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8 **TITLE: The cost and payout of age on germline regeneration and sexual**
9 **maturation in *Platynereis dumerilii***
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23 **ABSTRACT**

24 Regeneration, regrowing lost and injured body parts, is an ability that generally declines
25 with age or developmental transitions (i.e. metamorphosis, sexual maturation) in many
26 organisms. Regeneration is also energetically a costly process, and trade-offs occur
27 between regeneration and other costly processes such as somatic growth, or sexual
28 reproduction. Here we investigate the interplay of regeneration, reproduction, and age in
29 the segmented worm *Platynereis dumerilii*. *P. dumerilii* can regenerate its whole
30 posterior body axis, along with its reproductive cells, thereby having to carry out the two
31 costly processes (somatic and germ cell regeneration) after injury. We specifically
32 examine how age affects the success of germ cell regeneration and sexual maturation
33 in developmentally young versus old organisms. We hypothesized that developmentally
34 younger individuals (i.e. lower investment state, with gametes in early mitotic stages)
35 will have higher regeneration success and reach sexual maturation faster than the
36 individuals at developmentally older stages (i.e. higher investment state, with gametes
37 in the process of maturation). Surprisingly, older amputated worms grew faster and
38 matured earlier than younger amputees, even though they had to regenerate more
39 segments and recuperate the more costly germ cells which were already starting to
40 undergo gametogenesis. To analyze germ cell regeneration across stages, we used
41 Hybridization Chain Reaction for the germline marker *vasa*. We found that regenerated
42 worms start repopulating new segments with germ cell clusters as early as 14 days post
43 amputation. In addition, *vasa* expression is observed in a wide region of newly-
44 regenerated segments, which appears different from expression patterns during normal
45 growth or regeneration in worms before gonial cluster expansion. Future studies will
46 focus on determining the exact sources of gonial clusters in regeneration.

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INTRODUCTION

Organisms have limited energy resources at any given developmental stage and therefore need to have trade-offs for where to allocate these resources. Highly regenerative organisms need to balance the costly processes of regeneration, somatic growth, and sexual reproduction (Fields and Levin, 2020; Harshman and Zera, 2007; Henry and Hart, 2005; Partridge et al., 2005). Moreover, these trade-offs may be affected by the developmental stage and age of the organism: older individuals may have different trade-offs than younger individuals due to aging, metabolic, and hormonal state differences. Understanding these trade-offs may shed light on mechanisms that lead to success and failure of regeneration.

Many organisms experience changes in regenerative capacity or reduction and entire loss of this ability as they progress in developmental stages and age (Brunet et al., 2023; Poss, 2010; Sousounis et al., 2014; Yun, 2015). For example, older sponges and corals regenerate less well (Henry and Hart, 2005), fetal mice can regenerate digit tips at faster rates than adult mice (Borgens, 1982; Han et al., 2003; Tower et al., 2022; Yu et al., 2010), and cardiac regeneration ability decreases with age in mice (Haubner et al., 2012; Santos et al., 2020). In *Xenopus laevis* tadpoles, limb and tail regeneration is highly dependent on developmental stage (Patel et al., 2022; Poss, 2010; Yun, 2015). These changes in frogs are thought to be related to hormonal state changes after metamorphosis (reviewed in Yun 2015). In *Ciona intestinalis*, rate of regeneration decreases as the adult body size increases (Dahlberg et al., 2009). While declining regeneration with increasing age seems to indicate younger means more regenerative, being too young may work against regeneration as well: larval stages lack regenerative ability in many animals that can extensively regenerate as adults (Boyd and Seaver, 2023; Henry and Hart, 2005; Jeffery, 2015). Therefore, age and developmental stage are important intrinsic factors affecting regenerative potential in a given species, but which developmental stage or what age is most permissive to regeneration depends on the species.

Injury is generally expected to have an energetic cost and consequently a negative effect on growth and reproduction outputs such as reduced fecundity, lower egg numbers, smaller egg size, or longer time to sexual maturation (Olive and Clark, 1978; Rennolds and Bely, 2023). Regenerating sponges and corals may have reduced fecundity or may remain infertile (Henry and Hart, 2005) due to resource trade-offs between regeneration and sexual reproduction. All together, previous investigations suggest that developmental stage and age generally impairs regeneration in many organisms, and other studies show that injury/regeneration may delay reproductive processes due to resource trade-offs. However, how age, injury/regeneration, and

92   reproduction interact with each other, and whether the trade-off decisions between
93   regeneration and reproduction change in the context of age have been poorly studied.
94

95   We use the marine segmented worm (annelid) *Platynereis dumerilii* for investigating the
96   interplay between regeneration, sexual reproduction, and age. *P. dumerilii* can
97   regenerate its posterior body axis upon bisection, allowing us to test the effect of
98   regeneration on reproduction (Fischer and Dorresteijn, 2004; Özpolat et al., 2021;
99   Vervoort and Gazave, 2022). These bisections remove the majority of the germ cells,
100   adding an additional cost to regeneration because the worms have to regenerate both
101   somatic and gametic tissues. Individuals can be staged easily by counting the number
102   of segments, because as most annelids, *P. dumerilii* grows throughout its life by
103   producing new segments at its posterior growth zone located near the tail end.
104   Therefore, longer worms are developmentally more progressed and aged. Reproductive
105   development and maturation is tightly correlated with the number of segments
106   (reminiscent of pupal weight thresholds in insects required for metamorphosis). For
107   example, worms need to reach about ~35 segments before they start producing
108   numerous germ cell (gonial) clusters, the earliest stage in gametogenesis (Kuehn et al.,
109   2021; Rebscher, 2014; Rebscher et al., 2007; Zelada González, 2005). As the worms
110   grow longer, gonial clusters start progressing in their gametogenesis, and in worms
111   longer than 60 segments, typically stages of oogenesis in females or spermatogenesis
112   in males are observed (Kuehn et al., 2021; Zelada González, 2005). The worms then
113   need to reach about 70 segments before they can start sexually maturing. Therefore,
114   the biology of *P. dumerilii* enables robust developmental staging in the post-embryonic
115   phase of life. Altogether, developmental staging, the high regenerative ability, and the
116   unique reproductive biology of the species allow testing questions on the interplay of
117   age, regeneration, and reproduction.
118

