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Storage cell proliferation during somatic growth establishes that tardigrades are not eutelic organisms

Gonzalo Quiroga-Artigas^{1,*} and María Moriel-Carretero^{1,*}

1: *Centre de Recherche en Biologie cellulaire de Montpellier (CRBM), Université de Montpellier, Centre National de la Recherche Scientifique, 34293 Montpellier CEDEX 05, France.*

* Correspondence: gonzalo.quiroga-artigas@crbm.cnrs.fr
maria.moriel@crbm.cnrs.fr

ABSTRACT

Tardigrades, microscopic ecdysozoans renowned for their resilience to extreme environments, have long been thought to maintain a constant cell number after completing embryonic development, a phenomenon known as eutely. However, sporadic reports of dividing cells have raised questions about this assumption. In this study, we investigated whether tardigrades truly exhibit a fixed cell number during somatic growth using the model species *Hypsibius exemplaris*. Comparing hatchlings to adults, we observed an overall increase in the number of storage cells, a tardigrade cell type involved in nutrient storage. To assess cell proliferation, we monitored DNA replication via the incorporation of the thymidine analog 5-ethynyl-2'-deoxyuridine (EdU). A significantly higher number of storage cells incorporated EdU while animals were still growing. Starvation halted both animal growth and storage cell proliferation, linking the two processes. Additionally, we found that EdU incorporation in storage cells is associated with molting, a critical process in tardigrade post-embryonic development, since it involves cuticle renewal to enable further growth. Finally, we show that hydroxyurea, a drug that slows down DNA replication progression, strongly reduces the number of EdU⁺ cells and results in molting-related fatalities. Our data not only provide a comprehensive picture of replication events during tardigrade growth but also highlight the critical role of proper DNA replication in tardigrade molting and survival. This study definitively challenges the notion of eutely in tardigrades, offering promising avenues for exploring cell cycle, replication stress, and DNA damage management in these remarkable creatures as genetic manipulation techniques emerge within the tardigrade research field.

37 **Keywords:** tardigrade, storage cell, eutely, EdU, cell proliferation, DNA replication

38

39 **Abbreviations:** DAPI (4',6-diamidino-2-phenylindole), BODIPY (Difluoro{2-[1-(3,5-dimethyl-
40 2H-pyrrol-2-ylidene-N)ethyl]-3,5-dimethyl-1H-pyrrolato-N}boron), EdU (5-ethynyl-2'-
41 deoxyuridine), HU (hydroxyurea).

42

43 **SIGNIFICANCE**

44 Tardigrades, microscopic invertebrate animals renowned for their resilience in
45 extreme conditions, have traditionally been considered eutelic, implying little to no somatic
46 cell proliferation during their growth. However, a few isolated reports challenged this notion.
47 In this study, using the emerging model *Hypsibius exemplaris*, we provide unequivocal
48 molecular evidence of DNA replication and proliferation in a specific tardigrade cell type
49 called 'storage cells', primarily involved in nutrient storage, throughout the animal's growth.
50 Furthermore, we demonstrate that this proliferation is associated with the timing of cuticle
51 molting, and we highlight the critical role of proper DNA replication in tardigrade molting and
52 survival. Our research definitively resolves the long-standing controversy surrounding
53 tardigrade eutely, opening up uncharted territories in tardigrade research.

54 INTRODUCTION

55 Tardigrades, often called water bears, are tiny animals (0.1-1mm long) that belong to
56 the superphylum Ecdysozoa (Fig. 1A). This clade is characterized by the presence of an
57 exoskeleton or a cuticle, which acts as a protection for their bodies (1). Tardigrades' cuticle
58 plays pivotal roles in their life cycle, enabling them to respond and adapt to diverse
59 environmental challenges (2). Like other ecdysozoans, tardigrades must undergo a process
60 called ecdysis (*i.e.*, molting), which involves producing a new cuticle and shedding the old one
61 (known as exuvium), in order to grow in size (1, 2). Tardigrades are renowned for their
62 exceptional capacity to withstand extreme conditions, leading to a significant surge in research
63 on this subject in recent years (3, 4). However, a more comprehensive understanding of
64 tardigrade biology under physiological conditions is also crucial, as it is necessary to really
65 understand the changes they undergo when exposed to harsh scenarios.

66 Among tardigrades, *Hypsibius exemplaris* (Fig. 1B) is established as an emerging
67 model organism in evolutionary developmental biology and extreme tolerance research (5, 6).
68 Like many other tardigrades, it exhibits parthenogenesis and synchronizes its molting period
69 with oviposition to provide additional physical protection to the developing embryos through
70 the exuvium (6, 7). *H. exemplaris* hatch from their eggshells upon completion of embryonic
71 development, measuring about 125 μm in length, and resembling small adults. They
72 progressively increase in size through multiple molts, eventually reaching a fully-grown adult
73 state measuring approximately 240 μm in length ((8), this work). Subsequently, they continue
74 to molt at a relatively constant rate until their death, although these subsequent molting events
75 no longer contribute to additional growth (Fig. 1C). *H. exemplaris* start producing eggs (*i.e.*,
76 reach sexual maturity) shortly after hatching, long before reaching their fully-grown size
77 (Fig.1D; (7, 9)). To simplify the terminology, we named 'adults' those tardigrades that had both
78 reached sexual maturity and their fully-grown size, and 'juveniles' those that were still growing,
79 irrespective of sexual maturity (Fig. 1C-D).

80 Tardigrades feature a particular cell type, the storage cells, found floating freely
81 throughout their body cavity fluid ((10, 11); Fig. 1B). Their primary function is to assist in
82 tardigrade nutritional maintenance by storing and distributing energy in the form of protein,
83 glycogen, and fat (11, 12). They also participate in vitellogenesis (11, 13), and potentially
84 contribute to immunity (14). A recent study has shown increased expression of genes
85 belonging to the SAHS (secretory abundant heat-soluble) family in storage cells (15), hinting
86 at their potential involvement in desiccation tolerance. In *H. exemplaris*, lipids constitute the
87 primary reservoir material within these cells (11). These lipids can be stained with vital dyes
88 such as BODIPY ((11, 16), see methods), revealing abundant fat in the form of lipid droplets
89 inside the cytoplasm of storage cells (Fig. 1E-F). Storage cells can be isolated from the

90 organism through dissection, enabling independent experimentation with them, separate from
91 the whole organism ((17); Fig. 1F).

92 Eutely refers to a biological phenomenon observed in certain organisms where somatic
93 cell division ceases once embryonic development is complete, implying a relative constancy
94 in cell numbers throughout an animal's growth (18). This suggests that subsequent growth
95 involves an increase in cell size rather than an increase in cell count. The prevailing belief,
96 both in peer-reviewed scientific literature (1, 9, 19, 20) and in tardigrade biology outreach
97 websites, is that tardigrades are eutelic animals. However, over 50 years ago, variations in
98 organ cell numbers and potential somatic mitoses among different tardigrade species were
99 already documented (21, 22). More recently, two studies pointed at cell proliferation in two
100 different somatic cell types. One study captured a limited number of storage cells presenting
101 condensed chromosomes using DNA staining approaches in the tardigrade *Richtersius*
102 *coronifer* (23). Another study showed molecular evidence of cell proliferation at the anterior
103 and posterior ends of the midgut in the tardigrade *H. exemplaris*, a process involved in
104 replacing the cells lining the gut rather than in increasing the overall cell numbers (24).
105 Discrepancies regarding which cell types proliferate were noted by the authors compared to
106 previous claims, possibly due to the starvation conditions in which the experiments were
107 performed (24). In a recent biology book (25), the authors wrote 'facts as basic as whether or
108 not tardigrades are eutelic required considerable investigation (*i.e.* active bibliographic search)
109 from our side'. Further, in a recent tardigrade review (26) the question 'Are tardigrades eutelic
110 animals?' was discussed, and the authors reasoned that this was not the case, due to the
111 isolated indications of cell divisions and the cell number inconstancy studies mentioned above.
112 The ongoing debate surrounding whether tardigrades are genuinely eutelic or not, and the
113 apparent difficulty for both the broader scientific community and the general public to reach a
114 consensus on this matter, underscores the need for a more definitive characterization of the
115 cell types that proliferate under normal physiological conditions within this enigmatic phylum.

