revealing a rapid and dramatic response to infection. (B) Monocle-based trajectory analysis  
207 separated nuclei along the two trajectories; colored by their cluster identity (from Figure 1B)  
208 show that only Fat Body nuclei are present in both trajectories. Other cell types such as  
209 oenocytes and muscle cells are present in Trajectory 1 and hemocytes are present in Trajectory  
210 2, indicating these cell types do not have a strong transcriptional response to infection. (C)  
211 UMAP of Module 13 (Trajectory 1) and Module 16 (Trajectory 2) showing low gene aggregate  
212 expression scores for Mated Uninfected (Trajectory 1) and Mated Infected (Trajectory 2)  
213 compared to Virgin Uninfected and Virgin Infected respectively. Gradient of color represents the  
214 aggregate expression score with bright color indicating higher aggregate expression score.



215 Each dot represents a single nucleus. GO term analysis showed enrichment for ribosome  
216 biogenesis in the two modules (Tables S8, S9, Fig. S8).

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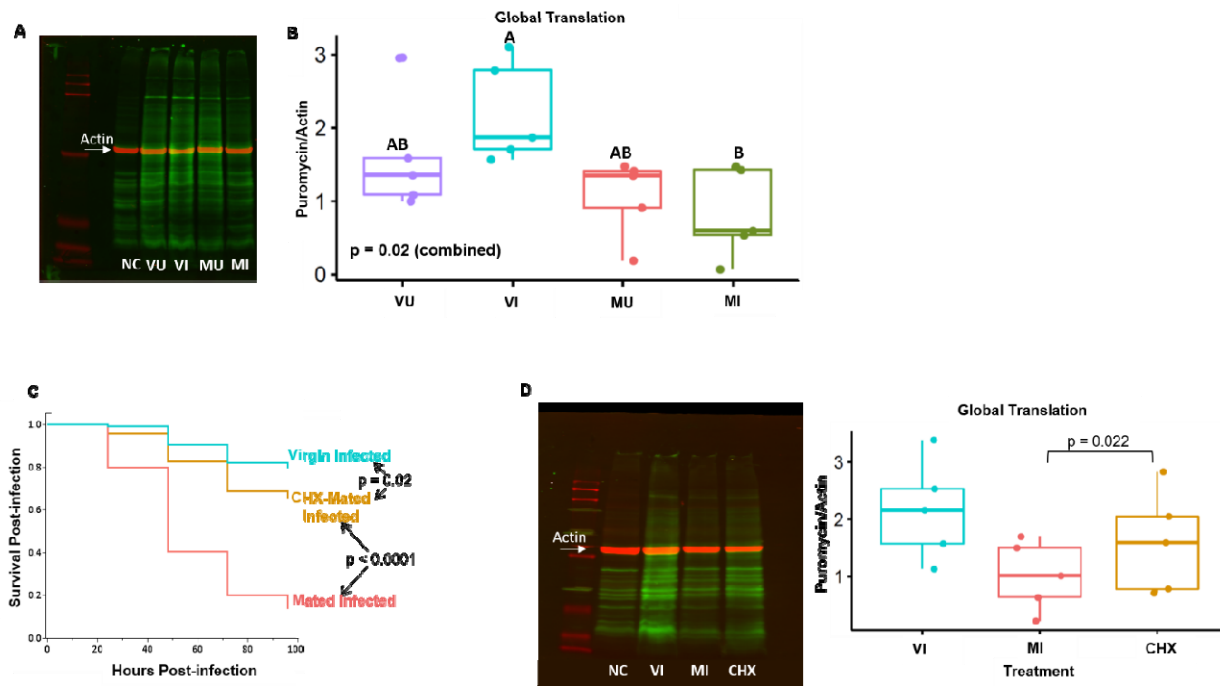
219 **Fig. 3 Electron micrographs of endoplasmic reticulum in the fat body (ER)**

220 Representative image (n = 4-5 images per treatment) showing dilation of ER membrane  
221 indicative of ER stress (right panel, red arrows) observed in fat body cells from Mated Infected  
222 females. Blue arrows show constricted ER membranes in Virgin Infected samples.

223

224 To test our hypothesis that mated infected (MI) females may lack sufficient capacity for  
225 translation in support of a full immune response to infection, we measured global protein  
226 synthesis in fat body tissues representing each of the four different treatments. We re-generated  
227 new female flies from each of the four factorial mating and infection treatments, dissected their  
228 fat bodies, and applied puromycin incorporation to label nascent polypeptides. Incorporated  
229 puromycin was then quantified on Western blots (20,21). We observed significant variability in  
230 global synthesis rates across the four treatments (one-way ANOVA,  $p=0.02$ , Table S11) with a  
231 spike in protein synthesis after infection in virgin females (VI) (Mean = 2.2, S.D. = 0.69) that fails  
232 to occur in mated (MI) females (Mean = 0.8, S.D. = 0.61) (Tukey's HSD,  $p = 0.0005$ , Fig. 4A,  
233 4B). These data are consistent with the reduction in ribosome biogenesis inferred from the  
234 sequencing data and with the hypothesis that the fat bodies of MI females are deficient in  
235 translation capacity. As the rapidity of an induced immune response is a critical determinant of  
236 infection outcome (22,23), a quantitative reduction or delay in the translation of immune  
237 response proteins such as antimicrobial peptides could contribute to the observed increased  
238 risk of death from infection in mated females.