119   Here in this context of the interplay of age, regeneration, and reproduction in *P. dumerilii*
120   we ask how the success of germ cell regeneration and sexual maturation compares
121   between developmentally younger individuals (i.e. lower investment state, with gametes
122   in early mitotic stages) with developmentally older stages (i.e. higher investment state,
123   with gametes in the process of maturation). We hypothesize that older animals that are
124   amputated would be more negatively affected in several ways (measurable by
125   fecundity, time to sexual maturation, regeneration success, and survival) compared to
126   younger amputated animals. We show that while older animals have a higher rate of
127   failure to regenerate, when they do regenerate, contrary to our expectations, they reach
128   sexual maturation significantly faster than younger animals and without any adverse
129   effects. We test whether this is due to higher rate of growth after regeneration, and
130   show that the oldest group has a higher growth rate. We also analyze the gonial cluster
131   regeneration timeline in the youngest and oldest groups using in situ Hybridization

132   Chain Reaction (HCR) using *vasa* mRNA detection as a marker. We show that worms

133   start repopulating regenerated segments with gonial clusters without having to reach the

134   ~35 segment threshold. We also show that there is a high and broader *vasa* expression

135   that is not typically observed in amputated worms at younger stages. We discuss these

136   results in the context of hormonal regulation that changes with age in *P. dumerilii*, and

137   present some future directions for how to address open questions regarding the

138   hormonal regulation of this process as well as cellular origins of regenerated gonial

139   clusters.

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142   ## METHODS

143   ### Github Resources

144   R code, protocols, and additional resources can be found in a GitHub repository

145   dedicated to this manuscript (ÖzpolatLab-GitHub-Metzger, 2024).

146   ### Animal Culturing

147   Animals were cultured as previously described (Kuehn et al., 2019) unless otherwise

148   noted. All samples used in this study were kept at light/dark photoperiodic conditions of

149   16:8h and 8 days of moonlight at night every 28 days. All boxes were checked for

150   deaths and maturations at minimum twice per week. For food, worms received a 1X

151   preparation of spirulina powder and sera micron as follows: 4g spirulina powder and

152   1.2g sera micron per 2 L of 0.22 μm filtered sea water. The amount of food depended

153   on the density of worms, size of the culturing container, or experiment (specified below

154   in each experiment). For experiments carried out at the Marine Biological Laboratory,

155   natural sea water was collected from the ocean water of Great Harbor and was 1 μm-

156   filtered at the lab for culturing animals (Filtered Natural Sea Water (FNSW)). For

157   experiments in St. Louis, Filtered Artificial Sea Water (FASW) was prepared with Red

158   Sea Salt (RSS, Red Sea Fish) to a salinity of 35 ppt. Prior tests by our lab and other

159   *Platynereis* labs established the suitability of RSS sea water and found no significant

160   difference in growth rate or time to maturation.

161   **Amputation & fecundity (A&F) experiment (batch culturing):** Animals were kept in

162   plastic sterilite culture boxes with 500mL of FNSW. At the beginning of the experiment,

163   each experimental and control box had between 13 and 18 animals. All boxes received

164   a complete water change once every two weeks, and death and mature checks were

165   performed at least three times per week. The number of worms per box was checked

166   and updated at least weekly, with additional updates each time a worm was discovered

167   dead or mature and removed. The amount of food was scaled to the current number of

168   worms in the box such that each worm received 333 µL of 1X spirulina and sera micron
169   mix per feeding.

170   **<u>Single worm growth rate experiment (individual culturing):</u>** Animals were kept
171   individually in 6-well plates with 7 mL of 0.22 µm FASW and final concentration of 1,000
172   U/mL penicillin and streptomycin (Fisher Scientific, 15140122). Feeding began 3 days
173   post amputation to allow for the gut to reform; unamputated controls also began feeding
174   3 days after the beginning of the experiment. Each worm received 333 µL of 1X
175   spirulina and sera micron prepared food on the regular feeding schedule. Once per
176   week, animals received a complete water change and were moved to fresh wells. To
177   protect against evaporation, well plates were kept in a humid chamber: a large plastic
178   box with a loose lid and a damp paper towel lining the bottom. To prevent mold growth,
179   the paper towel was changed out weekly alongside water changes.

180   **Amputations**

181   We amputated worms within three ranges: 40-49 segments, 50-59 segments, and 60-69
182   segments. In later sections, these range groups are referred to as 40s, 50s, and 60s.
183   These ranges were selected to encompass the range between germline expansion
184   (40s) and just prior to the onset of sexual maturation (60s). Segments were counted
185   according to 'Segment Counts' below.

186   For all amputations, worms were anesthetized for 15 minutes in 1:1 7.5% MgCl2:0.22
187   µm FNSW or FASW, then rinsed briefly in sea water before being transferred to a glass
188   slide. In most experiments amputations were performed between the 8th and 9th
189   chaetigerous segment. For the experiment testing the effect of amputation location, the
190   amputations were performed between the 7th and 8th segment, between the 8th and
191   9th segment, or roughly 2/3rds down the body of the worm. For the experiment
192   assessing fecundity after amputation, any worms that died before posterior regeneration
193   was completed (5 DPA) were discarded and noted, but not factored into the long-term
194   total mortality counts of the experiment. For experiments involving single worm cultures,
195   mortalities from the start of the experiment (0 DPA) were factored into total mortality.

196   **<u>A&F experiment:</u>** Most amputations were performed over the course of three days: all
197   40-segment amputations on the first day, followed by all 50-segment amputations on
198   the second and all 60-segment amputations on the third to minimize any effect of
199   uncontrollable fluctuations in culture conditions on recovery. In the case of 60-segment
200   amputations, one fertilization group showed fungal contamination and mass mortality.
201   All individuals from this fertilization group were discarded and a fresh series of 60-
202   segment amputations was performed 4 weeks later and incorporated into the
203   experiment. To recover, the amputees were kept individually in 6-well plates with 7mL of
204   0.22 µm FNSW/FASW and a final concentration of 1,000 U/mL penicillin and

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205 streptomycin. After 6 days in 6-well plates, the worms were transferred into 750 mL
206 culture boxes and cultured as described above.