116 In this study, we used *H. exemplaris* to determine whether somatic cell proliferation and
117 an increase in cell numbers occur during tardigrade post-embryonic growth. Our findings
118 reveal that overall cell numbers increase when comparing hatchlings to adults, mainly
119 attributed to a prominent rise in storage cell numbers. Notably, EdU, a molecular tool for
120 monitoring DNA replication, incorporates into storage cells at significantly higher rates during
121 tardigrade growth than in fully-grown adults. We also show that starvation arrests growth and
122 blocks EdU incorporation in storage cells, altogether linking storage cell proliferation to somatic
123 growth. Moreover, we found a direct association between storage cell DNA replication and
124 ecdysis, and reveal that hampering DNA replication provokes animal death during molting. Our
125 results offer a comprehensive insight into DNA replication patterns during tardigrade growth,
126 decisively demonstrating that tardigrades cannot be categorized as eutelic animals.

127 RESULTS

128

129 Tardigrade growth is accompanied by an increase in storage cell number

130 We first collected and fixed animals at both extremes of body size: hatchlings and fully-
131 grown adults (Fig. 1C). We utilized the fluorescent DNA marker DAPI (4',6-diamidino-2-
132 phenylindole) to quantify the number of cell nuclei present at each growth stage (see methods;
133 Fig. 2A; SI Appendix, Video S1). Our analysis revealed approximately 1140 ± 60 nuclei in
134 hatchlings and approximately 1400 ± 118 in adults, pointing at a significant increase in cell
135 numbers as the animals attained larger sizes (Fig. 2A-B). As we compared adult animals to
136 hatchlings, we observed a notable increase in the area stained by the lipid vital dye BODIPY
137 (Fig. 2C, in green). This led us to hypothesize that this change could be associated with an
138 augmented number of storage cells. Leveraging the nuclei's position within the animal, their
139 small size, and the intense BODIPY staining surrounding these nuclei, we successfully
140 identified and quantified the number of storage cells in both hatchlings and adult tardigrades.
141 While hatchlings contained 72 ± 10 storage cells, the counts for adult *H. exemplaris* showed
142 they bear 281 ± 64 storage cells, indicating a significant rise in storage cell numbers (Fig. 2C-
143 D; SI Appendix, Video S2). Accordingly, we occasionally observed storage cells that appeared
144 to be in the process of division (SI Appendix, Fig. S1). Intriguingly, the difference in the total
145 number of cells, and the difference in the total number of storage cells between hatchlings and
146 adults was similar, suggesting that the overall increase in cell number primarily results from
147 storage cell proliferation. Our results show that the number of cells increases during tardigrade
148 somatic growth and suggest that storage cells are the main cell type contributing to this raise.
149

150 Storage cells predominantly proliferate during animal growth

151 To investigate cell proliferation at the molecular level, we monitored DNA replication
152 via the incorporation of the thymidine analog EdU, a marker previously employed successfully
153 in tardigrades (24). Initially, we collected hatchlings and incubated them in EdU for three weeks
154 (Fig. 3A), approximately the duration required for a hatchling to develop into a fully-grown adult
155 when kept at 15°C and fed *ad libitum* (Fig. 1C). This approach had the potential of allowing us
156 to identify any DNA replication that may take place during *H. exemplaris* growth. We detected
157 EdU incorporation in storage cells, germ cells inside the ovary, and gut cells (Fig. 3B-B').
158 Interestingly, there was an absence of EdU incorporation in any other organ, including
159 epidermal cells, brain cells, ganglia, buccal apparatus, Malpighian tubules, and claw glands
160 (Fig. 3B). While it is known that gut cells incorporate EdU in *H. exemplaris* (24), our observation
161 represents the first molecular evidence demonstrating DNA replication, and thus cell
162 proliferation, in tardigrade storage cells.

163 Tardigrade germ cells reside in the anterior end of the ovary, and derive from primordial
164 germ cells that are specified during embryonic development (27). They form germ cell clusters
165 by undergoing mitosis followed by incomplete cytokinesis (28, 29), thus being expected to
166 incorporate EdU. Within these clusters, the cell completing meiosis develops into an oocyte,
167 while the remaining differentiate into trophocytes (nourishing cells) (28, 29). Accordingly, in
168 addition to EdU incorporation in germ cells, we observed EdU signal in trophocytes and
169 oocytes of *H. exemplaris* (SI Appendix, Fig. S2), corroborating the notion that these cell types
170 are the progeny of germ cell clusters.

171 Next, we explored whether storage cell proliferation varied between (growing) juveniles
172 and (fully-grown) adults. Juveniles and adults were incubated in EdU for seven days (Fig. 3C),
173 a duration sufficient for all animals to experience at least one molting event when kept at 15°C.
174 We observed that growing juveniles incorporated EdU in a large number of storage cells,
175 whereas we could only detect a limited number of EdU⁺ storage cells in adults (Fig. 3D – green
176 asterisks), rendering the differences highly significant (Fig. 3E). Juveniles' EdU incorporation
177 in a substantial number of storage cells occurred regardless of the animals' reproductive status
178 at the time of fixation (non-gravid – Fig. 3D; gravid, or laying eggs – SI Appendix, Fig. S3). Our
179 findings first show that the majority of storage cell number expansion takes place during
180 tardigrade growth. Second, they evidence that storage cell proliferation also happens, although
181 to a much-lessened extent, after tardigrades have reached their fully-grown status.

182

183 **Starvation halts animal growth and suppresses storage cell proliferation**

184 Tardigrades exhibit remarkable starvation tolerance, enduring several weeks without
185 food (12). Although prior studies noted that starvation reduces storage cell size (12), little is
186 known about its potential effects on tardigrade growth and cell proliferation. To assess this,
187 we first collected small juveniles of *H. exemplaris* and transferred them to dishes with and
188 without *Chlorococcum* algae ("Fed" and "Starved" conditions). We observed that the majority
189 of starved animals entered a contracted, resting state (Fig. 4A) shortly after food deprivation.
190 Upon reintroducing algae after one week, they resumed activity within minutes, and their guts
191 were filled with algae within 24 hours (SI Appendix, Fig. S4A), indicating reversibility of the
192 'starvation state'. We also noticed a marked reduction in lipid staining inside the storage cells
193 of starved individuals after one week (SI Appendix, Fig. S4B), suggesting that the fat
194 reserves of storage cells were utilized to sustain the animals during starvation. Additionally,
195 we collected small juveniles again, measured their length, and exposed them to the
196 aforementioned conditions. After seven days, fed individuals exhibited significant growth,
197 while starved ones showed no substantial increase in size (Fig. 4B-C), implying that
198 starvation induces growth arrest in tardigrades.