239 Since we observed a reduction in protein synthesis in mated infected (MI) flies compared  
240 to all other groups, and especially compared to virgin infected (VI) females (Fig. 4B), we  
241 hypothesized that the high demand of producing reproduction-related proteins in mated females  
242 reduced capacity to translate new proteins in response to infection, even leading to ER stress in  
243 MI females. We predicted that the reproduction-immunity tradeoff could be alleviated if  
244 translational investment in reproductive proteins was reduced. To test this hypothesis, we mated  
245 females and then placed them on food containing cycloheximide (CHX) for 18 hours. CHX  
246 reversibly suppresses production of proteins in eukaryotes such as *Drosophila* (24). We  
247 subsequently transferred flies to food without CHX for six hours to allow them to clear the drug,  
248 and then gave them bacterial infections. Females that were treated with CHX after mating  
249 survived infection significantly better than mated females that were not treated with CHX  
250 ( $p < 0.0001$ , Fig. 4C). We also observed an increase in post-infection protein synthesis in mated  
251 females pre-treated with CHX compared to non-treated females at six hours after infection ( $t(4)$   
252 = 3.63,  $p = 0.02$ , Fig. 4D, Table S12). Additional experiments confirmed that CHX has no direct  
253 role in the survival of infection (Fig. S10, see Supplemental Material for experimental details).  
254 Therefore, the observed tradeoff between reproduction and immunity is determined to be due to  
255 limited capacity for immune-related protein synthesis as a consequence of prior reproductive  
256 investment.



257

258 **Fig. 4 Effect of mating and infection on protein synthesis**

259 (A) Representative image of puromycin incorporation in nascent polypeptides of the fat body  
260 tissue detected using anti-puromycin antibody and Western Blotting. Secondary antibodies  
261 labelled with different fluorophores detected puromycin (Green, 800nm) and Actin (Red, 700  
262 nm). The fat bodies from Mated Infected (MI) produce noticeably less protein than those of the  
263 other treatments. Virgin Uninfected (VU), Virgin Infected (VI), Mated Uninfected (MU), and  
264 Mated Infected (MI) represent the four treatments. Negative Control (NC) (Figure A and D)  
265 shows proteins from fat body tissues which were not incubated with puromycin. (B)  
266 Quantification of relative protein synthesis using puromycin incorporation from four treatments  
267 (VU, VI, MU, and MI) ( $n = 5, 10$  flies per treatment). Treatments not connected by same letter  
268 are significantly different (Tukey's HSD,  $p < 0.005$ ). Virgin Infected females synthesize  
269 significantly more protein than Mated Infected females. (C) Cox proportional hazard analysis  
270 showing rescued post-infection survival ( $p < 0.0001$ ) of cycloheximide (CHX) pre-treated mated  
271 females (CHX- Mated Infected) compared to non-treated Mated Infected ( $n = 35-40$  flies per  
272 treatment per replicate, three independent replicates). (D) Representative Western Blot image  
273 of puromycin incorporation from CHX pre-treatment of mated females (CHX). Results suggest  
274 CHX treatment partially rescues protein synthesis in response to infection compared to non-  
275 treated mated females (MI) (Paired Student's t-test,  $p = 0.02$ ). Puromycin incorporation was  
276 measured after six hours of infection.

277 The impaired ability of the fat body to synthesize proteins in response to infection while  
278 simultaneously investing in reproduction illustrates a tradeoff driven by physiological constraint.  
279 The fact that immunity can be partially rescued with CHX treatment suggests the potential for  
280 plasticity in this tradeoff. Flies could, in theory, sustain greater immune capacity by reducing  
281 their commitment to reproductive investment. Thus, genetic variation for reproductive  
282 investment could allow evolutionary adaptation to infection risk. Reduced translation specifically  
283 in the fat body (25) extends lifespan in flies (26) through evolutionarily conserved mechanisms  
284 (27) observed across other organisms such as *C. elegans* (28,29) and mouse (30), whereas  
285 mating and reproduction are costly and negatively affects lifespan in fruit flies and other  
286 organisms (31). Translation in the fat body could therefore additionally be a mechanism  
287 mediating reproduction-longevity tradeoff. It seems likely that environmental factors, such as  
288 amino acid nutrition, may also influence the shape of these tradeoffs.

289 Managing competing physiological demands is a critical challenge for any polyfunctional  
290 tissue. We find here that the *Drosophila melanogaster* fat body executes diverse basal functions  
291 via heterogeneous cellular subpopulations. However, the whole tissue becomes engaged in an  
292 immune response. The gene expression markers that we have identified as defining the cellular  
293 subpopulations can serve to develop tags for future research into the dynamism and spatial  
294 structure of the *Drosophila* fat body. The fat body is a remarkable tissue that is highly  
295 responsive in regulating multiple aspects of physiology. However, while the fat body is  
296 enormously flexible, the shared reliance of multiple functions on a single tissue will inherently

297 lead to constraints and tradeoffs. As we have shown in defining a reproduction-immunity  
298 tradeoff, compound stresses can overwhelm the tissue and lead to adverse outcomes.  
299 Understanding strategies that polyfunctional tissues use for balancing critical functions at the  
300 whole-tissue and sub-tissue levels can elucidate general mechanisms of physiological and  
301 evolutionary tradeoffs that underpin life history theory.

302

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