207 **Single worm segment addition rate experiment:** All 40-segment worms were
208 amputated on the first day, followed by all 60-segment worms the day after to minimize
209 any effect of uncontrollable fluctuations in culture conditions on recovery. 40- and 60-
210 segment group amputations were performed in triplicate with each set of amputations
211 occurring ~1 month apart. Animals were kept individually in 6-well plates for the duration
212 of the experiment and cultured as described above. Worms were staged and segment-
213 counted weekly, which allowed us to track individual trajectories of posterior
214 regeneration, growth, and mortality. Segment counts were tracked until 63-64 days (9
215 weeks) after amputation. We chose this time frame based off of the segment counts
216 observed in our previous experiment (Suppl. Fig. 2), which suggested a rapid period of
217 growth around 28 DPA and a slower period of growth by 63 DPA. We expected this time
218 frame to be most useful for revealing age-specific differences in segment addition that
219 could impact time to maturation.

220 **Segment Counts**

221 Worms were anesthetized for 15 minutes in 1:1 7.5% MgCl2:0.22 μm FNSW or FASW,
222 rinsed briefly in sea water, then imaged using a Zeiss Stemi 305 CAM BODY
223 stereoscope with a built-in camera and an iPad with Lab Scope. These images were
224 used to conduct segment counts using the Cell Counter plugin in FIJI. We counted each
225 chaete-bearing segment, excluding segment primordia that had not yet formed chaete.

226 In rare cases, worms auto-amputated close to the beginning of the experiment. If a
227 worm had a visible amputation plane or a reduction in segments counted from one time
228 point to the next, it was removed from analysis. One 40 segment control worm and
229 three 60 segment control worms that auto-amputated were removed from analysis.

230 All segment counts are provided as csv files (see Suppl. File 1 for description of files,
231 Suppl. File 2 for R codes, and Suppl. File 3 for the csv files).

232 **A&F Experiment Analyses**

233 **Assessment of Maturation:** For each mature individual, we recorded the date of
234 maturation, the total number of segments at maturation, and the location of the
235 atokous/epitokous boundary, determined by the last segment displaying atokous
236 chaetae (Schulz et al., 1989). Mature animals were anesthetized and imaged as
237 described above.

238 **Assessment of Fertility and Fecundity:** After imaging, mature animals were paired for
239 fertilizations. When possible, mature animals from the experiment were paired with

240   mature animals from our stock cultures. This pairing allowed us to separate any sex-
241   specific effects on fertility and fecundity, assuming a stable rate of fertility in the stock
242   cultures. For example, if all fertilizations between amputated females and stock males
243   failed but all fertilizations between amputated males and stock females succeeded, we
244   would conclude that amputation impairs female, but not male, fertility. Fertilizations
245   between amputated animals would obscure potential sex-specific differences. In one
246   instance, no mature animals were available from the stock culture and one (successful)
247   fertilization was staged between two 50-segment amputated mature animals.

248   Infrequently, a female spawned but a male failed to release sperm naturally; in these
249   cases, the male was prodded with a pipette to release sperm, and failing that, cut open
250   with a scalpel on a glass slide so that the contents could be rinsed into the fertilization
251   dish to assist fertilization. Males from stock cultures as well as amputated and control
252   groups occasionally failed to spawn without assistance, so failure to spawn naturally
253   does not necessarily indicate infertile or defective individuals, as spawning behavior
254   itself is ruled by complex mating cues that are yet to be fully understood.

255   1-4 hours after fertilization, the eggs were collected and de-jellied in an 85 μm mesh
256   filter and rinsed a minimum of 10 times with 0.22 μm FNSW, with additional rinses if jelly
257   remained. Then, we prepared the eggs for quantification and assessment of fecundity.
258   The eggs were resuspended in 50 mL of 0.22 μm FNSW in a 50mL plastic beaker, then
259   poured rapidly between two 50 mL beakers to achieve an even suspension. A 1 mL
260   aliquot was removed from this suspension and dispensed into a watch glass. The eggs
261   were viewed under magnification and counted using a watch glass. Fertilized eggs were
262   distinguished from unfertilized eggs based on their color: unfertilized eggs became
263   yellowed, clouded, and finally opaque, while fertilized eggs became clear and began
264   cleaving. The total number of eggs, the number of fertilized eggs, and the number of
265   unfertilized eggs were recorded for each individual assessed.

266   All scores and counts are provided as csv files (see Suppl. File 1 for description of files,
267   Suppl. File 2 for R codes, and Suppl. File 3 for the csv files).

268   **Fixations**

269   Worms were anesthetized as described above and briefly rinsed in FNSW/FASW. Then,
270   they were submerged in fixative pre-chilled to 4℃. The fixative was composed of 1 part
271   16% paraformaldehyde, 2 parts 2X PBS made with DEPC-treated water, and 1 part
272   0.22 μm FSNW/FASW. The worms were fixed on an orbital shaker for 2 hours on ice or
273   overnight at 4℃. Following fixation, worms were dehydrated through 15-minute washes
274   of 25%, 50%, 75%, and 100% methanol in DEPC-treated PBSt, chilled to 4℃. Worms
275   were stored in fresh 100% methanol at -20℃.

276   **Hybridization Chain Reaction (HCR)**

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Probe design: Probes were designed through the Probe Maker software we developed (Kuehn et al., 2021). The sequences generated by the software were used to order a single, batched DNA oligo pool (50 pmol DNA oPools Oligo Pool) from Integrated DNA Technologies, resuspended to 1 pmol/µl in 50 µL nuclease-free water (Ambion, 10977015). B3 hairpins conjugated to Alexa-647 were purchased from Molecular Instruments. Probe sequences are provided in the supplementary information (Suppl. File 04).

Tissue collection and HCR: To visualize gonial cluster regeneration in worms of different ages, we amputated worms between 40-49 (40s) and 60-69 (60s) segments and allowed them to regenerate until 7 DPA, 14 DPA, or 23 DPA. At each of these timepoints, a subset of worms were fixed, dehydrated in a methanol series, and stored in 100% methanol at -20℃.