199 To assess cell proliferation during food deprivation, we simultaneously exposed
200 juveniles and adults to EdU and starvation for seven days (Fig. 4D). In both cases, we found
201 a complete absence of EdU incorporation in storage cells, with rare exceptions where a few
202 storage cells were EdU⁺ (Fig. 4E-F). Essentially, in most individuals of either growth stage we
203 could only detect EdU⁺ gut cells (Fig. 4E – red asterisks). These results align with a previous
204 study, which showed that starved adult *H. exemplaris* incubated with EdU for up to four days
205 only exhibited EdU incorporation in gut cells (24). Altogether, these findings demonstrate that
206 starvation arrests tardigrade growth and inhibits EdU incorporation in storage cells,
207 unambiguously linking storage cell proliferation to growth.

208

209 **EdU incorporation in storage cells takes place during molting**

210 To investigate whether DNA replication in storage cells occurs at specific stages of *H.*
211 *exemplaris* post-embryonic development or is a continuous process throughout growth, we
212 conducted 24-hour EdU incubations in juvenile animals, including those in proximity to
213 molting (Fig. 5A). The vast majority of animals that did not molt during the 24-hour EdU
214 incubation exhibited no EdU incorporation in storage cells (Fig. 5B-C); only gut cells
215 displayed EdU labeling (red asterisks – Fig. 5B). In contrast, juveniles fixed during molting
216 displayed a significant number of EdU⁺ storage cells (green asterisks in Fig. 5B; Fig. 5C), in
217 addition to EdU⁺ gut and germ cells (red and white asterisks, respectively; Fig. 5B). These
218 results strongly associate storage cell proliferation with the molting process (Fisher's exact
219 test: $p < 0.0001$; SI Appendix, Table S1). Hence, our findings indicate that storage cell (and
220 germ cell) proliferation does not occur constantly during tardigrade growth but rather in
221 bursts at each ecdysis.

222

223 **Hampering DNA replication progression results in tardigrade death during** 224 **molting**

225 This prompted us to assess the potential relevance of DNA replication for the molting
226 process. To this purpose, we used hydroxyurea (HU), a drug known to effectively slow down
227 the progression of replication forks and, consequently, EdU incorporation, in other aquatic
228 invertebrates (30, 31). First, to ensure that HU successfully reduces EdU incorporation in
229 replicative cells of *H. exemplaris*, we simultaneously exposed juvenile animals to HU and
230 EdU for seven days (Fig. 5D). We used a 20mM HU dose, intermediate between the doses
231 typically used in yeast (~100mM) and human cells (1-5mM) (32). We observed an overall
232 decrease in EdU incorporation in animals subjected to 20mM HU compared to control
233 animals (Fig. 5E). Quantification of the number of EdU⁺ storage cells, gut cells, and germ
234 cells (including their progeny: trophocytes and oocytes) in both control and HU-treated

235 condition revealed a highly significant reduction in all cases for animals under HU treatment
236 (Fig. 5F-H). Having confirmed that HU disrupts replication progression in *H. exemplaris* by
237 significantly reducing EdU incorporation in various replicative cell types, we then turned to
238 assess its effects on molting.

239 In these first experiments, we already observed that, by day 7, a significant number
240 of animals had died. To gain a more comprehensive insight into tardigrade survival under HU
241 exposure, we incubated *H. exemplaris* specimens in 20mM HU and monitored mortality for
242 up to 13 days. After nine days of incubation, allowing sufficient time for all treated animals to
243 molt, we found that $96\% \pm 4\%$ of the HU-treated animals had perished (Fig. 5I). Remarkably,
244 all dead animals died during ecdysis, irrespective of their size or whether they had undergone
245 egg laying during the process (Fig. 5E), indicating that ecdysis-associated deaths were
246 unrelated to oviposition or egg viability during molting. Altogether, we conclude that hindering
247 DNA replication leads to molting-related fatalities, emphasizing the critical role of accurate
248 DNA replication in allowing effective tardigrade molting and survival.

249 DISCUSSION

250 This study addresses the controversy in the current literature regarding whether
251 tardigrades maintain a constant number of cells during post-embryonic development, and we
252 demonstrate unequivocally that this is not the case. Using the emerging model *H. exemplaris*,
253 we illustrate how tardigrade storage cells primarily undergo proliferation during animal growth,
254 leading to an increase in their numbers. We reveal that food deprivation induces a reversible
255 'starvation state' in *H. exemplaris*, during which both growth and storage cell proliferation are
256 halted. Our 24-hour EdU pulse experiments establish a clear association between molting and
257 storage cell DNA replication, and allowed us to illustrate a comprehensive representation of
258 the DNA replication events occurring during tardigrade post-embryonic life for the three main
259 replicative cell types we identified (Fig. 6). Furthermore, we establish that proper DNA
260 replication is essential for tardigrade survival, as its disruption results in mortality during
261 molting, the sole phase in which gut, germ, and storage cells undergo replication
262 simultaneously (Fig. 6).

263 *H. exemplaris* does not present a defined number of cells, typical of eutelic animals, as
264 its cell count increases with age and body growth (Fig. 2B). This is reinforced by the observed
265 variability in the total number of cells among individuals of the same species (Fig. 2B). While
266 we have demonstrated that this increase in cell number is primarily attributed to storage cell
267 proliferation (Fig. 2D; Fig. 3), we also detected cell proliferation in the gut and ovary. However,
268 these proliferative events are involved in cell replacement and the formation and sustenance
269 of oocytes, respectively, rather than in overall cell number increase (24, 28, 29). In contrast to
270 observations from decades ago in various species of *Macrobiotus*, where some ganglion and
271 claw gland cells were interpreted as mitotic (21), our investigations did not reveal any evidence
272 of cell proliferation in any other organs or tissues of *H. exemplaris* (Fig. 3B, SI Appendix, Fig.
273 S2). Thus, storage cells represent the primary cell type in *H. exemplaris* that undergoes
274 proliferation and results in a cell number expansion.

275 Based on our findings, *H. exemplaris* cannot be categorized as entirely eutelic.
276 However, the absence of cell proliferation in specific organs, such as the epidermis and the
277 nervous system, suggests that some degree of cell constancy may exist in various tardigrade
278 organs. To gain a more comprehensive understanding, it would be valuable to reevaluate the
279 proliferative capacities of different cell types in a range of tardigrade species using
280 contemporary molecular techniques like EdU incorporation. This approach could provide
281 insights into whether the absence of cell proliferation in certain organs is a common feature
282 shared across the phylum.

283 The presence of cell proliferation in a limited number of cell types implies that the
284 growth of most tardigrade organs primarily occurs through an increase in cell size rather than

285 through cell proliferation. In this context, an increment in the number of storage cells may serve
286 as a mechanism to support a larger body size and the associated higher demand for stored
287 materials. This hypothesis has been previously suggested (23), and our data offer two pieces
288 of evidence to support it. First, we show that growth arrest induced by starvation in juvenile
289 tardigrades leads to the inhibition of storage cell proliferation (Fig. 4). Second, in adult *H.*
290 *exemplaris*, where full growth has been achieved, the majority of storage cells within the animal
291 no longer undergo proliferation (Fig. 3 D-E). From another perspective, considering that
292 storage cells also contribute to vitellogenesis (11, 13), and given that a larger body size allows
293 for a greater number of eggs to develop in the ovary, we postulate that an increase in storage
294 cell numbers throughout tardigrade growth could further enhance the capacity to produce and
295 nourish a larger number of eggs per individual. This aligns with our unreported observations
296 that adult animals consistently produce more eggs than juveniles.

