

Cell cycle re-entry in the aging *Drosophila* brain.

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Abstract:

The brain is an organ comprised mostly of long-lived, quiescent cells that perform vital functions throughout an animal's life. Due to the brain's limited regenerative ability, these long-lived cells must engage unique mechanisms to cope with accumulated damage over time. We have shown that a subset of differentiated neuronal and glial cells in the fruit fly brain become polyploid during adulthood. Cell cycle re-entry in the brain has previously been associated with neurodegeneration, but there may be a more complex relationship between polyploidy and cell fitness in the brain. Here, we examine how known lifespan modifiers influence the accumulation of polyploidy in the aging fly brain. Flies aged at a low temperature, or with a low protein diet, accumulate polyploid cells in the brain more slowly than expected if this phenotype were solely regulated by lifespan mechanisms. Despite the slower accumulation of polyploid cells, animals under conditions that extend lifespan eventually reach similar levels of polyploidy in the brain as controls. Our work suggests known lifespan modifiers can influence the timing of cell cycle re-entry in the adult brain, indicating there is a flexible window of cell cycle plasticity in the aging brain.

Introduction:

Cells in the brain accumulate DNA damage during aging which can lead to cell death when too severe (Fishel et al., 2007; Folch et al., 2012; Kruman, 2004). Consequently, cell death triggered from age related DNA damage contributes to neurodegeneration (Folch et al., 2012). This is problematic because the brain is comprised mostly of cells thought to be in a permanent, non-proliferative state of G0 (Aranda-Anzaldo, 2012; Aranda-Anzaldo & Dent, 2017). Therefore, other mechanisms besides proliferation may compensate for cell loss in the aging brain.

Cells in G0 are metabolically active and carry out various physiological functions but are not actively cycling. As research in the cell cycle field has progressed, flexibility in the G0 state has been realized, even for terminally differentiated cells in vivo (Borowik et al., 2023; Losick, 2016; Ma et al., 2019; Nandakumar et al., 2021; Yao, 2014; Zanet et al., 2010). In some cases, terminally differentiated cells can re-enter the cell cycle and undergo variant cell cycles to compensate for tissue damage, demonstrating surprising plasticity in the cell cycle and G0 state (Cohen et al., 2018; Dehn et al., 2023).

The flexibility of G0 in differentiated cells has not been extensively examined in the brain. It is generally believed that cells in an adult brain, especially neurons, are in a permanent G0 state (Folch et al., 2012; Kruman, 2004). Despite being in a stable non-proliferative state, multiple studies report cells with a more than 2C DNA content (polyploid cells) in the brain and variant cell cycle gene reactivation (Jungas et al., 2020; Khurana & Feany, 2007; Lu et al., 2004; McCarroll et al., 2004; Sigl-Glockner & Brecht, 2017; Sosunov et al., 2020; Yang & Herrup, 2007). This suggests cells in the brain,

47 including neurons, thought to be in a stable G0 state, retain the ability to re-enter the cell
48 cycle. Consistent with this, we have shown that under normal, physiological conditions,
49 the adult fly brain accumulates a small proportion of polyploid cells, likely due to cell
50 cycle re-entry in adults (Nandakumar et al., 2020). The presence of polyploid terminally
51 differentiated cells in the aged brain under normal and diseased conditions indicates an
52 intriguing, but poorly understood cell cycle plasticity in the brain.

53 The evidence for quiescent cells in the adult *Drosophila* brain that re-enter the
54 cell cycle is relatively sparse, and there are many unknowns, including how polyploid
55 neurons and glia might arise. Neuro-glial progenitors have been reported in the optic
56 lobes (Fernandez-Hernandez et al., 2013; Simoes et al., 2022), central brain (Crocker et
57 al., 2021; Kato et al., 2009), and antennal lobes (Fernandez-Hernandez et al., 2021;
58 von Trotha et al., 2009), that engage DNA replication when the adult brain is injured
59 (Foo et al., 2017; Kato et al., 2009; Li et al., 2020; von Trotha et al., 2009). Evidence for
60 mitotic divisions exists but they appear to be rare, suggesting daughters of progenitors
61 could undergo mitotic slippage or mitotic skipping to result in polyploid cells.
62 Alternatively, differentiated diploid neurons or glia may re-enter a variant cell cycle
63 lacking mitosis in response to tissue damage or cell loss, in a manner similar to wound-
64 induced polyploidization, observed in the adult *Drosophila* epithelium (Besen-McNally et
65 al., 2021; Grendler et al., 2019; Losick, 2016).

66 Regardless of whether the source of polyploid cells in the adult brain is neuro-
67 glial progenitors, differentiated cells, or a combination of both, the very low levels of
68 polyploidy in brains of newly eclosed adults indicates most cell cycle re-entry from a G0
69 state occurs during the first few weeks of adulthood. We previously showed that
70 polyploid cells accumulate most rapidly during the first three weeks of adulthood,
71 reaching a plateau by 21 days of age (Nandakumar et al., 2020). The steady state of
72 polyploidy after 3 weeks may be due to reduced cell cycle re-entry, or continued cell
73 cycle re-entry balanced by the loss of polyploid cells through latent reductive cell
74 divisions or cell death, or a combination of both. The window of polyploid cell
75 accumulation therefore is an age-associated trait, that can be explored to decipher
76 factors influencing cell cycle plasticity in the brain.

77 Here, we show that known lifespan modifiers can influence the timing and rate of
78 the accumulation of polyploid cells in the adult fly brain. Manipulations that extend
79 lifespan slow the accumulation of polyploid cells in the brain, but in a manner that
80 cannot be explained by the prolonged lifespan alone. When we scale polyploid cell
81 accumulation to the percentage of maximum lifespan under each condition, normalizing
82 for lifespan extension does not fully resolve the slowed dynamics of polyploid cell
83 accumulation. Collectively, our data suggests there is a relationship between low
84 temperature responses, protein intake and the window of flexibility of G0 in the adult
85 brain. We propose that polyploidy in the brain is the result of a highly regulated process
86 controlled by multiple physiological inputs that results in a reproducible proportion of
87 polyploid cells in the brain to be achieved and maintained during aging.

88 89 **Materials and Methods**

90
91 *Fly Strains and husbandry*

92 The *Drosophila melanogaster* *w¹¹¹⁸* fly strain (BDSC 5905) was used for all
93 experiments. Virgin female and male flies collected within 48 hours of eclosion were
94 considered age matched and placed in separate vials containing 20 flies per vial. Flies
95 were housed in a 25°C incubator on Bloomington Cornmeal food and exposed to 12-
96 hours of ambient light per day unless otherwise noted.

97

98 *Flow Cytometry*

99 The proportion of polyploid cells in whole brains was determined using flow
100 cytometry. For region specific experiments the optic lobes (OLs) and central brain (CB)
101 from the brain were manually separated. Unless otherwise stated, one female brain and
102 one male brain was used per biological replicate. 3-6 biological replicates were
103 performed per group. DNA content was measured using the live DNA stain, DyeCycle
104 violet. Cell death was measured using permeability assays with either propidium iodide
105 (PI) or Sytox Green. The flow rate was 500 µl per minute for each sample and a
106 minimum of 10,000 events were gated as non-doublets. Flow cytometry experiments
107 were performed as previously described (Nandakumar et al., 2020) on an Attune NxT
108 flow cytometer.

109 Briefly, fly brains were dissected and dissociated using trypsin:EDTA and then
110 stained with DyeCycle violet and PI or 7-AAD. Brains were incubated for twenty minutes
111 in the dark and then triturated using a p200 pipette tip. The solution was moved to an
112 Eppendorf tube containing 400 µl of the trypsin:EDTA, PBS, and dye solution. The
113 sample was incubated at room temperature for 45 minutes in the dark. After incubation,
114 the sample was diluted with 500 µl of 1X PBS and gently vortexed prior to running on
115 the Attune NxT flow cytometer for analysis.

116 To identify polyploid cells in our sample we used a stringent gating strategy that
117 was used previously (Nandakumar et al., 2020). Debris was eliminated based on size
118 (FSC) and complexity (SSC). We identified cells by gating around events that were
119 positively stained with DyeCycle Violet. The DNA-stained events were then gated based
120 on area (VL1-A) and height (VL1-H) four doublet elimination. Next, we analyzed the
121 DNA content of non-doublet, DNA-stained events and examined cell death using PI or
122 7-AAD positivity.

123

124 *Fixing, Immunostaining, and Imaging*

125 *Drosophila* brains were dissected in 1X phosphate buffered saline (PBS). Per
126 condition, 4 brains were dissected within 20 minutes to minimize tissue damage.
127 Tissues were fixed in 4% paraformaldehyde (PFA) in 1X PBS for 30 minutes. Tissues
128 were permeabilized in 1X PBS+ 0.05% Triton X-100 and blocked with 1X PBS, 1% BSA,
129 0.1% Triton X-100 (PAT). Lamin (DSHB ALD67.10) (1:500 dilution) staining was
130 performed in PAT solution overnight at 4°C with rotation. After the overnight incubation,
131 the samples were washed, blocked with PBT-X (1X PBS, 2% Normal goat serum,
132 0.03% Triton X-100) and incubated with secondary antibody overnight at 4°C. After the
133 secondary antibody incubation, samples were washed with 1X PBS and DAPI was
134 applied for ten minutes. Afterwards, the samples were washed again with 1X PBS and
135 then mounted in Vectashield H1000 (Vector Labs).

136 All images were taken on a Leica SP8 at 63x magnification with 0.5 micron Z-
137 sections. The OLs and the CB were imaged at age day 7, day 14, and age day 21.

138 Image quantifications were performed as previously described (Box et al., 2024;
139 Grendler et al., 2019). Raw integrated intensity measurements were taken from diploid
140 cells, which comprise most of the brain. Polyploid cells were identified based on nuclear
141 size and circularity of the Lamin stain. The raw integrated intensity was measured, and
142 the background fluorescence was subtracted from each measurement to ascertain the
143 corrected fluorescence intensity. The intensity of haploid DNA content was calculated by
144 dividing the average intensity of the diploid cells by 2. Polyploid cells identified were
145 then confirmed based on the following binning: 2N (1.9–2.9), 4N (3.0–6.9), 8N (7–12.9).
146 Four brains per time point were examined, with 4 OLs images and 2 CB images being
147 analyzed from each brain. Data from all brain regions is pooled together for each
148 timepoint.