Probeset for *vasa* were designed using the HCR Probe Maker (Kuehn et al., 2021). Probe hybridization buffer, probe wash buffer, amplification buffer were sourced from Molecular Instruments (https://www.molecularinstruments.com) (Choi et al., 2014). HCR 3.0 was performed according to the published methodology (Choi et al., 2018) with a few modifications to suit our samples as previously described (Kuehn et al., 2021). Additionally, in place of proteinase K digestion followed by post-fixation, permeabilization was achieved through a 30 min wash in a Tween-based detergent solution (Bruce et al., 2021). At the amplification step, DAPI was added to a final concentration of 2 µg/ml. The full protocol adapted for *Platynereis* is provided in the GitHub repository (ÖzpolatLab-GitHub-Metzger, 2024).

Imaging: HCR samples were mounted in Slowfade Glass with DAPI (Thermo-Fischer S36920-5X2ML), kept at 4°C until imaging, and imaged using Zeiss Laser Scanning Confocal Microscope LSM 900 using Z-stacks and tiling. For each experiment, a no-probe, hairpin-only control was included to support differentiation of true signal from baseline background and autofluorescence. To survey all possible gonial clusters in original and regenerated segments, we acquired tiled Z stacks encompassing the region immediately posterior to the jaw to the pygidium. A step size of 8 µm was chosen so that all germline structures would be captured in a minimum of one slice. All stages of female germline structures exceed 8 µm (Fischer, 1975). Male germline structures exceed 8 µm up through the spermatocyte stage, which average 6.7 µm in diameter (Meisel, 1990), and no male worms were observed past the spermatogonia ball I stage.

Image analysis: Images were processed for levels and annotated with scale bars in FIJI (Schindelin et al., 2012) or Imaris (Bitplane). Panels were assembled in Adobe Illustrator. For segment counts, only chaetae-bearing segments were counted (new segments without chaetae were not counted). For gonial cluster counts, if the anterior cluster was the dispersed phenotype, these were counted as gonial clusters, otherwise,

315 if the anterior cluster was the ring or stripe phenotype, these were not counted as gonial

316 clusters. Single oocytes were included in gonial cluster count. However, cells within the

317 broad *vasa* expression, or cells that do not have gonial cluster or gamete phenotype

318 were not included in the count. For regeneration stages, previously-established staging

319 for *P. dumerilii* was used (Planques et al., 2018). Scoring table of results is provided as

320 supplementary material (Suppl. File 5)

Phalloidin Staining

322 28 DPA, a 60s-amputated worm from the segment addition rate experiment was

323 observed to be dying, signified by minimal response to stimuli, tissue deterioration, and

324 depigmentation. To explore potential factors in 60s mortality, this worm was removed

325 from the experiment and placed into fixative (4% PFA in ASW-PBSt, 2 weeks nutating

326 at 4C). The worm was then stained with DAPI (1 ug/100 µL) and Phalloidin (Alexa-647,

327 1X) together in PBS for 45 mins at room temperature. To aid in mounting, the head and

328 pharynx, including the first four segments, were removed. The tail segments were

329 mounted and imaged as above ('Imaging').

Plots and Statistical Analyses

331 Line graphs, violin plots, and bubble plots were generated in RStudio (R Development

332 Core Team, 2011; RStudio Team, 2020) using ggplot 2 (v3.3.3) (Wickham, 2016).

333 Visuals were collected and edited in Adobe Illustrator for clarity, shape, axis labels, and

334 text size adjustment. ANOVAs, paired t tests, and Wilcoxon non-parametric tests were

335 performed in RStudio.

336 For the time to maturation and number of segments at maturation, univariate and

337 multivariate ANOVAs were performed by the Biostatistics Consulting Service at

338 Washington University in St Louis. The data analysis was generated using SAS

339 software, version 9.4 of the SAS System for Windows (SAS Institute Inc., Cary, NC,

340 USA). In univariate models of segment group, amputation, and sex, differences among

341 segment groups were highly significant (p<0.0001), differences between amputated and

342 control groups were marginally significant (p=0.0857), and males and females were not

343 significantly different (0.2857) We then ran a multivariate model including predictors in

344 univariate models with p<0.1, which included segment group, amputation, and their

345 interaction. In the multivariate model, the interaction between segment group and

346 amputation was marginally significant (p=0.0584). We performed all planned

347 comparisons. P-values for multiple comparisons were adjusted with the Tukey-Kramer

348 adjustment.

349 All R scripts and datasets are available as csv files (Suppl. File 1 for description of files,

350 Suppl. File 2 for R codes, and Suppl. File 3 for the csv files) and also via GitHub

351 (ÖzpolatLab-GitHub-Metzger, 2024).

352

353

RESULTS

355

Amputated *Platynereis dumerilii* reach sexual maturation, are fertile, and show normal reproductive morphology following regeneration

Platynereis dumerilii can regenerate the whole posterior body axis when bisected, but they fail to regenerate anteriorly. Worms also fail to regenerate if the head is smaller than 8 chaetigerous segments (Suppl. Fig. 1). Amputations leaving 8-segment or longer head pieces will typically result in body axis regeneration. As the germ cells are stored along the body cavity, such amputations also remove the majority of the germ cells, which regenerate along with body axis regeneration (Fig. 1A-B). We wanted to test if worms that had to regenerate their germline were impacted on their sexual maturation, morphology, and reproductive success. To characterize the success of sexual reproduction following amputation and regeneration, we compared the maturation of amputees and controls of different initial segment lengths that span the period between germline expansion (i.e. when germ cell clusters start appearing in the coelomic cavity) at ~35 segments (Kuehn et al., 2021) and sexual metamorphosis at ~70 segments (Fig. 1A). We split this period into three segment ranges to capture approximate phases in germline development: 40-49s (the stage when small gonial clusters are becoming numerous), 50-59s (when gonial clusters are generally in the early stages of gametogenesis), and 60-69s (when the gonial clusters are generally in late stages of gametogenesis, or full maturation is imminent) (Fig. 1B). For each individual, the amputation was performed after the 8th pair of chaetae, the most anterior possible cut site for which regeneration proceeds without excessive mortality in previous works (Planques et al., 2018). Our pilot experiments on worms shorter than 50 segments showed that, by 7 DPA, worms amputated after the 8th segment regenerated comparably to worms amputated after the ~30th segment, while worms amputated after the 7th segment were significantly delayed or unable to close the wound (Suppl. Fig. 1A). Therefore we proceeded with amputations after the 8th segment to maximize the removal of segments containing the germ cells. However, it did not remove the anterior germline cluster, which is typically located between the 5th and 6th segment. Amputated worms were allowed to regenerate, and both amputated worms and their controls were raised to sexual maturation (see Methods for details).