297 While anterior and posterior midgut cells maintain a constant rate of proliferation (Fig.
298 6, (24)), germ and storage cells appear to be arrested in G₁ or in G₀, only resuming cycling to
299 undergo DNA replication during molting (Fig. 5B-C; Fig. 6). Correspondingly, a similar
300 association between storage cell mitotic activity and molting has been reported in the
301 tardigrade species *R. coronifer* (23). These observations would imply that a specific cue,
302 received at the time of molting, prompts these cell types to resume the cell cycle towards DNA
303 replication. What could be the nature of such a signal? Tardigrades share orthologs of several
304 genes associated with arthropod molting processes, including the ecdysone receptor and
305 early-activated genes driven by ecdysone (33). This indicates a certain level of conservation
306 of molting determinants within the Panarthropoda (33, 34). Notably, recent research has shown
307 that low ecdysone levels can stimulate cell proliferation in *Drosophila* wing imaginal discs (35).
308 Downstream of ecdysteroid hormones, a cascade of evolutionarily ancient ecdysis-related
309 neuropeptides (ERNs) plays crucial roles during ecdysis (34). Originally responsible for
310 regulating life cycle transitions in both molting and non-molting phyla, these ERNs have been
311 co-opted to orchestrate the molting process in ecdysozoans (34, 36). Some of the genes
312 encoding these ERNs are conserved in tardigrades, including eclosion hormone (EH) and
313 crustacean cardioactive neuropeptide (CCAP), which are upregulated at hatching (36).
314 Intriguingly, *H. exemplaris* presents five EH and two CCAP paralogs (36, 37). Along these
315 lines, a neuropeptide-receptor couple has been found to induce meiotic progression in jellyfish
316 oocytes arrested at prophase I (38), suggesting that ERN-receptor couples could also regulate
317 cell cycle progression. Collectively, we propose that ecdysteroid-type molting hormones and/or
318 ERNs may serve as triggers for the resumption of the germ and storage cell cycle in
319 tardigrades.

320 The question emerges as to why HU kills *H. exemplaris* during molting, irrespective of
321 whether they are juveniles or adults. Indeed, even though adult tardigrades exhibit minimal

322 storage cell proliferation (Fig. 3D-E; Fig. 6), they still perish during molting when exposed to
323 HU treatment. Tardigrade molting events are likely the most energy-demanding stages of their
324 post-embryonic development, since they involve replacing the buccal apparatus, claws, and
325 the entire cuticle (2), synchronization with egg laying (7), and bursts of storage and germ cell
326 proliferation (Fig. 5B-C). Given that gut cells undergo permanent renewal (24), animals' death
327 during molting could reflect a profound impact of disrupting gut cell proliferation, consequently
328 affecting overall energy absorption. In this scenario, the decrease in gut cell proliferation would
329 have a more deleterious effect on survival than that of storage cells.

330 Alternatively, yet not exclusively, molting-associated fatalities could be ascribed to
331 more direct effects of HU. HU slows down the progression of replication forks and inhibits the
332 firing of late origins (39). Consequently, the coordination of the replicative program with other
333 DNA-related processes, such as transcription, will be perturbed (40). As shown in other
334 ecdysozoans, ecdysis is a highly transcriptionally-active process (33). Moreover, it has been
335 recently demonstrated that successful molting in the nematode *Caenorhabditis elegans*
336 requires rhythmic accumulation of transcription factors, which, in turn, relies on rhythmic
337 transcription (41). Therefore, HU treatment could enhance tardigrade lethality during molting
338 due to profound alterations in the execution of the ecdysis transcriptional schedule.
339 Additionally, HU induces DNA damage through oxidative stress (42), and also leads to DNA
340 break accumulation when replication fork stalling is long-lasting (43), two DNA damage-
341 associated signals that trigger a robust DNA damage response (44). In the context of *H.*
342 *exemplaris*, where there is no storage cell proliferation between molting cycles, DNA damage
343 signals may be managed or controlled. However, they may translate into a systemic death
344 signal when this insult occurs during a burst of storage cell proliferation. In agreement, DNA
345 damage in proliferative cell types has recently been shown to trigger an alert in the whole
346 animal that accelerates inflammatory and aging outputs (45).

347 In summary, our study provides the first molecular evidence of storage cell proliferation
348 in a tardigrade species. It determines that tardigrades do not present cell constancy during
349 post-embryonic development, contrarily to several previous claims, thus resolving a long-
350 standing controversy in the field. Moreover, our data shed new light on tardigrade cell biology
351 and establish a clear connection between tardigrade ecdysis and cell cycle transitions. The
352 finding that storage cells undergo replication opens new possibilities for *in vitro* culture of this
353 cell type and suggests its potential as a valuable model for studying cell cycle dynamics,
354 responses to replication stress, and DNA damage control in tardigrades. With the advent of
355 transgenesis (15) and CRISPR (46) technologies in tardigrades, along with the established
356 RNAi methods (47, 48), *Hypsibius exemplaris* is increasingly becoming a promising genetic
357 experimental model, altogether marking an exciting era in tardigrade research.

358 MATERIAL AND METHODS

359

360 Animal husbandry and drug treatment

361 *H. exemplaris* (Z151 strain) husbandry was conducted as previously described (49),
362 with minor modifications. In brief, tardigrades were maintained in an incubator at 15 °C,
363 placed inside 55 mm diameter plastic Petri dishes filled halfway with spring water (Volvic)
364 filtered through a 0.2 µm mesh. To facilitate tardigrade movement, the bottom of the dishes
365 was scratched with sandpaper. Photoperiod and relative humidity inside the incubator were
366 not monitored. Animals were fed *ad libitum* with *Chlorococcum* algae, cultivated in 50 ml
367 tubes with BG-11 Growth Media (Gibco, A1379901). Water changes were performed every
368 two weeks. All experimental incubations lasting ≥24 hours were carried out under these
369 same conditions in 35 mm diameter dishes, placed inside cardboard boxes to avoid
370 degradation of potentially photosensitive compounds.

371 Tardigrades were subjected to incubation with the ribonucleotide reductase inhibitor
372 hydroxyurea (HU; Sigma-Aldrich, H8627) at a concentration of 20 mM, dissolved in filtered
373 spring water (FSW) for the duration of the experiment. Renewal of HU took place every 2-3
374 days to ensure its continued effectiveness.

375

376 DAPI and BODIPY staining

377 Animals were collected and filtered through a 40 µm mesh, then transferred to a glass
378 depression slide within a humid chamber using a glass Pasteur pipette. Whenever possible,
379 samples were kept in darkness for the duration of the experiment. To label neutral lipids, live
380 specimens were incubated in 10 µg/ml BODIPY (Difluoro{2-[1-(3,5-dimethyl-2H-pyrrol-2-
381 ylidene-N)ethyl]-3,5-dimethyl-1H-pyrrolato-N}boron; Sigma-Aldrich, 790389) diluted in FSW
382 for 30 minutes. Subsequently, samples were rinsed three times with FSW and fixed in 4%
383 PFA in 1x PBS for 1 hour at room temperature (RT). After fixation, three 15-minute washes in
384 1x PBS were performed. Samples were then incubated in the DNA fluorescent stain DAPI
385 (4',6-diamidino-2-phenylindole; Sigma-Aldrich, D9542) at a concentration of 1 µg/ml in 1x
386 PBS for 30 minutes. Following this step, the samples were washed three times in 1x PBS for
387 15 minutes, transferred to a slide, and mounted in ProLong Gold Antifade Mountant
388 (Invitrogen, P36930) prior to confocal imaging.