149

150 *Hydroxyurea Feeding*

151 10mg/ml Hydroxyurea (HU) in 50% sucrose was used to block DNA synthesis in
152 adult brains (Elkahlah et al., 2020). Briefly, HU (ThermoFisher, AAA1083106) was
153 dissolved in 50% sucrose to create a stock of 50 mg/ml. 600 μ l of the stock solution was
154 added to reheated Bloomington Cornmeal food for a total volume of 3 ml for a final
155 concentration of 10 mg/ml. Food was made one day prior to the addition of flies. w^{1118}
156 flies were collected the day they eclosed. Virgin female and male flies were placed on
157 HU or vehicle containing food. Vials were flipped every other day and flow cytometry
158 measurements were taken at age day 7, day 14, and age day 21.

159 For larvae experiments, yeast paste containing 5 mg/ml of HU dissolved in ddH₂O
160 was used instead of solid food (Elkahlah et al., 2020). w^{1118} flies laid eggs on grape agar
161 plates the day before for two hours. 24-hours post egg legging, the agar plates were
162 checked hourly. Any larvae that emerged within the hour were immediately moved to
163 yeast paste containing 5mg/ml of HU. Larvae were left on the yeast paste for eight
164 hours and then moved to regular cornmeal food. The vehicle group was treated in a
165 similar way, accept the yeast paste contained no HU, only ddH₂O. For the control group,
166 eggs were laid on the standard Bloomington Cornmeal food. Flow cytometry
167 experiments were performed on adult brains exposed to HU on age day 7.

168

169 *Lifespan assays*

170 Female and male w^{1118} flies were collected over a 24-hour period and separated
171 into different vials to prevent mating. 13 vials of males and 13 vials of female flies
172 containing 20 flies of the same sex per vial were collected per condition. A total of at
173 least 230 flies per condition were measured. Conditions included 25°C, 18°C, 29°C,
174 reduced yeast protein (RY), 25°C + RY, 18°C + RY, and 29°C +RY. Lifespan measurements
175 were performed by changing the food every Monday, Wednesday, and Friday with the
176 number of dead flies recorded each day (Damschroder et al., 2018; Linford et al., 2013).
177 Flies were housed based on the appropriate condition in the dark, which does not influence
178 polyploid cell accumulation (Nandakumar et al., 2020). Two separate cohorts for each
179 condition were examined in parallel. Flies from the different cohorts had different parents.
180 Significance was tested by a log-rank test, or one-way ANOVA of the average median and
181 maximum lifespan.

182

183 *Measuring polyploidy under different conditions and scaling to total lifespan.*

184 Flies were housed in the various conditions described above. Polyploidy
185 measurements started on the day of eclosion (age day 0), day 7, and then every two days
186 thereafter. Data was scaled to the total lifespan for each condition. The percent lifespan
187 was determined by taking the age in days of the measurement divided by the maximum
188 lifespan of that group then multiplied by 100 (% lifespan = (age in day / maximum lifespan)
189 * 100).

190

191 *Graphs and Statistics*

192 Graphs and statistical analysis were created and performed using GraphPad Prism 10.
193 Analysis for all experiments is reported in the figure legends.

194

195 **Results**

196 **Resolving the window for cell cycle re-entry in the adult *Drosophila* brain.**

197 Incidences of cell cycle re-entry have been reported in *Drosophila* adult brains of
198 mutants and disease or damage models (Crocker et al., 2021; Fernandez-Hernandez et
199 al., 2013; Khurana et al., 2006; Simoes et al., 2022). Cell cycle re-entry also occurs in
200 healthy adult fly brains but is less understood (Foo et al., 2017; Kato et al., 2009; Li et
201 al., 2020; Nandakumar et al., 2020; von Trotha et al., 2009). We previously identified
202 polyploid neuronal and glial cells in the adult *Drosophila* brain under normal
203 physiological aging conditions without mating (Nandakumar et al., 2020). Here we
204 determined whether there is an adult-specific window of cell cycle re-entry in the
205 *Drosophila* brain under these normal physiological conditions.

206 The proportion of cells containing more than a diploid (2C) DNA content
207 (polyploid cells) increases in female and male wild-type flies during normal physiological
208 aging at 25°C on standard cornmeal and agar food (Fig. 1A, 2-way ANOVA, Aging
209 factor, $p < 0.0001$). The proportion of polyploid cells in the whole brain is similar between
210 female and male flies (Fig. 1A, 2-way ANOVA, Sex factor, $p = 0.931$), and peaked on
211 average at 21-28 days of age (Fig. 1A. Sidak's multiple comparison tests, $p > 0.05$),
212 which may indicate there is a physiological requirement for a precise proportion of
213 polyploidy in the brain. The proportion of cells with a more than 4C DNA content
214 (greater than tetraploid) is similar in female and male fly brains during aging and peaked
215 on average at 28 days of age (Fig. 1B., 2-way ANOVA, Aging factor, $p < 0.0001$, Sex
216 factor, $p = 0.652$). We measured cell death in the brain using a propidium iodide (PI) cell
217 permeability assessment. The fraction of cells incorporating PI increases during aging
218 with an increase after 21 days of age (Fig. 1C., 2-way ANOVA, Aging factor, $p < 0.0001$)
219 with similar proportions in female and male flies (Fig. 1C., Sex factor, $p = 0.652$). This
220 level of cell death in the adult brain is similar to the cell death rates measured using
221 other permeability assays such as 7-AAD (SF. 1A.).

222 After observing no sex-specific effects on polyploidy or cell death, one female
223 and one male brain were combined in a biological replicate for all subsequent
224 experiments. Even though polyploidy in the brain increases with age, most cells in the
225 brain remain diploid at all time points (Fig. 1D., 2-way ANOVA, Ploidy effect, $p < 0.0001$).
226 Thus, only a small proportion of cells re-enter the cell cycle in adult brains under normal
227 physiological aging conditions. The majority of dead and dying cells in the brain are also
228 diploid (Fig. 1E., 2-way ANOVA, Ploidy effect, $p = 0.0006$), with the proportion of dying
229 diploid and polyploid cells increasing with age (Fig. 1E., 2-way ANOVA, Aging effect,

230 p<0.0001). Consistent with our previous observations, the proportion of dying cells that
231 are polyploid is less than diploid cells at several timepoints (Fig. 1E.) (Nandakumar et
232 al., 2020). Differences in cell death rates between diploid and polyploid cells may be
233 explained by the fact that diploid cells comprise most of the brain.

234 We verified our flow cytometry results by performing fixed imaging and DAPI
235 intensity measurements on brains at days 7, 14, and 21. These measurements were
236 done similarly to prior studies, except that we used a nuclear lamin staining to manually
237 segment individual nuclei (Besen-McNally et al., 2021; Box et al., 2024) in the optic
238 lobes and central brain using confocal microscopy. Most cells in the brain are diploid
239 and uniform in nuclear circularity and size, while polyploid cells are visually
240 distinguishable with enlarged nuclei, increased DAPI intensity and decreased nuclear
241 circularity (Fig. 1D.). Integrated DAPI intensity measurements from similar-sized z-
242 stacks of the optic lobe and central brain reveal an increase in the number of polyploid
243 nuclei with age, consistent with our flow cytometry data (Fig. 1G. one-way ANOVA,
244 p<0.0001).

245 On the day of eclosion (day 0), less than 2% of cells are polyploid in the brain,
246 but by day 21 around 10% of cells in the brain are polyploid. Polyploidy can arise
247 through cell fusion, as well as a variant cell cycle known as an endocycle, which
248 includes S-phase (DNA synthesis phase) but lacks mitosis and cytokinesis resulting in
249 increased nuclear ploidy (Nandakumar et al., 2021) . To confirm that cell cycle re-entry
250 resulting in endocycling gives rise to polyploid cells in the adult fly brain, we fed adult
251 flies the DNA synthesis inhibitor hydroxyurea (HU) to block S-phases (Koc et al., 2004).
252 Flies fed HU from the day of eclosion develop significantly fewer polyploid cells than
253 vehicle-fed flies (Fig. 1H. 2-way ANOVA, Drug effect p<0.0001) and do not continue to
254 accumulate polyploid cells during aging (Fig. 1H., Tukey's multiple comparisons test,
255 p>0.05). Therefore, reducing DNA synthesis limits polyploidy in the aging brain and
256 supports the hypothesis that some polyploid accumulation relies on cells re-entering the
257 cell cycle to replicate their DNA.

258 To ensure the cell cycle- re-entry window is adult specific, we fed HU to larvae for
259 the first 8 hours of life. Flies fed HU as larvae develop adult-specific polyploidy normally
260 during their first week of life (Fig. 1I., One-way ANOVA, p=0.154), demonstrating that
261 blocking DNA synthesis before adulthood does not influence polyploid cell
262 accumulation. In addition, this demonstrates that cell types in the brain depleted by
263 larval HU feeding such as the mushroom body neuroblasts in the central brain, are not a
264 significant source of polyploid cells in the adult brain. While we cannot rule out the
265 possibility that neural or glial progenitor cells observed by others (Fernandez-
266 Hernandez et al., 2013; Kato et al., 2009; von Trotha et al., 2009) contribute to the
267 accumulation of polyploidy in the brain, these cells are believed to be largely quiescent
268 in the adult brain under normal physiological conditions (Fernandez-Hernandez et al.,
269 2013; Ito & Hotta, 1992; Kato et al., 2009; Li et al., 2020; Li & Hidalgo, 2020; Siegrist et
270 al., 2010; Truman & Bate, 1988; von Trotha et al., 2009). Our data therefore supports a
271 model where some of the age-associated accumulation of polyploid cells under normal
272 physiological conditions is due to cells re-entering the cell cycle during the first 3 weeks
273 of adulthood.