Overall, amputated worms did not show any significant differences from control worms in fecundity, fertility, and reproductive morphology (Fig. 1C-J). We assessed the production of functional gametes after regeneration, by scoring for the number of eggs released and the percentage of eggs fertilized (Fig. 1F). Mature animals from within the experiment were paired for fertilization with matures from the wild type cultures when

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391 possible. There were no significant differences in the number of eggs released or the

392 percentage of eggs fertilized (Fig 1I-J; Wilcoxon signed rank test, p =0.8596 for eggs

393 released, p =0.4787 for eggs fertilized). From these fertilizations, F1 and F2 offspring

394 were raised to maturation and reproduced successfully as well (data not shown).

395 Previous literature indicates that *Platynereis dumerilii* typically undergoes sexual

396 metamorphosis between 65-75 segments (Fischer, 1975, 1974; Legras et al., 2023).

397 However, whether the number of segments at maturation differ in amputated-

398 regenerated worms have not been analyzed systematically. It is possible that

399 amputated worms would reach a similar segment threshold for maturation, or

400 alternatively, amputation and subsequent regeneration would alter or decouple the

401 relationship between segment number and transition to sexual maturation. We

402 compared a subset of our control and amputated groups by counting total segment

403 number at maturation. In both control and amputated worms we observed a more

404 flexible segment range at maturation than previously-reported results, with worms

405 being between 60 and 82 segments at maturation (Fig. 1E). Univariate models showed

406 no significant difference in the number of segments at maturation among different

407 segment groups (p=0.3115), between the control and amputated groups (p=0.9127) or

408 between males and females (p=0.2257; Suppl. Fig. 1B-C). These data indicate that

409 regenerated worms reach a similar number of segments for sexual metamorphosis as

410 control worms.

411

412 We also assessed whether the segment-specific location of a mature characteristic, the

413 atokous-epitokous border, would be maintained in mature regenerated animals. In *P.*

414 *dumerilii*, sexual metamorphosis involves dramatic restructuring of the somatic tissues.

415 A sex-specific number of parapodia at the anterior end of the worm maintain an

416 "atokous" phenotype, while all parapodia posterior of this boundary take on an

417 "epitokous" phenotype with transformed muscles and knife-like chaetae. This boundary

418 is easily distinguishable in males by the boundary between the white, sperm-rich

419 anterior and the red muscular posterior (Fig. 1D''). In females, morphology of the

420 parapodia is sufficient to identify the border (Fig. 1C''). In males, the atokous-epitokous

421 boundary occurs after the 14th-16th segments, while in females, the atokous-epitokous

422 boundary occurs between the 20th-24th segments (Schulz et al., 1989). For all

423 amputated groups, we found that the atokous-epitokous border was within the correct

424 sex-specific segment range (Fig. 1G, 1H). For the control groups, one 40s male had the

425 atokous-epitokous border one segment anterior to the normal location, after the 13th

426 segment (Fig. 1G). All others were within the sex-specific segment range.

427

428 **Amputated worms show stage-specific timelines of regeneration and maturation**

429   We next wanted to analyze if stage of development (measured by segment number)
430   had an effect on time to maturation after amputation. We expected that worms of
431   different developmental stages (40-49 segments, 50-59 segments, and 60-69
432   segments) would vary in the time required to regenerate all lost somatic and germ cells
433   and reach sexual metamorphosis (Fig. 2A-A'). For brevity, we will refer to these groups
434   as 40s, 50s, and 60s groups. Our rationale was that overall, older worms (e.g. 60s)
435   would have invested more resources into germ cells such as having vitellogenic
436   oocytes, compared to younger worms (e.g. 40s). Therefore, these older worms had to
437   recover a more "expensive" loss, and a longer body axis upon amputation. We expected
438   a gradually-increasing delay to maturation in older worms compared to younger worms
439   (Fig. 2B).

440   Strikingly however, our analyses revealed the opposite: we found that the older
441   amputated worms matured earlier than younger control and amputated worms, and this
442   shift is gradual as indicated by the 50s amputees maturing sometime between 40s and
443   60s amputees (Fig. 2B', C). We ran univariate and multivariate analysis of variance to
444   determine if the days to maturation were different between the 2 treatments (amputated
445   vs control), 3 segment groups (40s, 50s or 60s) and sex at maturation (Male vs
446   Female). In univariate models, there were overall significant differences among the 40s,
447   50s, and 60s groups (p<0.0001). The treatment groups (amputated vs control) were
448   marginally different (p=0.0857), and there was no significant difference between males
449   and females (p=0.2857). With segment group and amputation as our two independent
450   variables, we ran a multivariate model with an additional interaction term. This allowed
451   us to assess effects of segment group and amputation alone, as well as effects that
452   emerge from combinations of these variables. In the multivariate model, the interaction
453   between segment group and treatment was marginally significant (p=0.0584), meaning
454   that amputation had different effects on different segment groups. Taken together, these
455   data indicate an inverse relationship between the age/developmental stage at
456   amputation and the length of time to regenerate and reach sexual metamorphosis.