389

390 EdU experiments

391 To detect DNA replication, animals were incubated in 100 µM EdU (EdU Click-iT™
392 Cell Proliferation Kit for Imaging, Alexa Fluor 488 Dye; Invitrogen, C10337) diluted in FSW
393 containing algae (except during experimental starvation) for specific durations (24 hours, 7

394 days, or 3 weeks). In the 3-week experiment, EdU was refreshed weekly. Following EdU
395 exposure, tardigrades were collected, transferred to a glass depression slide, and fixed with
396 4% PFA in 1x PBS + 1% Triton X-100 (PTx) for 1 hour at RT. After fixation, the samples
397 underwent three 15-minute washes in PTx. Subsequently, they were blocked for 2 hours in a
398 0.2 μ m-filtered blocking solution containing 10% Bovine Serum Albumin (BSA) in 1x PBS.
399 The Click-iT EdU detection reaction was carried out for 1 hour at RT according to the
400 manufacturer's instructions. After the detection reaction, four 10-minute PTx washes were
401 conducted. DAPI was used to stain nuclei, and samples were mounted in ProLong Gold for
402 confocal imaging, as described above. In some cases, tardigrades were euthanized with
403 10% EtOH before fixation to prevent the animals from contracting during exposure to PFA.

404

405 **Imaging, length measurements, cell counting and statistics**

406 To capture live images of tardigrades, animals were anesthetized using 20mM
407 levamisole hydrochloride (MedChemExpress, HY-13666) in mqH₂O and mounted on slides,
408 covered with coverslips. Clay was positioned in each corner of the coverslip to prevent
409 compression, except for the intentionally compressed specimen in Fig. 1B. Differential
410 interference contrast (DIC) color images were taken with a Leica K3C digital color camera
411 attached to a compound light microscope (Leica Thunder). For measuring the body length of
412 tardigrades, measurements were conducted using ImageJ (50). The measurement was
413 taken from the head to the juncture on the posterior-most segment with legs (9). For starved
414 tardigrades, measurements were obtained immediately after animals came back to an active
415 state, ensuring their full body extension.

416 Fluorescently labeled images were acquired using a Zeiss LSM980 confocal
417 microscope, equipped with an Airyscan2 module. To ensure consistency when comparing
418 samples within a given experiment, identical scanning parameters were applied to all
419 conditions in each independent experiment. Confocal Z-stacks and projections were
420 processed and adjusted for brightness and contrast in ImageJ, using K. Terretaz's
421 visualization toolset (https://github.com/kwolbachia/Visualization_toolset).

422 To count the total number of cells in hatchlings and adults, Imaris software (Oxford
423 instrument) was employed. Nuclei were highlighted using custom thresholding to allow for
424 quantification. Quantifications of BODIPY-stained storage cells and EdU⁺ cells were
425 performed manually in ImageJ. In all instances, z-stacks encompassing the entire depth of
426 the animals were utilized. Supplementary videos S1 and S2 were assembled using Imaris
427 and ImageJ software, respectively. Tardigrade schematics and figure compilation were
428 generated using Adobe Illustrator.

429 To determine statistical significance, normality of all datasets was first assessed using
430 the Shapiro-Wilk test. Two-tailed Student's *t*-test was conducted for the dataset concerning

431 total number of storage cells (Fig. 2D). For all other comparisons, Mann-Whitney *U*
432 nonparametric tests were employed for two-way comparisons, and the Kruskal-Wallis test
433 was used for statistical analyses in Fig. 4C. Fisher's exact test was selected for the
434 association assessment, based on a 2 × 2 contingency table. Statistical significance for all
435 quantitative comparisons is represented as ****, where *p*-value ≤ 0.0001. Graphs and
436 statistical analyses were performed using GraphPad Prism 9.

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439 *Chlorococcum* samples, and for giving us advice on tardigrade manipulation. We also thank
440 Benjamin Lacroix for helping us to establish the model by sharing his equipment, and
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447

448

449 **AUTHOR CONTRIBUTIONS**

450

451 Conceptualization: G.Q.A. and M.M.C

452 Formal Analysis: G.Q.A.

453 Funding acquisition: M.M.C

454 Investigation: G.Q.A.

455 Methodology: G.Q.A.

456 Project administration: G.Q.A. and M.M.C

457 Supervision: M.M.C

458 Validation: G.Q.A.

459 Visualization: G.Q.A.

460 Writing – original draft: G.Q.A. and M.M.C

461 Writing – review & editing: G.Q.A. and M.M.C

462 The authors declare no competing interest.

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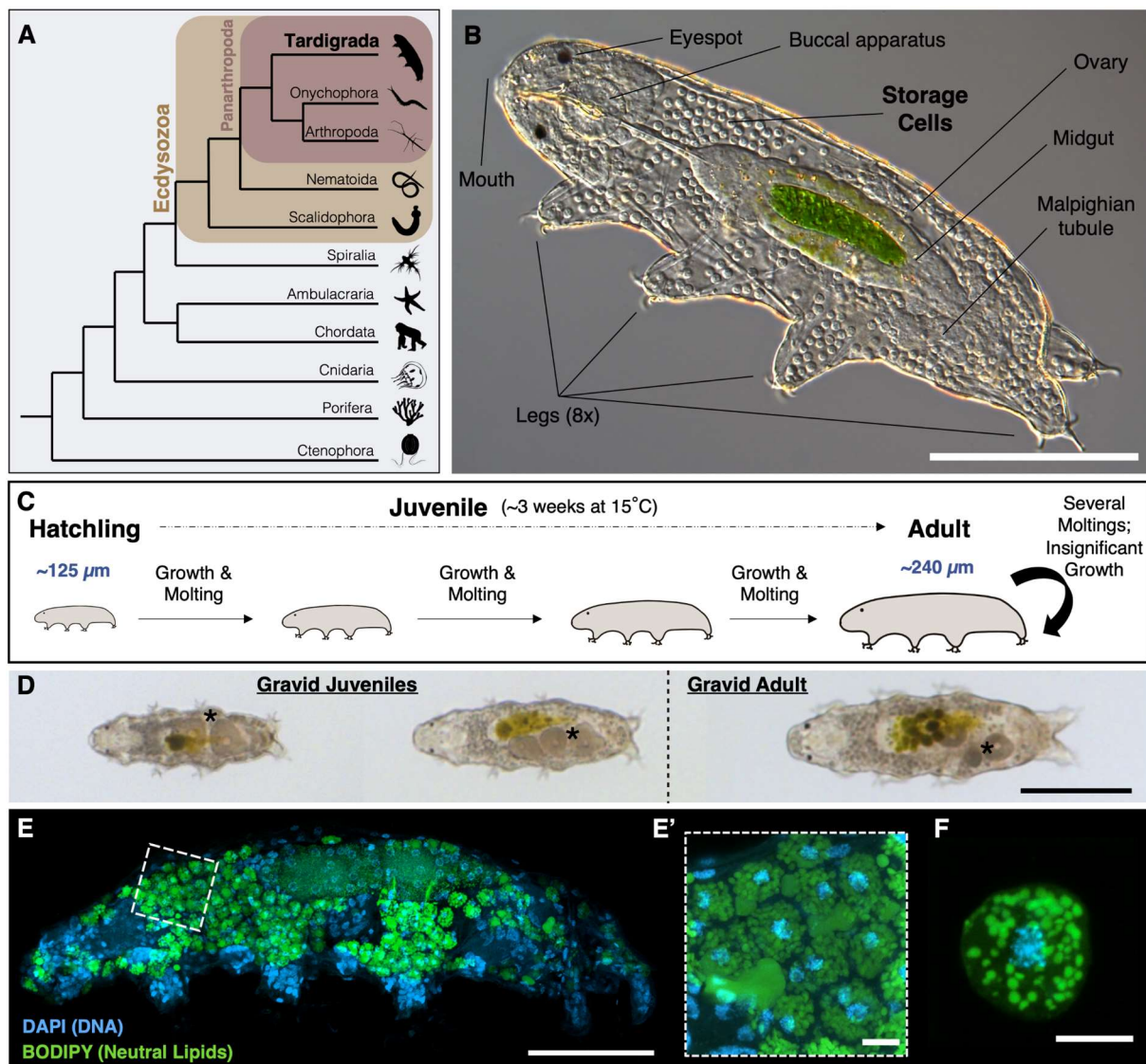
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590