274

275 **Extending lifespan delays cell cycle re-entry in the adult brain.**

276 Due to the association between aging and the accumulation of polyploidy in the
277 brain, we next examined whether known lifespan modifiers could influence the timing or
278 rate of polyploid cell accumulation. Housing temperature and protein content in diet are
279 known lifespan modifiers (Grandison et al., 2009; Mair et al., 2005; Molon et al., 2020;
280 Shaposhnikov et al., 2022; Skorupa et al., 2008; Tatar et al., 2014). We first confirmed
281 the effects of altered housing temperatures on lifespan, specifically a low (18°C) and a
282 high (29°C) temperature. Consistent with previous reports (Miquel et al., 1976;
283 Shaposhnikov et al., 2022) female and male flies housed in 18°C on the day of eclosion
284 (age day 0) live longer than their age-matched siblings housed at the control
285 temperature (25°C) (Fig. 2A.), while flies housed at 29°C have a significantly shorter
286 lifespan than their age-matched siblings housed at 25°C (Fig. 2B.).

287 We next confirmed the effects of feeding a reduced protein diet by restricting yeast
288 content, (Grandison et al., 2009; Steck et al., 2018) which is the main source of amino
289 acids and protein in the standard cornmeal and agar fly food
290 (<https://bdsc.indiana.edu/information/recipes/bloomfood.html>). Our reduced yeast (RY) diet
291 contains 30% less yeast than control food (1.1% total yeast in RY compared to 1.58%
292 yeast in control diet). Feeding rate decreases with age (SF2) (Min & Tatar, 2006) and there
293 is a small, but significant, increase in feeding rate in young flies fed a reduced yeast diet
294 (SF 2A., 2-way ANOVA, Diet effect, $p=0.020$). At age day 21 there is no difference in
295 feeding rate (SF 2B., 2-way ANOVA, Diet effect, $p=0.80$). Flies fed the RY diet from age day
296 0 live longer than their age-matched siblings fed the control diet (Fig. 2C.), although this
297 lifespan extension is subtle, which may be explained by the fact that our control diet is
298 already a lower yeast diet (1.58%) compared to other common diets (Ren et al., 2020;
299 Skorupa et al., 2008).

300 When replicate lifespans were combined and averaged, flies aged at a low
301 temperature had the largest average median and maximum lifespan (Fig. 2D. & E.) while
302 flies aged at a higher temperature had the lowest average median and maximum lifespan
303 (Fig. 2D & E.), as expected based on prior work (Miquel et al., 1976; Shaposhnikov et al.,
304 2022; Zajitschek, 2023). The effects from combining modifiers on lifespan is unclear, with
305 some groups finding additive effects (Phillips, 2024; Shaposhnikov et al., 2022) while others
306 do not (Zajitschek, 2023). We observed no additive effects to the average median and
307 maximum lifespan when combining these different lifespan modifiers. For example, housing
308 flies at 18°C while on a low protein diet has no effect on median (Fig. 2D. Tukey's multiple
309 comparison test, $p=0.083$) or maximum lifespan (Fig. 2E., Tukey's multiple comparison test,
310 $p=0.272$), compared to 18°C on our normal diet. Furthermore, there is no rescue to the
311 median (Fig. 2D. Tukey's multiple comparison test, $p=0.995$) or the maximum (Fig. 2E.,
312 Tukey's multiple comparison test, $p=0.993$) lifespan in flies housed at 29°C and fed a low
313 protein diet. Nevertheless, altered temperature and RY diet alone were reproducible
314 lifespan modifiers.

315 We then examined the effects of these lifespan modifiers on polyploid cell
316 accumulation in the adult brain. Flies housed at 18°C accumulate polyploid cells in their
317 brain significantly slower than flies housed at 25°C (Fig. 3A., 2-way ANOVA, Temperature
318 factor, $p<0.0001$) and the proportion of cells with a more than 4C DNA content is also
319 reduced (Fig. 3B. 2-way ANOVA, Temperature factor, $p<0.0001$). Cell death in flies housed
320 at 18°C is not significantly altered (Fig. 3C., 2-way ANOVA, Temperature factor, $p=0.173$),
321 suggesting the changes in polyploid cell accumulation at 18°C cannot be explained by

322 differences in cell death in the brain. By contrast, flies housed at 29°C accumulate
323 polyploidy similarly to flies at 25°C (Fig. 3D., 2-way ANOVA, Temperature factor, $p=0.641$).
324 However, the proportion of cells containing more than 4C DNA content is partially
325 influenced by aging at a high temperature (Fig. 3E., 2-way ANOVA, interaction, $p=0.0003$),
326 with flies at 29°C exhibiting more cells with higher (>4C) DNA content by day 35 (Fig. 3E.,
327 Sidak's multiple comparison test, $p=0.0002$). This increase in cells with higher ploidy is
328 not due to significant changes in cell death however (Fig. 3F. Temperature factor, $p=0.914$).
329 This suggests there could be a relationship between the stress of aging at a high
330 temperature and developing cells of higher ploidy than tetraploid at later timepoints in
331 adulthood.

332 Like reduced temperature, flies on a low protein diet (RY) accumulate polyploidy
333 slower in the brain (Fig. 2G., 2-way ANOVA, Diet factor, $p<0.0001$) and fewer cells with a
334 DNA content of more than 4C (Fig. 2H., 2-way ANOVA, Diet factor, $p<0.0001$). Slower
335 polyploid accumulation in the brain is not due to altered cell death in flies fed a low protein
336 diet (Fig. 2I., 2-way-ANOVA, Diet factor, $p=0.171$). In fact, the proportion of polyploid cell
337 death in the whole brain is significantly reduced in flies fed a reduced protein diet (SF3 E.,
338 2-way ANOVA, Diet factor, $p<0.0001$). The slower accumulation of polyploid cells during the
339 first 2 weeks of adulthood may contribute to the reduced number of cells with higher
340 ploidy more than 4C at later ages, beyond 2 weeks (Fig. 3G, H.).

341 Combining lifespan modifiers indicate an interaction between a high housing
342 temperature and protein availability. Flies housed at 18°C and fed a low protein diet and
343 flies housed at 29°C and fed a low protein diet had less polyploid cell death than controls
344 (SF. 3G., 2-way ANOVA, Temperature and diet effect, $p=0.0314$, SF 3. I, 2-way ANOVA,
345 Temperature and diet effect, $p=0.001$).

346 Flies housed at 18°C and fed a low protein diet accumulate the same proportion of
347 cells that contained a more than 4C DNA content as flies housed at 18°C (Fig. 4B., 2-way
348 ANOVA, Temperature and Diet factor, $p=0.0056$). Therefore, combining a low protein diet
349 with a low housing temperature does not further reduce the development of cells with
350 higher ploidy. In contrast, when we compared the proportion of cells with a more than 4C
351 DNA content at 29°C and 29°C with a low-protein RY diet, we observe a significant
352 reduction of higher ploidy at 4 weeks of age (Fig. 4E., 2-way ANOVA, Temperature & Diet
353 factor, $p=0.007$).

354 Flies housed at 29°C exhibit an increased number of cells with higher ploidy (Fig.
355 3E.), while flies fed a low-protein diet at a normal temperature (25°C) accumulate fewer
356 cells with higher ploidy (Fig. 3H.). Our data suggests that a lack of sufficient protein in the
357 diet hinders the development of highly polyploid cells in the adult brain at higher
358 temperatures, indicating that the cell cycle re-entry process in the adult brain may be
359 partially dependent upon protein and amino acid sensitive signaling pathways.

360

361 **The cell cycle re-entry window in the adult brain is flexible.**

362 Collectively, our data demonstrates that conditions that extend lifespan delays cell
363 cycle re-entry in the adult brain resulting in the slower accumulation of polyploid cells and
364 reduced levels of cells with higher ploidy. However, despite the slower rate of polyploid
365 cell accumulation, the proportion of polyploid cells in the brain eventually reaches control
366 levels. The underlying reason for this is unclear but implies the proportion of polyploidy in
367 the brain may be physiologically relevant. This also suggests the window of cell cycle re-

368 entry is not dependent upon chronological age but may scale with physiological age.
369 Therefore, we next examined whether the accumulation of polyploid cells under lifespan
370 alterations scaled with percent of maximum lifespan which effectively normalizes the
371 phenotype to physiological age, rather than chronological age (number of days after
372 eclosion).

373 When we plot polyploid cell accumulation based on percent maximum lifespan,
374 we see that after an initial increase immediately after eclosion, polyploid cell
375 accumulation is still significantly slower under conditions of reduced temperature or
376 reduced protein (RY), even when normalized to the total lifespan under these conditions
377 (Fig. 4B.). If the window of cell cycle re-entry in the adult brain is entirely dependent
378 upon physiological aging cues, we would expect the levels to overlap when normalized
379 to total lifespan. The reduced levels of polyploid cells in the brain at 18°C or on a
380 reduced protein diet suggests that the window for cell cycle re-entry in the adult brain is
381 flexible and responds to signaling impacted by temperature and protein levels in a
382 manner that is more sensitive to, or independent of the downstream pathways
383 responsible for lifespan extension.