457   Interestingly, when amputated groups were compared with their unamputated controls,
458   we found that for 40s, there was no significant difference in the number of days to reach
459   maturation between the amputated group and the controls (p=1.0) (Fig. 2C). The same
460   was observed for 50s amputees vs controls (no significant difference, p=0.9941), even
461   though the time to maturation showed greater variance for 50s amputees than for 50s
462   controls (Fig. 2C). Effectively, having an amputation that removed the major percentage
463   of the body, and having had to regenerate these missing segments cause no overall
464   delay to maturation in the 40s and 50s amputees compared with their controls. In
465   contrast, 60s amputees were significantly delayed to maturation relative to unamputated
466   controls (p=0.0309), with amputees maturing at an average of 134 days and controls
467   maturing at an average of 65 days (Fig. 2C). As noted earlier, however, even though

13

468   60s amputees matured later than their 60s controls, they still matured earlier than both
469   40s and 50s controls, despite needing to regenerate 52 segments along with their germ
470   cells.

471

472   **Segment addition rates differ between 40s and 60s amputees**

473   Since the 60s amputees were maturing earlier, one explanation could be that worms in
474   this group start maturing without growing to 70-80 segments, and instead they can
475   mature at shorter segment numbers compared to the 40s and 50s groups (i.e. adding
476   segments at a comparable rate and just reaching fewer segments before maturing).
477   However, all groups reached a similar segment number threshold before maturing (Fig.
478   1E, p=0.07946), and there was not a significant correlation between the length at
479   maturation and the time to maturation (Suppl. Fig. 1D). Another explanation for the 60s
480   amputated group reaching maturation faster could be faster rate of regeneration and
481   segment addition after amputation, allowing the worms to grow to sexual maturation
482   faster. For this experiment, we had collected data on the number of segments at
483   different checkpoints (28, 63, 96 DPA) between amputation and maturation in these
484   batch cultures (Suppl. Fig. 2C-D). The number of segments in amputated groups
485   differed as early as 28 DPA, at which point 40s amputees were significantly shorter than
486   both 50- and 60 segment amputees (p<0.001, p<0.001;). By 84 DPA, 40s amputees
487   were still significantly shorter than 50s and 60s amputees (p<0.001) as well as their
488   unamputated controls (p<0.001), while 50- and 60s amputees had caught up to their
489   controls (p=0.06031, p=0.1909). These data suggest that differential rates of segment
490   addition, established at least as early as 28 DPA, could at least partly underlie the
491   observed differences in the time to maturation. However, because these worms were
492   kept in collective culture, the growth rates of individual worms could not be assessed.
493   Furthermore, collective culture increases the incidence of tail breakage and territorial
494   encounters (and potentially cannibalism, or differential access to food resources due to
495   social and territorial behaviors).

496   To get a clearer understanding of stage-specific differences in the rate of segment
497   addition following amputation, we performed a follow-up experiment with single-cultured
498   worms, and observed their growth individually across time. We amputated 40s and 60s
499   worms and kept them in individual wells, counted their segments at several time points
500   to assess the rate of growth, and noted mortality (see Methods for details). The growth
501   trajectories of single worms reveal that by 63 DPA, controls grow at a much slower rate
502   than amputees (Fig. 3A-B), and 60s amputees added more segments than 40s
503   amputees (Fig. 3B, F, Tukey-Kramer test, p adj <0.05). Notably, the rate of segment
504   addition is not constant throughout the 63 day time period. Both amputated groups
505   show an earlier, faster period of growth around 20 DPA as well as a later, slower period

14

506 of growth by 63 DPA (Fig. 3B, D). The period of highest segment addition for both
507 amputated groups appears to occur until ~30 DPA (Supp. Fig. 3). Until this point, 60s
508 amputees added segments at a higher rate than 40s amputees. After ~30 DPA,
509 segment addition rates declined and appeared to converge.
510
511 Interestingly, a finer-scale analysis of segment numbers shows that 60s amputees did
512 not immediately begin adding more segments than 40s amputees. In contrast, they
513 were significantly delayed early on (Fig. 3C). At 7 DPA, 40s amputees have added
514 significantly more segments than 60s amputees; 40s amputees have added an average
515 of 2.6 segments compared to an average of 0.55 segments for 60s amputees (Fig. 3C,
516 p=2.4e-06). Proportionally, 93% of 40s amputees have added at least one segment,
517 indicating successful regeneration of the posterior growth zone by 7 DPA, compared to
518 only 39% of 60s amputees. Previous work has established that the normal timeline for
519 regeneration of the posterior growth zone occurs within 5 days, culminating in late
520 patterning/visible segment formation. Images of 7 DPA regenerates show that 40s
521 amputees are reaching the expected stage of regeneration by 7 DPA, but 60s amputees
522 show delayed initiation of segment addition as well as high mortality (see below).
523 Despite this initial delay, by 14 DPA, surviving 60s amputees caught up to 40s
524 amputees and the differences between amputated groups were no longer significant
525 (Fig. 3D, p=0.20). By 28 DPA, 60s amputees have added more segments than 40s
526 amputees (Fig. 3E, p=0.01058) and continue to outpace 40s amputees until
527 approximately 35 DPA, at which point the rates appear to converge (Suppl. Fig. 3).
528 Overall, 60s amputees generally show a faster segment addition rate compared with the
529 40s controls, which could partly explain the faster timeline of getting to sexual
530 maturation in this group.
531
532 We expected amputated worms to have an elevated segment addition rate compared to
533 controls, given that regenerating worms have been shown to add segments faster than
534 unamputated controls (Gazave et al., 2013). Our data exemplify this in both age groups:
535 by 63 DPA, 40s amputees have added an average of 37.0 segments in comparison to
536 an average of 11.0 segments in 40s controls (Fig. 3F). The difference between 60s
537 amputees and controls is even greater, with 60s amputees adding an average of 44.2
538 segments and 60s controls adding an average of 3.2 segments over the experimental
539 time course (Fig 3F). By the end of the 63 DPA, segment addition rates of amputated
540 worms are declining, but still slightly elevated compared to controls (Supp. Fig. 3).
541
542

543 **Amputated worms show age-specific mortality, regeneration defects and delays**

544 While the surviving 60s amputees grow and mature faster, this group also had a higher
545 rate of mortality and regeneration defects. The mortality rate of 60s amputees was

546 highly elevated compared to 60s controls and 40s amputees. We carried out
547 comparisons in the single-worm culture experiment and found that over the 63 day
548 course of the experiment, 57.5% of 60s amputees died compared to 16.67% of 60s
549 controls (Fig. 4A). The mortality rate for 40s amputees was only 6.25%, and no 40s
550 controls died (Fig. 4A). Instances of death were elevated earlier within 7 DPA, but
551 otherwise distributed across the experimental timeline. 8 of 23 total deaths (34.8%)
552 among 60s amputees occurred on or before 7 DPA (Fig. 4B). This early elevated
553 mortality occurred alongside delays and failures of posterior regeneration, and seemed
554 to coincide with or were possibly precipitated by the first feeding after amputation.