591 **FIGURES AND FIGURE LEGENDS**

592

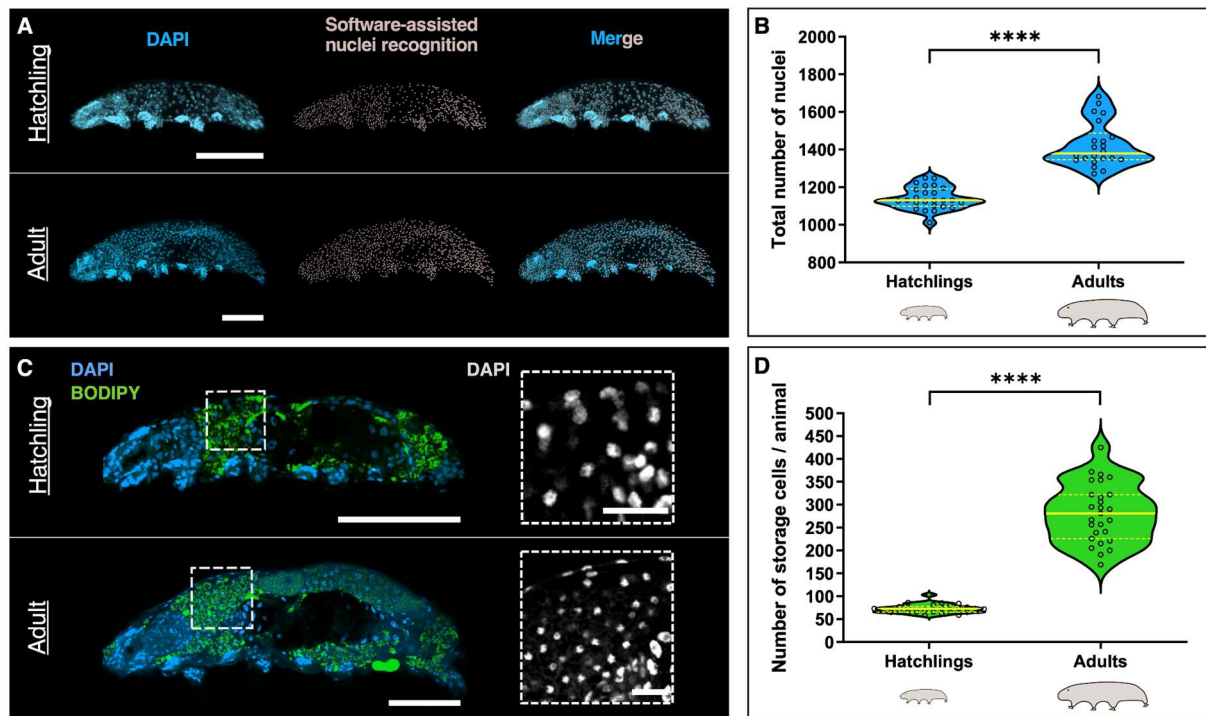


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595 **Figure 1. The tardigrade *Hypsibius exemplaris* and its storage cells.** (A) A simplified
596 animal phylogeny, highlighting the position of tardigrades (Tardigrada) within the Ecdysozoa.
597 Tardigrades share common ancestry with the Onychophora + Arthropoda group, collectively
598 forming the Panarthropoda clade (after Schultz *et al.* (51), Wu *et al.* (52), Laumer *et al.* (53),
599 Dunn *et al.* (54), and Campbell *et al.* (55)). Certain groups like Placozoa and
600 Xenacoelomorpha are omitted for simplicity. Images sourced from Phylopic.org. (B) Anatomy
601 of *H. exemplaris*. Internal organs are revealed in a smushed specimen. The cuticle refracts
602 light and envelops the animal, while the green patch inside the midgut indicates the digestion
603 of *Chlorococcum* algae. (C) Schematic representation of *H. exemplaris* post-embryonic
604 development. (D) Images of two gravid juveniles at different sizes and a gravid fully-grown

605 adult. Black asterisks indicate the location of oocytes. (E) Representative image of a gravid
606 juvenile showing cell nuclei (DAPI, blue) and neutral lipids (BODIPY, green), primarily visible
607 in lipid-rich storage cells. (E') Magnification of the region outlined in (E), emphasizing lipid-
608 filled storage cells. (F) One storage cell isolated from the animal, stained as in (E). The full
609 depth of the tardigrade (E) and of the storage cell (F) was captured in confocal z-stacks, and
610 the images shown are maximum projections. In (E'), a sub-stack was used to facilitate lipid
611 droplet visualization in the storage cells' cytoplasm. Scale bars: 100 μm in (B, D); 50 μm in
612 (E); 5 μm in (E'-F).
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616 **Figure 2. Somatic growth involves overall cell number increase, mainly driven by**

617 **storage cell number expansion.** (A) Representative images of a hatchling (top) and an

618 adult (bottom) of *H. exemplaris*, showing nuclei (DAPI, blue) and software-assisted nuclei

619 recognition (in gray; see methods). Rightmost panels show merged images. The full depth of

620 the animals was captured in confocal z-stacks, and the images shown are maximum

621 projections. (B) Violin plots displaying the total number of nuclei in hatchlings (n = 25) and

622 adults (n = 22). Center yellow lines show the medians; discontinuous yellow lines show the

623 quartiles; the width of the violin at any given point represents the density of data points at

624 that value; each quantified sample is represented by an empty circle. (C) Representative

625 images of a hatchling (top) and an adult (bottom) of *H. exemplaris*, showing DNA (DAPI,

626 blue) and neutral lipids (BODIPY, green). Insets show magnifications of the regions outlined

627 in C, corresponding to the antero-dorsal region of the tardigrade body, where a big proportion

628 of storage cells are normally found (Fig. 1E; (10)). Nuclei in insets are shown in gray.