384 385 **Polyploidy accumulation is brain region specific.**

386 Different regions of the *Drosophila* brain accumulate different levels of polyploid cells
387 (Nandakumar et al., 2020). We therefore examined whether polyploidy in different brain
388 regions may be affected differentially by aging at a low temperature or being fed a low
389 protein diet. To examine this, we physically separated optic lobes (OLs) from the central
390 brain (CB) and performed flow cytometry under the indicated lifespan extending conditions
391 to assess whether effects on cell cycle re-entry may be brain region specific. We
392 consistently observe the levels of polyploidy in the OLs are more affected by temperature
393 and protein levels relative to the central brain. The OLs of flies housed at 18°C accumulate
394 significantly fewer polyploid cells (Fig. 6A., 2-way ANOVA, Temperature factor, $p < 0.0001$)
395 and fewer cells of high ploidies (Fig. 6B., 2-way ANOVA, Temperature factor, $p = 0.0031$),
396 than the CB, which showed no significant difference in the accumulation of polyploid cells
397 under these conditions (Fig. 6D. 2-way ANOVA, Temperature factor, $p = 0.420$, Fig. 6E., $>4C$
398 DNA content, 2-way ANOVA, Temperature factor, $p = 0.295$). Unexpectedly, cell death in the
399 CB is significantly increased at a reduced temperature for a single timepoint (Fig. 6F.) but is
400 not significantly affected in the OLs at any timepoint (Fig. 6C.), suggesting the changes in
401 the levels of polyploid cells of the OLs are not caused by changes in cell survival. The OLs
402 of flies on the low protein diet also accumulate fewer polyploid cells than flies on a control
403 diet (Fig. 7A., interaction $p = 0.0021$). while the CB shows no effect of a low protein diet on
404 polyploid cell accumulation (Fig. 7D. Diet factor, $p = 0.314$). Interestingly, like flies aged at a
405 low temperature, flies fed a low protein diet have significantly more cell death in their CB at
406 one timepoint (Fig. 7F., 2-way ANOVA, Diet factor, $p = 0.0068$). While the reasons why low
407 temperature and low protein diet may affect cell death in the central brain is unclear, this
408 finding again suggests the effects on polyploid cell accumulation in the OL are not
409 indirectly due to changes in cell survival.

410 Our data supports the possibility that cell cycle re-entry in the adult fly brain and
411 the polyploid cell accumulation that results, is responsive to temperature and amino acid
412 signaling, possibly through metabolic activity, specifically in the OL. This important result
413 separates the effects of these pathways on polyploidy from their effects on lifespan and

414 suggests the cells that respond to these cues to induce cell cycle re-entry are specific
415 to, or enriched in, the optic lobes.

416

417 **Discussion**

418

419 *An adult-specific window for cell cycle re-entry in the brain.*

420 We previously found an onset of polyploidization in the adult fly brain
421 (Nandakumar et al., 2020). Here, we resolve the window for cell cycle re-entry in the
422 adult brain, which relies on DNA replication during the first three weeks of adult life.
423 When flies are housed under conditions that extend lifespan, we see the rate of
424 polyploid cell accumulation in the brain is slowed at a rate independent of lifespan
425 extension. Furthermore, we show that the brain region influences polyploid cell
426 accumulation. Collectively, our data points to an adult-specific program in the brain that
427 integrates information from signaling pathways impacted by temperature and protein
428 content in the diet, to establish a window for cell cycle re-entry in the optic lobes.

429 The mechanistic Target of Rapamycin (mTOR) network, specifically mTOR
430 complex 1 (mTORC1), could be a key regulator for this adult-specific cell cycle re-entry
431 program in the aging brain. mTORC1 integrates nutrient availability, growth factor
432 signals, and cellular energy to induce various pathways that drive cell growth and
433 proliferation (Weichhart, 2018). To achieve increased cell growth and proliferation,
434 mTORC1 activity increases nucleotide synthesis, protein synthesis, and DNA replication
435 licensing through S6K, 4E-BP1, and Cdc6 respectively (He et al., 2021), all of which are
436 also required for cell cycle re-entry. Interestingly, in flies aged at 18°C, mTOR activity is
437 reduced (Scialo et al., 2015), which could explain the slowed rate of polyploid cell
438 accumulation we observed in flies aged at 18°C. Future studies will illuminate if the mTOR
439 signaling network impacts the cell cycle plasticity of the aging brain.

440

441 *Some cells in the adult brain are in a flexible state of G0.*

442 Work over the past decade supports the non-proliferative G0 state of
443 differentiated cells is not a binary on or off switch, but more of a spectrum with some
444 cells having more flexibility to re-enter the cell cycle, while other cells maybe in a stable,
445 robust G0 (Borowik et al., 2023; Losick, 2016; Ma et al., 2019; Nandakumar et al., 2021;
446 Yao, 2014; Zanet et al., 2010). Our work suggests there is heterogeneity in the flexibility
447 of G0 among cells in the brain and reveals that environmental conditions influence the
448 plasticity of the G0 state.

449 In this study, we focused on how polyploid cell accumulation changes in the
450 whole brain, OL, or CB under different housing conditions during aging. We see that
451 housing adults at a reduced temperature or feeding a reduced protein diet slows the
452 accumulation of polyploidy in the whole brain. These manipulations do not block cell
453 cycle re-entry, since polyploid cells still accumulate with age. Additionally, feeding the
454 DNA synthesis inhibitor, hydroxyurea, during adulthood significantly reduced the age-
455 associated polyploid cell accumulation (Fig. 1H.). The slower accumulation under low
456 temperature or reduced protein could be due to a reduced number of cells that re-enter
457 the cell cycle, a slower speed of the variant cell cycle, or some combination of these two
458 possibilities. Further work will be needed to resolve these important open questions.

459 When polyploid cell accumulation is examined by brain region, we see the OLs
460 specifically accumulate polyploid cells slower when housed at a reduced temperature or
461 fed a reduced protein diet. This suggests either the flexibility of the G0 state is brain
462 region specific, or that the cell type(s) poised to re-enter the cell cycle in adult brains are
463 specific to or enriched in the OLs. Several neuronal and glial cell types become
464 polyploid in adults (Nandakumar et al., 2020), so further work will be needed to resolve
465 the sources and lineages of adult-specific polyploid cells in the brain.

466
467 *Polyploid cells in the brain may arise from multiple origins and different triggers.*

468 The adult fly brain is mostly post-mitotic and does not normally retain
469 developmental neuroblasts (Siegrist et al., 2010). However, after direct physical injury to
470 the brain in either the optic lobe (OL) (Fernandez-Hernandez et al., 2013; Simoes et al.,
471 2022) or the central brain (CB) (Crocker et al., 2021; Kato et al., 2009), cell division and
472 neurogenesis ensue, suggesting quiescent neuro-glial progenitors exist in these
473 regions. BrdU (Kato et al., 2009) and EdU (Foo et al., 2017) labeling shows DNA
474 replication occurring in these same regions in young wild-type brains without injury up to
475 age day 10, but the mitotic marker PH3 is very rarely present (Kato et al., 2009). In our
476 previous work, we also saw no convincing evidence of mitosis in aged wild-type flies
477 (Nandakumar et al., 2020). The positive S-phase labeling and lack of PH3 labeling is
478 indicative of cells entering a non-mitotic variant cell cycle. Currently, it is unclear
479 whether adult neuro-glial progenitor cells are contributing to the physiological age-
480 dependent accumulation of polyploid cells, or whether cell cycle re-entry of terminally
481 differentiated cells occurs. Alternatively, a combination of differentiated and progenitor
482 cells may contribute to polyploidy in different regions of the brain. Further work will
483 require lineage tracing to decipher the potentially complex potential sources of polyploid
484 cells in the adult fly brain. Regardless of the source of polyploid cells, our data indicates
485 there is adult-specific cell cycle re-entry occurring in the brain.

486 The stimuli that trigger polyploidization in the brain also remain unclear.
487 Acute physical injury to the fly brain (Crocker et al., 2021; Fernandez-Hernandez et al.,
488 2013; Foo et al., 2017), acute DNA damage in the fly brain (Nandakumar et al., 2020),
489 and overexpression of known neurodegeneration-inducing proteins (Khurana et al.,
490 2006; Rimkus et al., 2008) can induce cell cycle activity. In this study, we did not cause
491 any acute physical injury, DNA damage, or express neurodegenerative proteins. Our
492 study specifically examines cell cycle re-entry during normal physiological aging. It is
493 known that DNA damage naturally accumulates in the brains of flies (Fishel et al., 2007;
494 Nandakumar et al., 2020). Relative to acute damage, DNA damage during aging may
495 be slower and more prone to engage adaptive mechanisms. As such, this may explain
496 why the increase in polyploidy in the brain, and by extension cell cycle re-entry, is less
497 dramatic during aging relative to the cell cycle activity that occurs from direct damage or
498 genetic manipulation paradigms.

499 A possible hypothesis is only specific stressors influence the rate of
500 polyploidization in the brain. For example, housing flies at a higher temperature, which
501 significantly reduces lifespan, does not accelerate the accumulation of polyploidy in the
502 brain but increases the proportion of cells exhibiting higher ploidies (>4C). This
503 suggests that the rate of cell cycle re-entry may be a response to specific triggers while
504 the degree of polyploidization may respond to chronic stress.

505

506 *Possible models to explain the accumulation of polyploidy in the aging brain.*

507 In control flies, the total proportion of polyploidy in the brain increases with age
508 and then plateaus after age day 21 (Fig 1A.). This suggests the brain obtains a
509 physiological proportion of polyploid cells that is maintained through an unknown
510 mechanism during aging. We establish that reduced temperature or reduced protein
511 slows the initial increase of polyploidy in the brain, in a manner partially independent of
512 the lifespan increase. The mechanisms involved in achieving and maintaining this
513 physiological proportion of polyploid cells in the brain are unresolved.

514 To maintain the plateau observed (Fig. 1A.), the polyploid cells may continue to
515 be produced but progress through mitotic or amitotic reductive divisions to become
516 diploid in the aged brain. We do not observe an increase in diploid cells in the aging
517 brain (Fig. 1D.), and find little evidence for mitosis in wild-type fly brains (Foo et al.,
518 2017; Kato et al., 2009; Li et al., 2020; Nandakumar et al., 2020; von Trotha et al.,
519 2009). As there are no molecular markers for amitosis (Lucchetta & Ohlstein, 2017) this
520 possibility will be difficult to address and live cell imaging in adult brains will be
521 challenging since polyploid cells accumulate over weeks.