555 Most 60s amputees that died after 7 DPA had added several segments, indicating
556 functional regeneration of the growth zone and gut structures (Fig. 4C). However, there
557 were also several 60s amputees that survived for weeks without adding segments (Fig.
558 4C). Among 60s amputees, there does not appear to be a correlation between original
559 segment number and the number of days surviving (Fig. 4D), suggesting that there is
560 not a sharp threshold for survival following amputation, or progressively early mortality
561 with increasing segment number. It is unclear why these worms, which had successfully
562 added several segments, died. We noted that in one of these dying 60s amputees there
563 were nearly mature oocytes in the regenerated region (Fig. 4E). Previous studies
564 indicate that submature oocytes may exert negative feedback on growth and
565 regeneration (Hofmann, 1975; Porchet and Cardon, 1976). Notably, many 60s
566 amputees that successfully regenerated and survived for the full 63 days were delayed
567 in regeneration relative to 40s amputees as well as published timelines for posterior
568 regeneration (Planques et al., 2018). The stages occur ~1 stage per day, with worms
569 typically reaching stage 5 (late patterning with segment primordia) at 5 DPA (Fig. 5A).
570 By 7 DPA, over 75% of 40s amputees surpassed stage 5 (chaetae-bearing segments
571 formed) compared to about 40% of 60s amputees . By 14 DPA, 100% of 40s amputees
572 and about 80% of 60s amputees were forming visible segments (Fig. 5B). Abnormalities
573 were more common among 40s amputees than 60s amputees during the early stages
574 of regeneration, at 3 DPA (Fig. 5B). These abnormalities included a failure to form a
575 blastema (Fig. 5C) and aberrant morphology (Fig. 5C'). By 7 DPA, 60s amputees had a
576 slightly higher percentage of abnormalities (Fig. 5B). Aberrant morphology at this stage
577 included improperly scaled and patterned regenerated regions (Fig. 5D-D') as well as
578 edema (Fig. 5D"). By 14 DPA, no abnormalities remained in 40s amputees (Fig. 5B),
579 one 60s worm had formed chaete-bearing segments but had an improperly formed
580 pygidium (Fig. 5E), and another had edema (Fig. 5E'). All 60s worms that still had
581 abnormal posterior regeneration at 14 DPA died between 19 and 31 DPA.

582

Analyses of gonial cluster regeneration in 40s and 60s amputees via HCRs

584

585 Our previous experiments established that amputated worms successfully regenerate
586 their gonial clusters and reach sexual maturation without any major differences in terms
587 of fecundity and fertility from unamputated control siblings. Next, we wanted to visualize
588 the early events of gonial cluster regeneration and interrogate age-specific differences
589 using *in situ* Hybridization Chain Reaction (HCR) for the germline marker *vasa*. We
590 focused on the 40s and 60s groups as these two groups had the most pronounced
591 difference from each other in time to maturation. In addition, at the physiological level,
592 these stages highly differ in their state of gametogenesis. 40s worms usually have a
593 variable number of small undifferentiated gonial clusters, while 60s worms have a mix of
594 numerous sexually differentiated clusters nearing maturation as well as some
595 undifferentiated clusters. A minority of 40s worms may have small oocytes due to the
596 asynchronous nature of oogenesis, but there is generally a clear difference in
597 gametogenesis and resource investment between these two ages both in terms of
598 number and maturation stage of the gonial clusters (i.e lower investment in 40s group
599 compared to 60s group). We amputated worms after the 8th segment in both 40s and
600 60s groups, and fixed samples at 7, 14, or 23 DPA to visualize general *vasa* expression
601 patterns, presence/absence of gonial clusters in the original versus regenerated
602 segments, and the general morphology of the anterior cluster and the gonial clusters.

603 In both 40s and 60s amputees, gonial clusters appeared in the original segments in the
604 majority of the worms, however the numbers were generally low for the 40s group
605 (median 1, average 2.1) compared to 60s (median 8, average 11.5). At 7 DPA, most
606 40s amputees had already regenerated new segments, while most 60s amputees were
607 still in mid or late stages of regeneration. Gonial clusters were observed in regenerating
608 and regenerated segments as early as 7 DPA but only 1 gonial cluster in 1 out of 5 in
609 40s and 2 gonial clusters in 1 out of 6 worms in 60s group (Fig. 6C, D). In both groups,
610 in a small number of samples, we observed gonial clusters right at the border of the
611 original and regenerated segments (Fig. 7B, D, F'). By 14 DPA, the majority of 60s
612 samples (5/6) had gonial clusters (median 3) in the regenerated region, while only one
613 40s sample (1/5) had 3 gonial clusters in the regenerated region, even though both
614 groups generally had a similar number of segment length (median 20 segments 40s;
615 17.5 segments 60s). By 23 DPA in both groups the majority of worms had numerous
616 gonial clusters in the regenerated segments (median 3.5 gonial clusters in 40s, 13 in
617 60s) even though many of the worms had not crossed the 35 segment threshold that is
618 required for normal gonial cluster expansion (median 24.5 segments in 40s, 26.5
619 segments in 60s). At this stage, 40s amputees still had relatively fewer gonial clusters
620 (median 0.5) in their original segments compared to 60s amputees (median 11) (Fig.
621 6C, D).