629 Individual images are shown, rather than maximum projections, to facilitate storage cell

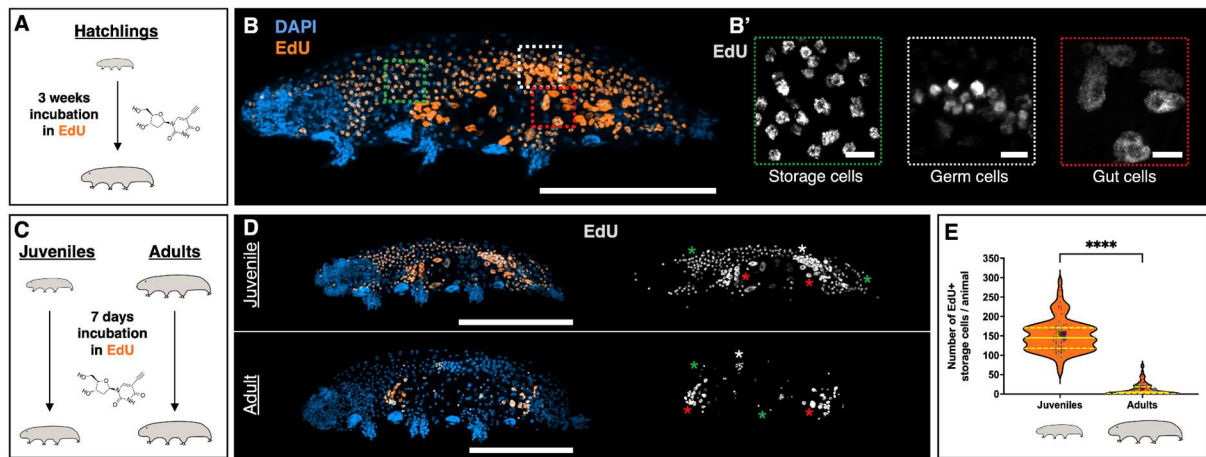
630 visualization within the animals. (D) Violin plots depicting the number of storage cells in

631 hatchlings (n = 26) and adults (n = 27). Violin plot description as in (B). ****, $p \leq 0.0001$.

632 Scale bars: 50 μm in (A, C); 10 μm in (C insets).

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637 **Figure 3. Storage cell proliferation predominantly occurs during somatic growth. (A)**

638 Schematic depicting the 3-week EdU incubation experiment, concerning (B). (B)

639 Representative image of tardigrade upon 3-week exposure to EdU showing nuclei (DAPI,

640 blue) and EdU⁺ cells (orange). (B') Magnification of regions outlined in (B), highlighting the

641 three main replicative cell types labeled with EdU (gray). (C) Experimental schematics for 7-

642 day EdU exposure (D-E). (D) Representative images of juvenile (top) and adult (bottom) after

643 1-week EdU exposure, displaying nuclei (DAPI, blue) and EdU⁺ cells (orange). Right images

644 show only EdU⁺ cells (gray). (E) Violin plots showing the number of EdU⁺ storage cells in

645 juveniles (n = 95) and adults (n = 90). Plotted values belong to three independent

646 experiments. Each quantified animal is represented by a colored dot, with distinct colors

647 corresponding to individual experiments. Larger dots represent each experiment's mean. Plot

648 description as in Fig. 2B. Images shown are maximum projections of the animals' full depth,

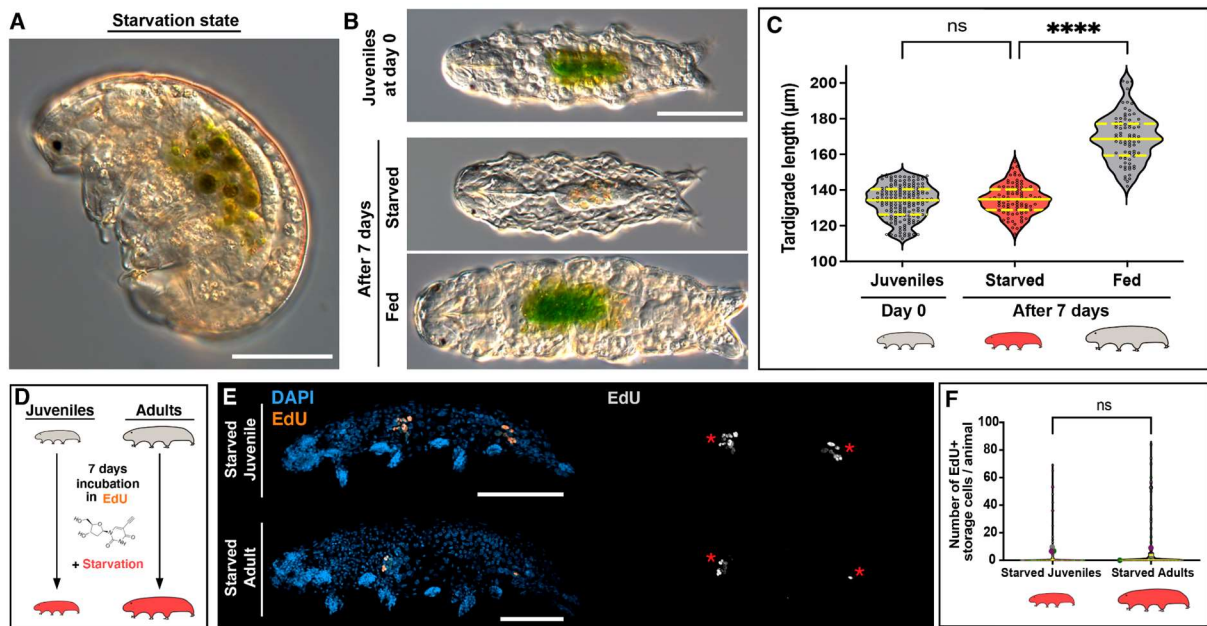
649 except for (B'), which focuses on specific cell types using sub-stack projections. Asterisks

650 denote EdU⁺ cell types (storage cells, green; germ cells, white; gut cells, red). ****, $p \leq$

651 0.0001. Scale bars: 100 μm in (B, D); 5 μm in (B').

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656 **Figure 4. Impact of starvation on growth and storage cell proliferation. (A)**

657 Representative image of *H. exemplaris* in the described 'starvation state'. (B) Representative

658 images of *H. exemplaris* juveniles at day 0 and at day 7 under starved and fed conditions.

659 (C) Violin plots illustrating tardigrade length in juveniles at day 0 (n = 172) and at day 7 under

660 starved (n = 92) and fed (n = 80) conditions. Plot description follows Fig. 2B. (D)

661 Experimental schematics for 7-day EdU exposure in starved juveniles and adults (E-F). (E)

662 Representative images of starved juvenile (top) and starved adult (bottom) after 1-week EdU

663 exposure, showing nuclei (DAPI, blue) and EdU⁺ cells (orange). Right images display only

664 EdU⁺ cells (gray). Red asterisks indicate EdU⁺ gut cells. The full depth of the animals was

665 captured in confocal z-stacks, and the images shown are maximum projections. (F) Violin