522 A different possibility is that a subset of cells re-enters the cell cycle, become
523 polyploid, and then no further cell cycle re-entry occurs later in adulthood. This
524 hypothesis suggests that the G0 state becomes more robust with age, which is
525 supported by other studies (Buttitta et al., 2010; Buttitta et al., 2007; Ma et al., 2019).
526 Interestingly, housing at a reduced temperature or feeding a reduced protein diet slows
527 the accumulation of polyploidy in the brain (Fig. 3) but not the overall total (Fig. 5),
528 demonstrating that the window of G0 flexibility is modifiable. Temperature or diet
529 manipulations influence overall metabolism in the fly (Asiimwe et al., 2023; Blanchard et
530 al., 2024; Klepsatel et al., 2016; Klepsatel et al., 2019). Therefore, metabolism and
531 metabolic mediators may be involved in determining the robustness of G0 in early
532 adulthood. A promising mediator between metabolism and the cell cycle re-entry in the
533 brain is the insulin-Akt-mTOR pathway, which is known to influence the endocycle in the
534 fly (Ohhara et al., 2017; Xiang et al., 2017; Zielke et al., 2011). Future work will
535 determine the impact this metabolic pathway and others have on G0 robustness in the
536 aging brain.

537 A third possibility is a portion of cells re-enter the cell cycle, become polyploid,
538 and then with age the cell death rate of the polyploid cells increases, such that the total
539 level of polyploidy remains constant. Our data is not fully consistent with this model
540 since, under control aging conditions, the proportion of diploid cell death also
541 significantly increases with age (Fig. 1E.) and at each timepoint measured is significantly
542 higher than polyploid cell death, which remains relatively constant during aging (Fig. 1E).

543 Alternatively, brain region differences in polyploid cell accumulation and cell death
544 may result in an overall net gain of zero polyploid cells in later adulthood. Cells in
545 different brain regions may be in different states of G0, since the proportion of
546 polyploidy is not the same in the OLs and CB (Fig. 6 & 7). Furthermore, cells in one
547 region of the brain may have a more flexible G0 state, since OLs of flies housed in
548 different conditions accumulate polyploidy slower (Fig. 6 & 7), suggesting their G0 state
549 became more robust under different conditions. Concurrent to altered states of G0 and
550 flexibility in the G0 state, cell death in the CB is higher than the OLs (Fig. 6 & 7) and is

551 influenced by different environmental conditions. Taken together, our data supports
552 more complex models where there is an intricate relationship between brain region
553 specific polyploid accumulation and brain region specific cell death, possibly mediated
554 by mTOR signaling. Future studies will be needed to elucidate how these variables all
555 contribute to polyploidy in the aging brain.

556

557 **Acknowledgments and Funding**

558 The authors thank the members of the Buttitta lab and Dr. Scott Pletcher for advice and
559 discussions. This work was supported by the National Institute on Aging Career Training
560 in the Biology of Aging Training Grant (NIH T32 AG000114) and by the National Institute
561 of General Medical Sciences of the National Institutes of Health (NIH R35 GM149273).
562 Stocks obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537)
563 were used in this study. The anti-Lamin (DSHB ALD67.10) antibody developed by Dr.
564 Paul Fisher (SUNY Stony Brook) was obtained from the Developmental Studies
565 Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of
566 Iowa, Department of Biology, Iowa City, IA 52242.

567

568 **Authorship**

569 D.D. performed most of the data acquisition, analysis, and manuscript preparation
570 with input from L.B. J.S. and M.D. aided with lifespans, flow cytometry and data
571 analysis.

572

573 **Conflict-of-interest disclosure**

574 The authors have no conflicts to declare.

575

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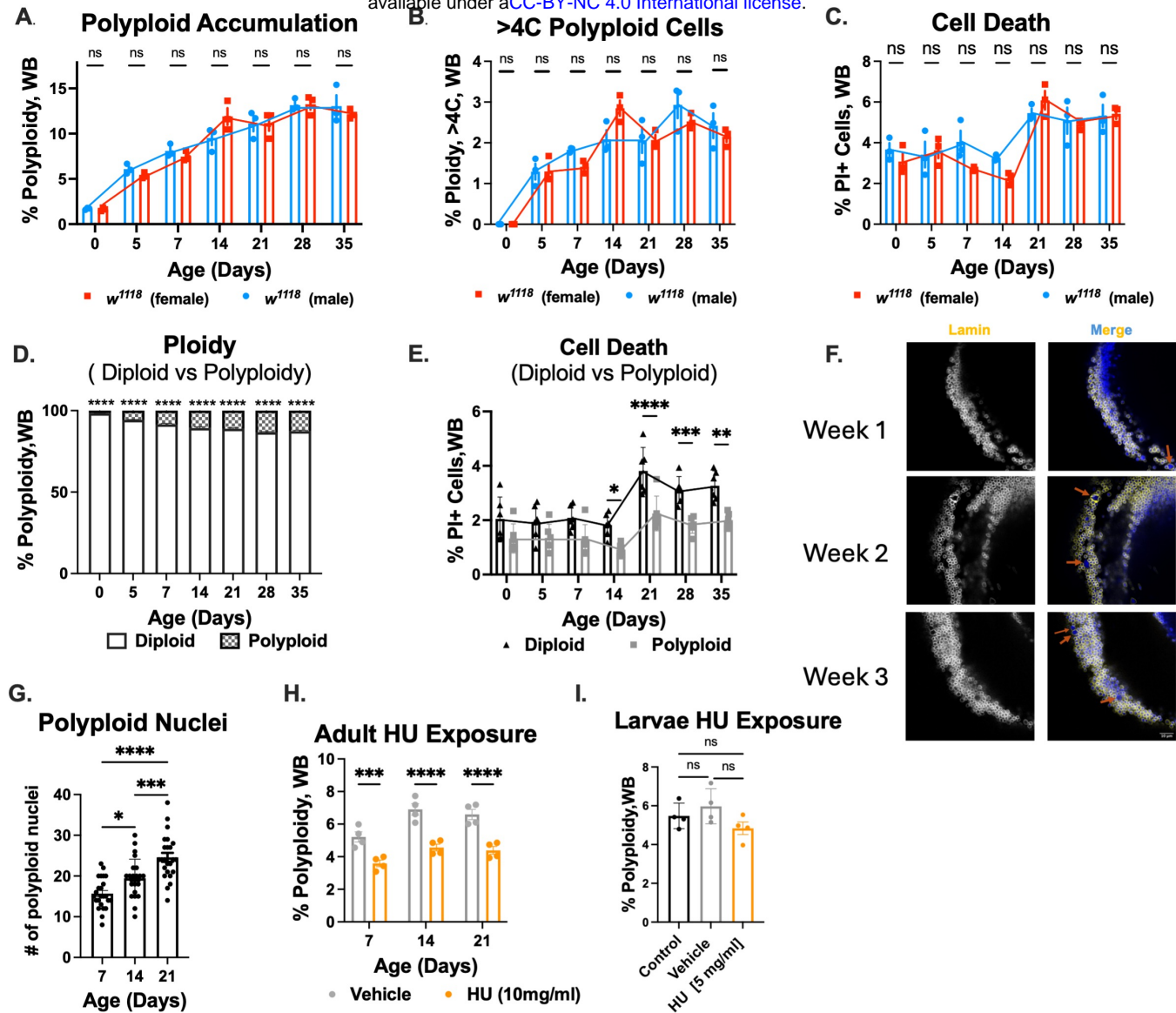


Figure 1: Cell cycle plasticity in the adult fly brain. (A.) Adult female and male flies accumulate polyploid cells similarly during aging (3 biological replicates per time point, 2 fly brains per biological replicate) (2-way ANOVA, Aging factor, $p < 0.0001$, Sex factor, $p = 0.931$). (B.) The proportion of cells that contain more than a 4C (tetraploid) DNA content changes with age similarly in female and male flies (2-way ANOVA, Aging factor, $p < 0.0001$, Sex factor, $p = 0.652$). (C.) Cell death is not significantly different in female and male aging brains (2-way ANOVA, Sex factor, $p = 0.159$), but does increase with age (2-way ANOVA, Aging factor, $p < 0.0001$). (D.) The brain remains mostly diploid during aging (Pooled data, 2-way ANOVA, Ploidy factor, $p < 0.0001$, Sidak's multiple comparisons test). (E.) Diploid cell death increases with age (2-way ANOVA, Aging factor, $p < 0.0001$, Ploidy factor, $p < 0.0001$, Sidak's multiple comparisons test) (F.) Representative images of the *Drosophila* optic lobe (OL) (63x magnification) at age day 7, day 14, and age day 21. The orange arrows indicate enlarged, polyploid cells. (G.) Dapi intensity measurements from fluorescent images of the OL and central brain (CB) at day 7, day 14, and day 21 show an increase in polyploid nuclei with age ($n = 4$ brains per timepoint with 4 OL images and 2 CB images per brain) (One-way ANOVA, $p < 0.0001$). (H.) Flies fed the S-phase inhibitor hydroxyurea (HU) from the day of eclosion accumulate fewer polyploid cells during aging (3 biological replicates per time point containing one male and one female brain per biological replicate) (2-way ANOVA, Drug factor, $p < 0.0001$, Aging factor $p = 0.003$, Tukey's multiple comparisons test). (I.) Feeding HU to larvae did not prevent polyploidy in adult brains age day 7 (3 biological replicates per aging point, 2 fly brains per biological replicate) (One-way ANOVA, $p = 0.154$). Graphs display individual biological replicates, with SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns, not significant.

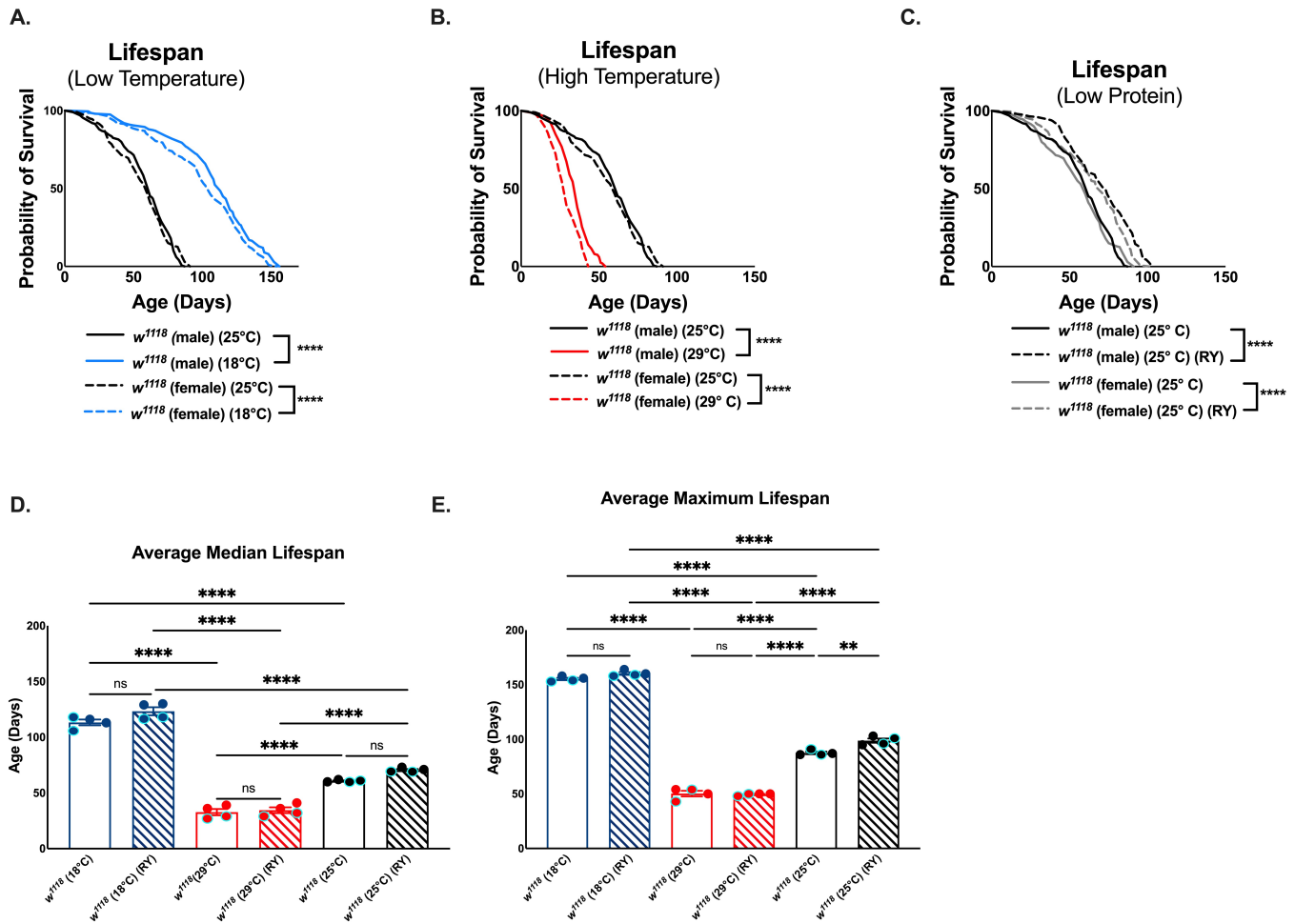


Figure 2: Temperature and protein levels in diet influence adult fly lifespan. (A.-C.) One replicate of lifespan assessment under different housing conditions. 260 flies were measured for each condition and lifespan curves were compared, using a pair-wise log-rank analysis. Significant differences are noted (**** $p < 0.0001$). (A.) Flies housed at 18°C live longer than their sex-matched siblings (male (25°C) vs male (18°C), $p < 0.0001$, female (25°C) vs female (18°C), $p < 0.0001$). (B.) Flies housed at 29°C live shorter than their sex-matched siblings (male (25°C) vs male (29°C), $p < 0.0001$, female (25°C) vs female (29°C), $p < 0.0001$). (C.) Reduced yeast (RY) diet increases lifespan similarly in males and females (male 25°C vs female 25°C, $p = 0.725$, male 25°C RY vs female 25°C RY, $p = 0.322$, RY vs. control diet $p < 0.0001$). (D. & E.) The average median and maximum lifespan from 2 replicate lifespan assessments were calculated (one-way ANOVA with Tukey's multiple comparisons test). Graphs display the average from all replicates with SEM. Replicates from adult females are outlined in turquoise. Each replicate contains 260 flies per condition. The effects of temperature on lifespan are much greater than RY diet. RY diet did not significantly increase average lifespan at any temperature, but did increase maximum lifespan at 25°C.

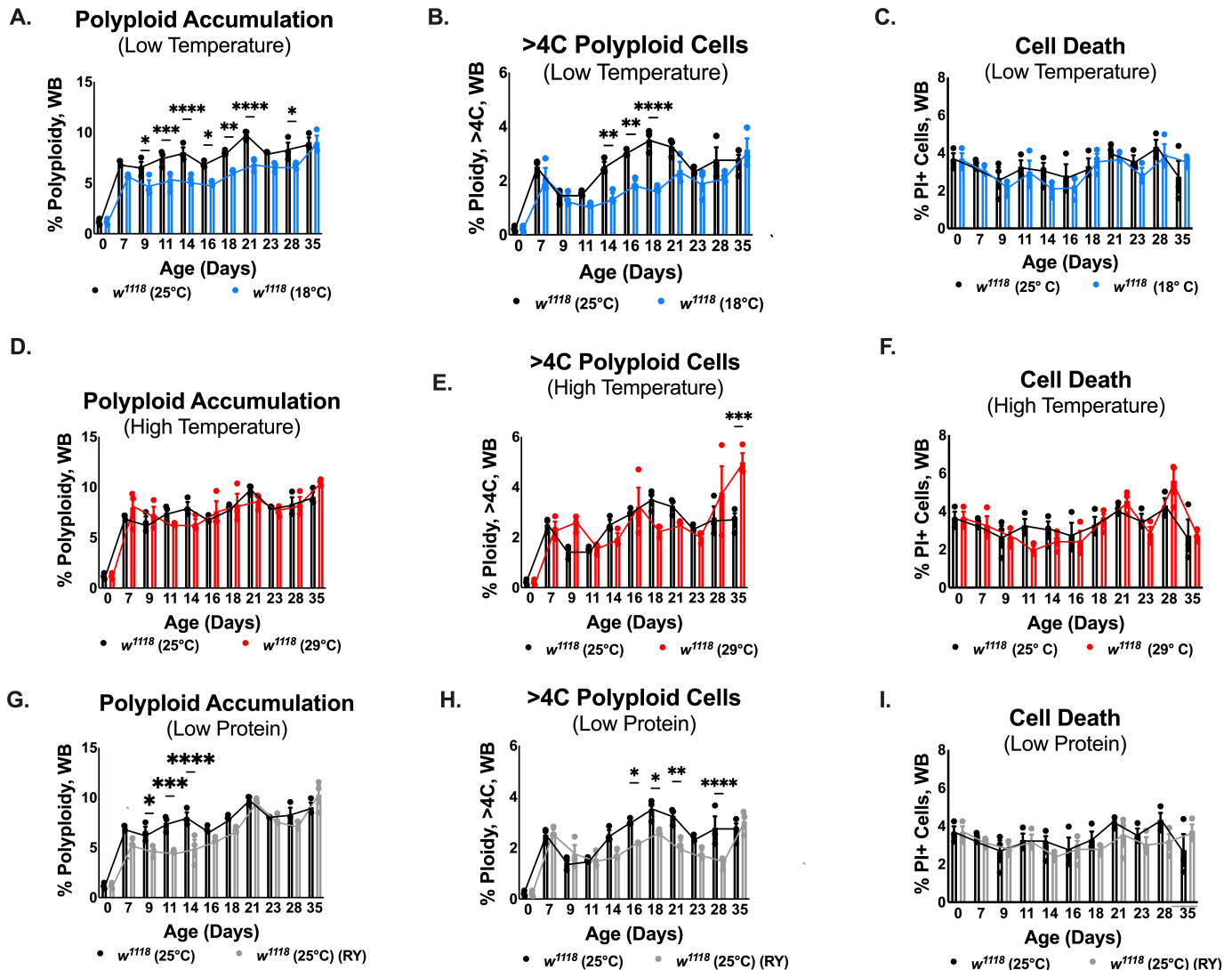


Figure 3: Conditions that increase lifespan delay cell cycle re-entry in the adult fly brain. (A.) Flies housed at 18°C accumulate polyloid cells in their brain slower than flies housed at 25°C (2-way ANOVA, Aging factor, $p < 0.0001$, Temperature factor, $p < 0.0001$, Sidak's multiple comparisons test). (B.) Flies housed at 18°C accumulate fewer cells with a DNA content exceeding 4C (2-way ANOVA, Aging factor, $p < 0.0001$, Temperature factor, $p < 0.0001$, Sidak's multiple comparisons test). (C.) Cell death is not significantly influenced by housing temperature (2-way ANOVA, Aging factor, $p = 0.001$, Temperature factor, $p = 0.173$). (D.) Flies aged at 29°C show no significant differences in polyloid cell accumulation in the brain compared to flies aged at 25°C (2-way ANOVA, Aging factor, $p < 0.0001$, Temperature factor, $p = 0.641$). (E.) However the proportion of cells with a DNA content exceeding 4C significantly increased with age at 29°C (2-way ANOVA, Aging factor, $p < 0.0001$, Interaction, $p = 0.0003$, Sidak's multiple comparisons test). (F.) Housing flies at 29°C did not significantly impact cell death in the adult brain (2-way ANOVA, Aging factor, $p < 0.0001$, Temperature factor, $p = 0.914$). (G.) Flies fed a low protein diet (30% reduced yeast, RY) exhibit slowed accumulation of polyloid cells in the brain (2-way ANOVA, Aging factor, $p < 0.0001$, Diet factor, $p < 0.0001$, Sidak's multiple comparisons test). (H.) Low protein-fed flies accumulate fewer cells with a DNA content exceeding 4C during aging (2-way ANOVA, Aging factor, $p < 0.0001$, Diet factor, $p < 0.0001$, Sidak's multiple comparisons test). (I.) Cell death was not influenced by the low protein diet (2-way ANOVA, Diet factor, $p = 0.171$). Graphs display individual biological replicates, with SEM. All significant differences are noted: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