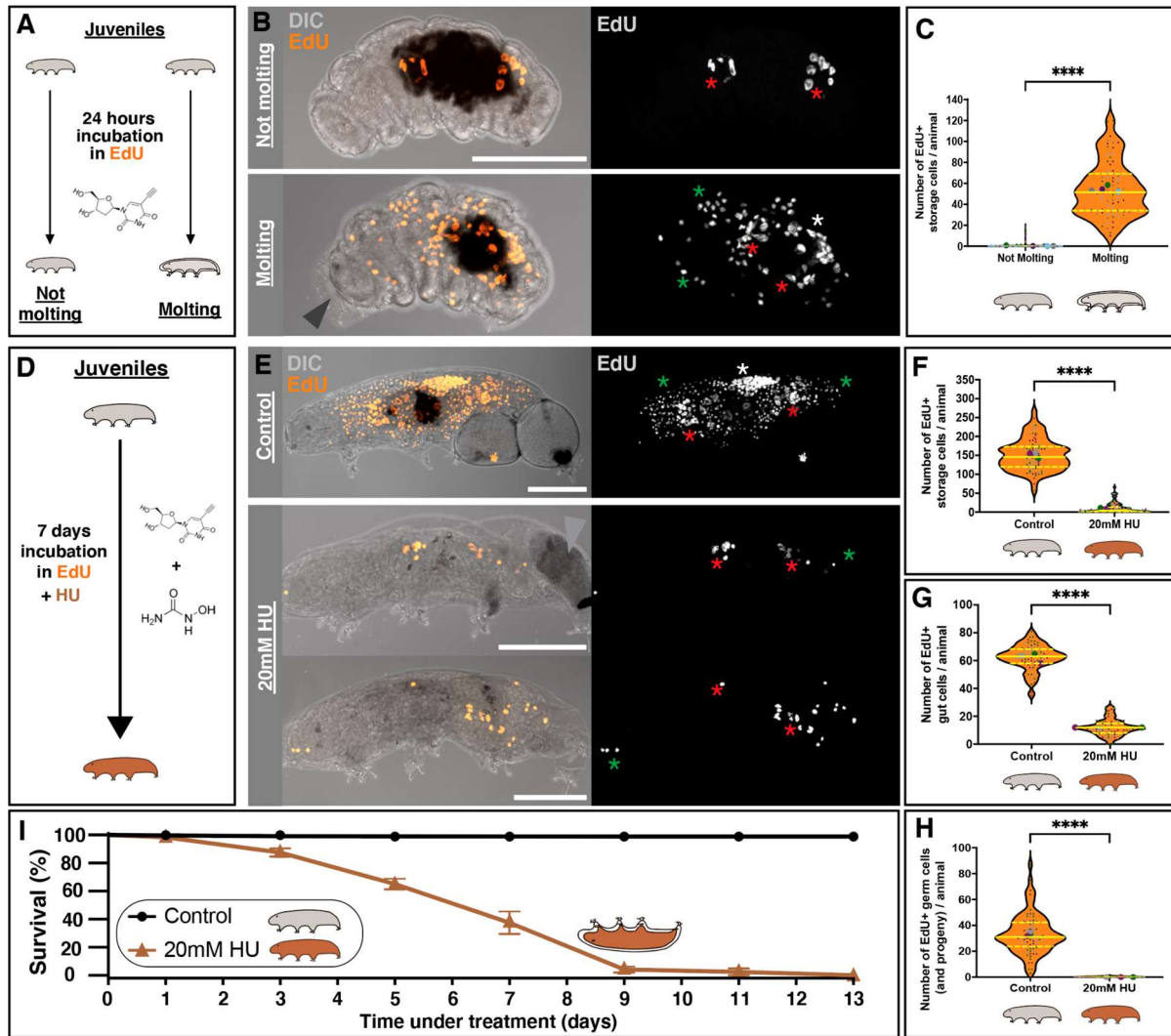
666 plots showing the number of EdU⁺ storage cells in starved juveniles (n = 115) and starved

667 adults (n = 112). Plotted values belong to three independent experiments. Plot conventions

668 are consistent with Fig. 3E. Red tardigrade schematics indicate starved condition. ns = non-

669 significant; **** = p ≤ 0.0001. Scale bars: 50 µm.

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673 **Figure 5. Storage cells undergo replication during molting, while blocking replication**

674 **results in molting-related fatalities.** (A) Experimental schematics for 24-hour EdU

675 exposure (B-C). (B) Representative images of non-molting (top) and molting (bottom)

676 tardigrades upon 24-hour EdU exposure, illustrating animal morphology (DIC, gray) and

677 EdU⁺ cells (orange). Arrowhead indicates the old cuticle being molted. Right images show

678 only EdU⁺ cells (gray). (C) Violin plots displaying the number of EdU⁺ storage cells in non-

679 molting (n = 452) and molting animals (n = 76). Plotted values belong to four independent

680 experiments. (D) Schematic illustrating the 7-day experiment exposing tardigrades to EdU

681 and HU simultaneously, concerning (E-H). (E) Representative images of control (- HU) and

682 20 mM HU-treated animals after 1-week EdU exposure, showing animal morphology (DIC,

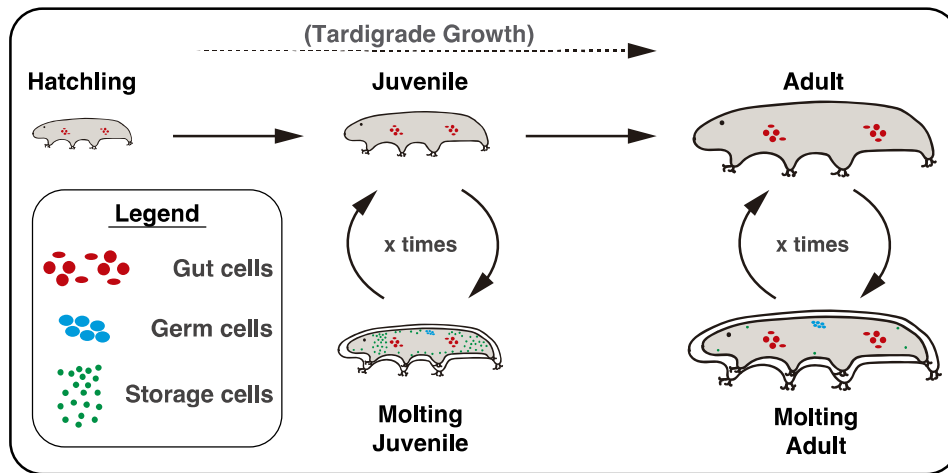
683 gray) and EdU⁺ cells (orange). Arrowhead indicates a laid egg that has degraded. Right

684 images display only EdU⁺ cells (gray). Maximum projections of the animals' full depth are

685 shown. Asterisks as in Fig. 3. Scale bars = 50 μm. (F-H) Violin plots showing the number of

686 EdU⁺ storage cells (F), gut cells (G), and germ cells (including their progeny: oocytes and

687 trophocytes; H) in control (n = 80) and 20 mM HU-treated animals (n = 93). Plotted values
688 belong to three independent experiments. All plot conventions as in Fig. 3E. ****, $p \leq 0.0001$.
689 (I) *H. exemplaris* 13-days survival curve in control (-HU) vs 20mM HU conditions (n = 120,
690 across three independent experiments). Means \pm standard errors of the mean (SE) are
691 plotted. Brown tardigrade schematics indicate 20mM HU condition. The upside-down brown
692 tardigrade schematic represents animal death during molting.
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Figure 6. Overview of DNA replication events in tardigrade post-embryonic

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development. Replicative gut cells are represented as red circles and ovals. Replicative

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germ cells are represented as blue circles. Replicative storage cells are represented as small

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green circles. In summary, gut cells in the anterior and posterior parts of the midgut replicate

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continuously, while germ and storage cells only undergo replication at each molting event.

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The number of replicative storage cells decreases markedly as tardigrades reach their fully-

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grown adult size, highlighting that most storage cell proliferation occurs during somatic

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growth.