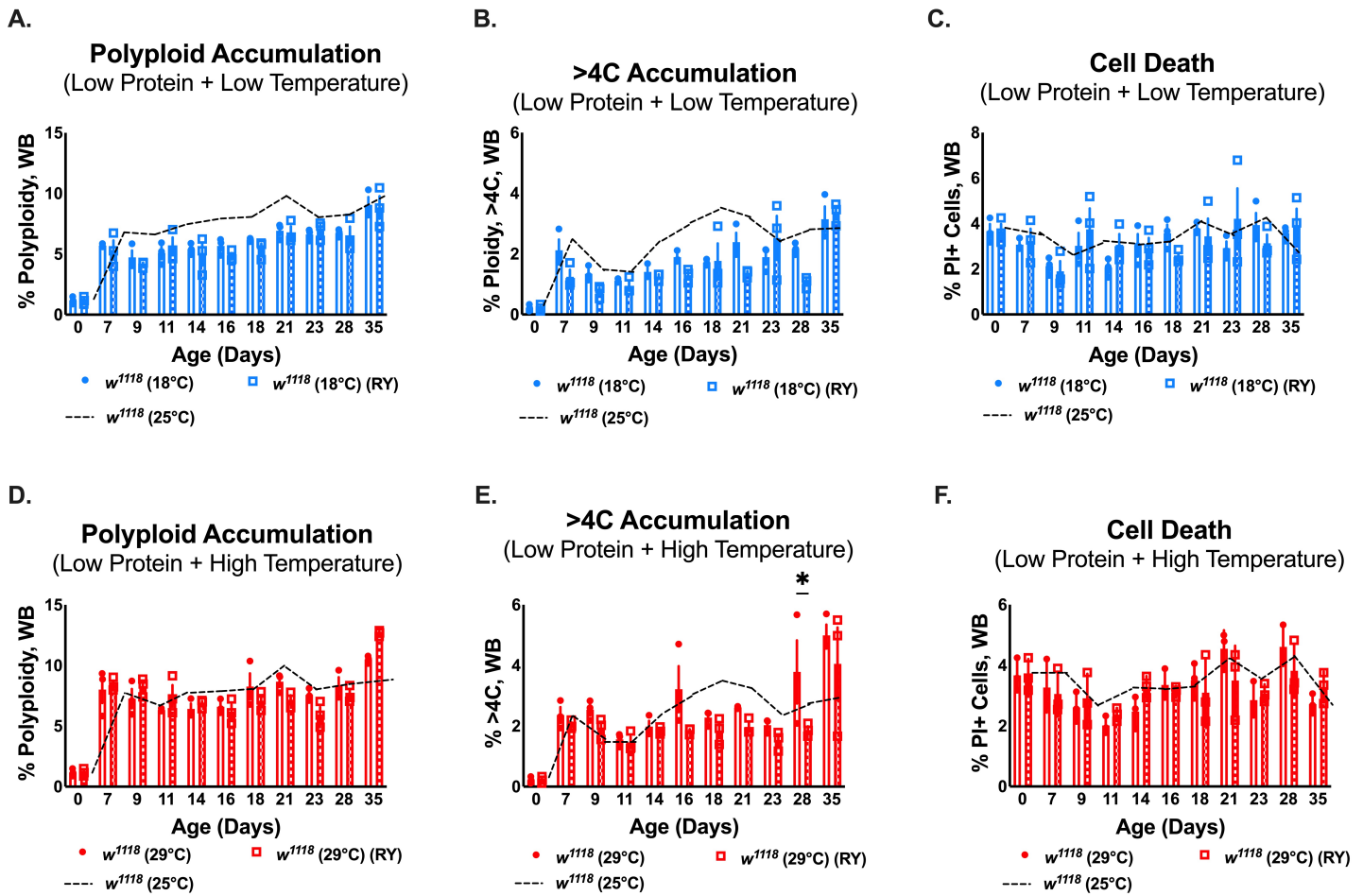


Figure 4: The accumulation of cells with a higher than 4C DNA content may rely on protein availability. (A.) overall polyploidy in the brain is similar between flies housed at 18°C and flies housed at 18°C and fed a low-protein diet (2-way ANOVA, Diet & Temperature factor, $p=0.423$). (B.) Combining a low temperature and a low protein diet mildly increases the accumulation of cells with a DNA content that is more than 4C relative to just housing at low temperature (2-way ANOVA, Aging factor, $p<0.0001$, Temperature & Diet factor, $p=0.0056$, interaction, $p=0.041$, Sidak's multiple comparisons test). (C.) Housing flies at a low temperature in addition to feeding a reduced protein diet has no impact on cell death (2-way ANOVA, Aging factor, $p=0.057$, Temperature & Diet factor, $p=0.669$). (D.) Feeding a low protein diet to flies housed at a high temperature does not influence polyploid accumulation (2-way ANOVA, Aging factor, $p<0.0001$, Temperature & Diet factor, $p=0.879$). (E.) However, the number of cells with more than 4C DNA content is influenced by housing flies at a high temperature with reduced protein (2-way ANOVA, Aging factor, $p<0.0001$, Temperature & Diet factor, $p=0.007$, Sidak's multiple comparisons test). (F.) Housing flies at a reduced temperature in addition to feeding a reduced protein diet has no significant impact on cell death (2-way ANOVA, Aging factor, $p=0.057$, Temperature & Diet factor, $p=0.876$). Graphs display individual biological replicates, with SEM. Each biological replicate contains one male and one female brain. All significant differences are noted: * $p<.05$, ** $p<.01$ *** $p<0.001$, **** $p<0.0001$.

A.

Control		Reduced Temperature		Reduced Protein	
Age in Days	% Lifespan	Age in Days	% Lifespan	Age in Days	% Lifespan
0	0	0	0	0	0
7	7.09	7	4.51	7	7.09
9	10.29	9	5.80	9	9.11
11	12.57	11	7.09	11	11.14
14	16.00	14	9.02	14	14.18
16	18.29	16	10.31	16	16.20
18	20.57	18	11.59	18	18.23
21	24.00	21	13.53	21	21.27
23	26.29	23	14.81	23	23.29
28	32.00	28	18.04	28	28.35
35	40.00	35	22.54	35	35.44

B.

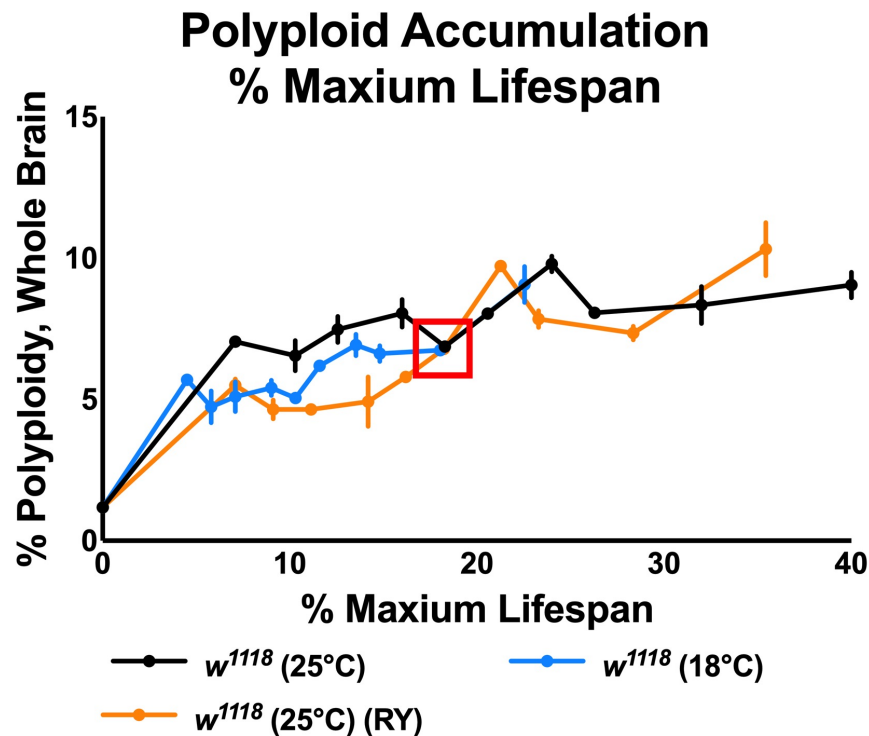


Figure 5: The cell cycle re-entry window for cells in the adult fly brain is flexible and impacted by temperature and diet. (A.) A table displaying chronological age with the percent of the maximum lifespan for conditions that prolong lifespan. (B.) The proportion of polyploid cells in the brain vs. percent of the maximum lifespan. Reduced temperature and reduced yeast slow the accumulation of polyploid cells, even when normalized to percent of maximum lifespan. Red box indicate when polyploidy levels under reduced temperature or reduced yeast reach control levels.

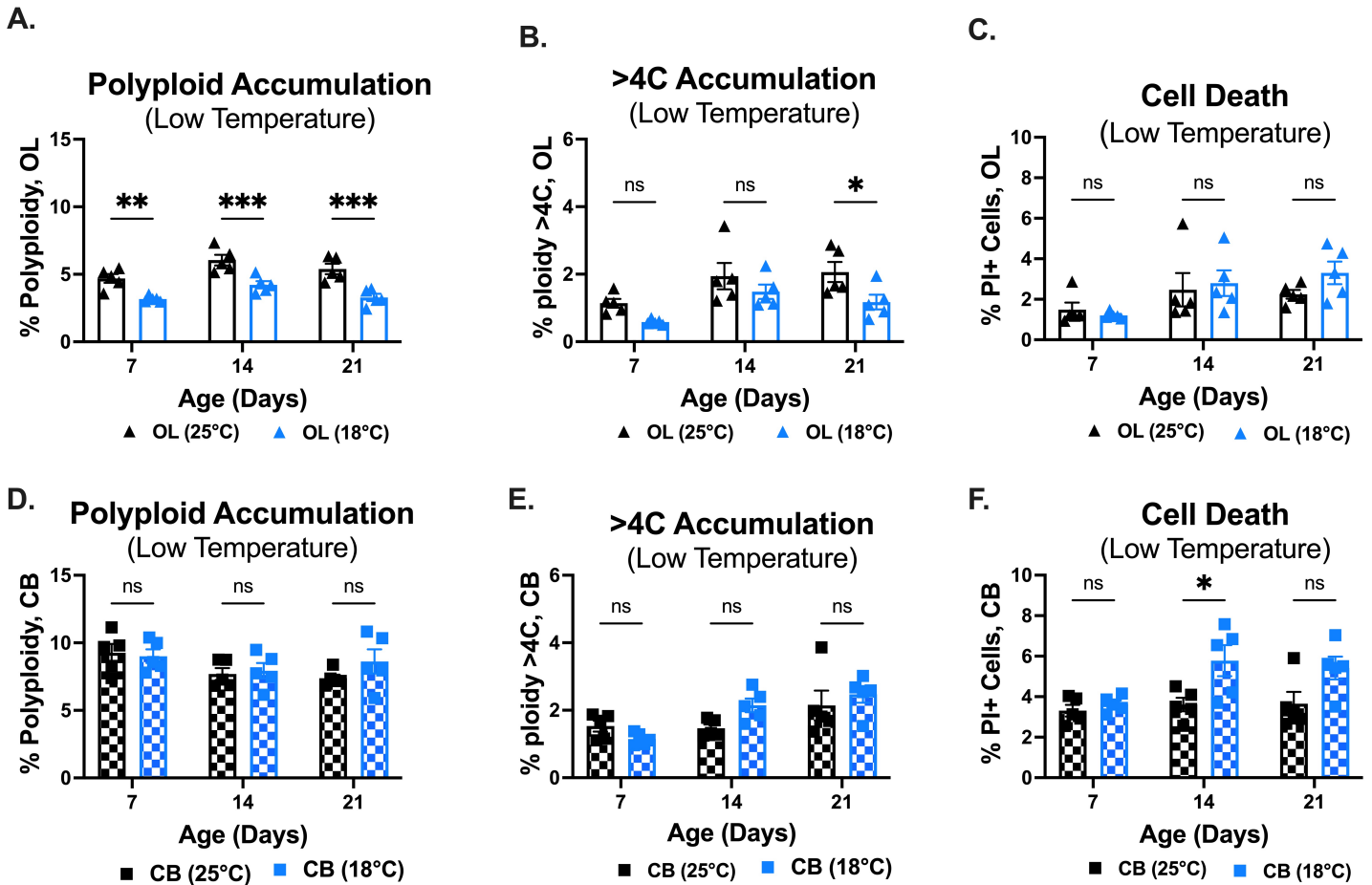


Figure 6: Housing flies at a low temperature impacts polyploidy in the optic lobes more than the central brain. (A.) Flies housed at 18°C accumulate fewer polyloid cells in their optic lobes (OL) (5 biological replicates per time point, 4 optic lobes per biological replicate 2-way ANOVA, Aging factor, $p=0.002$, Temperature factor, $p<0.0001$, Sidak's multiple comparison's test). (B.) The proportion of cells with more than 4c DNA content is reduced in flies housed at 18°C (5 biological replicates per time point, 4 OL per biological replicate 2-way ANOVA, Aging factor, $p=0.0031$, Temperature factor, $p=0.0037$, Sidak's multiple comparison's test). (C.) Housing temperature does not significantly impact cell death (5 biological replicates per time point, 4 OLs per biological replicate, 2-way ANOVA, Aging factor, $p=0.018$). (D.) Temperature does not affect polyloid accumulation in the central brain (CB) (2-way ANOVA, Temperature factor, $p=0.420$). (E.) The proportion of cells that exceed a 4C DNA content increases with age regardless of housing temperature (2-way ANOVA, Aging factor, $p=0.002$). (F.) Low temperature impacts cell death in the CB (2-way ANOVA, Aging factor, $p=0.042$, Temperature factor, $p=0.0018$). Graphs display individual biological replicates, with SEM. All significant differences are noted: * $p<.05$, ** $p<.01$ *** $p<0.001$, **** $p<0.0001$. Abbreviations: Optic Lobe (OL), Central brain (CB)

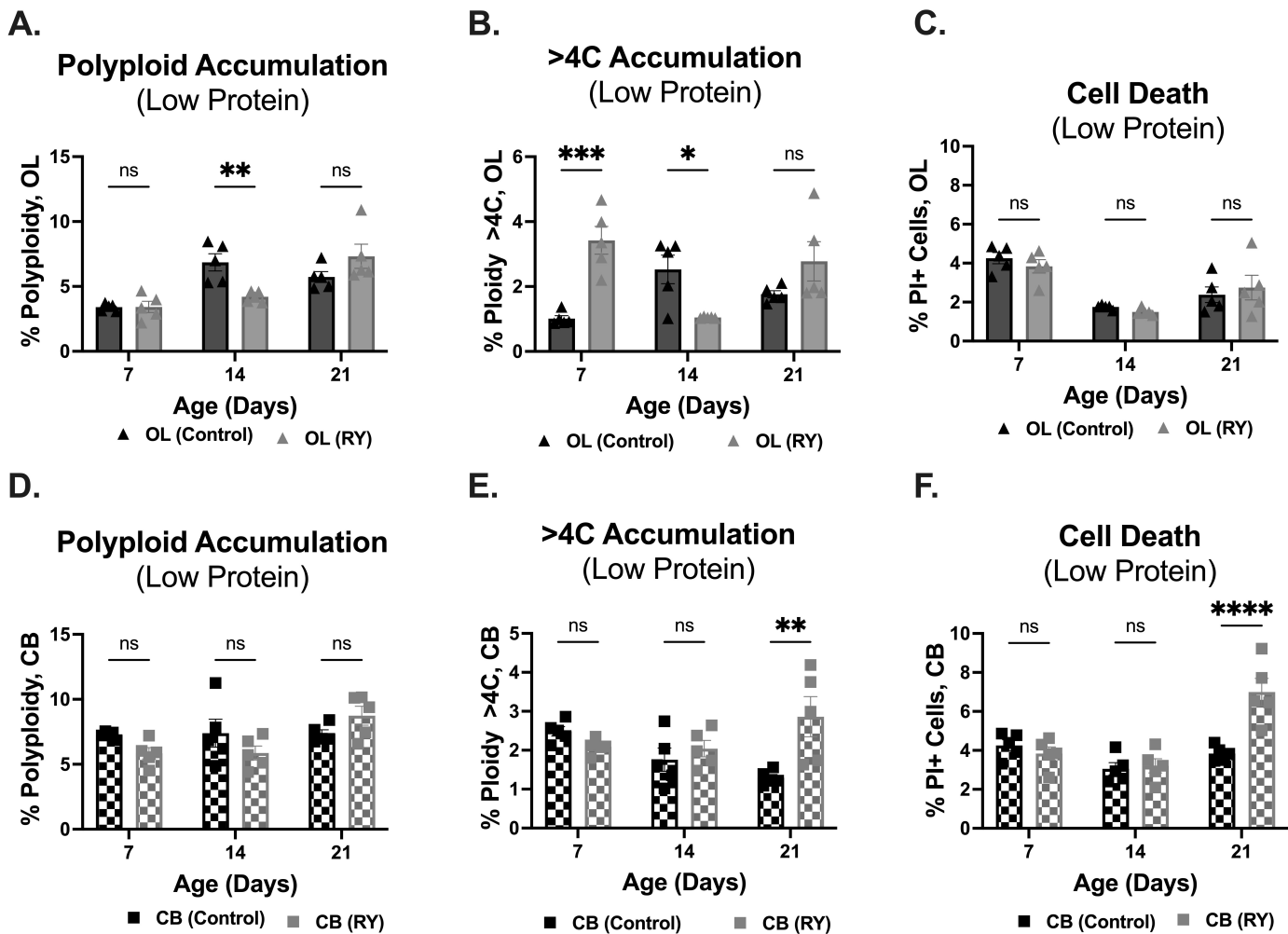


Figure 7: The impact of a low-protein diet on polyplody is brain region specific. (A.) At age day 14, flies fed a low-protein diet have fewer polyploid cells in their optic lobes (OL) (5 biological replicates per time point, 4 OL per biological replicate, 2-way ANOVA, Aging factor, $p < 0.0001$, Interaction, $p = 0.0021$, Sidak's multiple comparison's test). (B.) A low-protein diet influences the number of cells that exceeded a 4C DNA content in the OL of flies fed (5 biological replicates per time point, 4 OL per biological replicate, 2-way ANOVA, Aging factor, $p = 0.347$, Interaction, $p < 0.0001$, Sidak's multiple comparison's test). (C.) Regardless of diet, cell death in the OL decreased during aging (2-way ANOVA, Aging factor, $p < 0.0001$). (D.) Polyploid accumulation in the central brain (CB) was not influenced by diet (5 biological replicates per time point, 2 CB per biological replicate, 2-way ANOVA, Aging factor, $p = 0.0394$, Interaction, $p = 0.042$, Sidak's multiple comparison's test). (E.) At age day 21, flies on a low-protein diet accumulated more cells with a DNA content exceeding 4C in the CB (5 biological replicates per time point, 2 CB per biological replicate, 2-way ANOVA, Diet, $p = 0.035$, Interaction, $p = 0.0042$, Sidak's multiple comparison's test). (F.) A low-protein diet alters cell death in the CB (5 biological replicates per time point, 2 CB per biological replicate, 2-way ANOVA, Aging factor, $p < 0.0001$, Interaction, $p = 0.0003$, Diet factor, $p = 0.0068$, Sidak's multiple comparison's test). Graphs display individual biological replicates, with SEM. All significant differences are noted: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Abbreviations: Optic Lobe (OL), Central brain (CB)