622 Next, we wanted to assess changes in the anterior cluster (AC) during regeneration in
623 either group. The AC starts forming when the primordial germ cells migrate anteriorly in

624   larval stages, and as the worms grow longer the AC forms (presumably from the
625   proliferation of the germ cells) between the 5th and 6th segments, around the pharynx
626   (Kuehn et al., 2021; Rebscher et al., 2012, 2007). The morphology of the AC changes
627   as the worms add segments, and these morphological phenotypes are classified as
628   follows (Fig. 6B): 'ring', where the clusters are arranged in a ring shape around the
629   gut/pharynx with a clear ventral and dorsal component; 'stripe', where the clusters are
630   arranged linearly, usually dorsally; and 'dispersed', where there is not a notable
631   organization of an anterior cluster in this specific region, although there may be some
632   individual clusters nearby in no particular pattern. When scored, 40s and 60s amputees
633   showed strikingly different AC phenotypes (Fig. 6C, D). For 40s, all samples at all
634   timepoints had a distinct anterior cluster (18/18; Fig. 6C, 7C). Many samples had a clear
635   ring phenotype (11/18), with a few samples with the stripe phenotype (7/18). There did
636   not appear to be a trajectory towards ring or stripe phenotypes as regeneration
637   progressed, and there were no samples with the dispersed AC phenotype. In contrast,
638   60s AC phenotypes were dramatically different: in most 60s amputees, the anterior
639   cluster was dispersed (13/18; Fig. 6D). This observation is consistent with anecdotal
640   experience in the lab in uninjured worms over 60 segments, we often observe no AC
641   (Kuehn et al., 2021).

642   In addition to gonial clusters, we noticed that in both 40s and 60s groups, there was a
643   broad *vasa* expression in the newly-regenerated segments in the region between the
644   gut and the epidermis (Fig. 7A', B', E, G'). This pattern was most pronounced at 14 DPA
645   in both groups, but it arose as early as 7 DPA in the 40s amputees (60s amputees were
646   still in the regeneration phases at this time point). Furthermore, in both 40s and 60s
647   samples we began to see single cells or small groups of cells expressing *vasa*, but their
648   overall morphology was distinct from gonial clusters or singlet oocytes (Fig. 7G, H, I).
649   These putative progenitor cells were first observed at 14 DPA and persisted to 23 DPA
650   when the broad *vasa* expression was no longer present except for in the posterior
651   growth zone. These putative progenitor cells were typically in the areas surrounding the
652   gut, the body wall musculature, or parapodia, but not in the coelom. They appeared
653   smaller than gonial cluster cells, with smaller and less round nuclei.

654

DISCUSSION

The effect of regeneration on reproduction, growth and survival

657   Many annelids, including *P. dumerilii* can regenerate germ cells and gonads (Özpolat,
658   2023). While there have been anecdotal notes on *P. dumerilii*'s ability to regenerate its
659   germline during posterior regeneration (Hauenschild and Fischer, 1969; Rebscher,
660   2014), systematic studies on this process to see whether removal and regeneration of

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661 germ cells have an impact on fertility and fecundity have not been carried out before. In
662 some annelids injury can negatively impact reproduction, measured by the production of
663 gametes, or gamete, cocoon, and brood numbers (Gibbs, 1968; Hill et al., 1982;
664 Rennolds and Bely, 2023; Zajac, 1985). To address this in *P. dumerilii*, we implemented
665 the most severe survivable amputation at three different developmental stages with
666 differential germline investment states. The oldest worms (60s) had extensive germline
667 investment (late stages of gametogenesis, e.g. with vitellogenic oocytes) and were on
668 the cusp of sexual metamorphosis, while the younger worms (40s or 50s) had only
669 undifferentiated germ cells, or were at the early stages of gametogenesis, respectively.
670 We expected that older worms that have high gametogenesis investment potentially
671 could show higher rates of adverse effects on fecundity upon injury and regeneration
672 compared with younger worms. However, we found that all 40s, 50s, and 60s amputees
673 which regenerated have normal fertility and fecundity, measured by the number of eggs
674 released, percentage of eggs fertilized, and successful maturation of the next
675 generation.

676 While reproductive success was not overall different in the 3 groups, we observed
677 differences in time to maturation. Here, we expected that the older group would be more
678 delayed in the time to maturation due to the larger loss experienced, however we found
679 the opposite to be the case: 60s amputees reached maturation earlier than 50s and 40s
680 amputees. A simple explanation for this could be the overall growth rate differences. As
681 previously shown, number of segments is a good metric of growth in annelids, and
682 especially in *P. dumerilii*, worms are known to have to reach a particular segment
683 number threshold for developmental transitions (Kuehn et al., 2021). Therefore we
684 reasoned that even though all amputees are reduced to a total of 8 segments, older
685 worms may be growing faster than younger worms, hitting the developmental thresholds
686 faster, and therefore reaching sexual maturation faster. Indeed, we found that older 60s
687 amputees had an overall higher segment addition rate compared with younger 40s
688 amputees. However, the growth rate difference was not drastic enough to be the only
689 factor explaining the difference in time to maturation. Other factors in the anterior 8
690 segments remaining after amputation could be physiological, morphological, and
691 molecular changes that occur in *P. dumerilii* before the visible onset of maturation
692 (Dahlitz et al., 2023; Fischer et al., 2010; Fischer and Hoeger, 1993; Schenk et al.,
693 2019, 2016). For example, there may be differential storage of nutrients, including lipids,
694 that could enable the rapid growth observed in older amputated worms. The older
695 worms tend to also get larger and they may be more efficient in feeding. The
696 neurohormonal state is also likely to play a major role, as with age, complex hormonal
697 shifts take place (see below for details). Overall, due to these changes, older worms
698 may be primed to return to the metabolic, hormonal states associated with maturation
699 compared to younger worms, therefore reaching maturation faster.

700 We also observed, however, that amputation and regeneration did not come without a
701 cost for the 60s amputees, which had a higher rate of mortality or abnormal
702 regeneration after amputation, relative to the 40s and 50s amputees. That is, if 60s
703 amputees could successfully regenerate the posterior body axis they also regenerated
704 germ cells without an adverse effect on their reproductive success, but on the other
705 hand some samples failed regeneration altogether, or had higher mortality even after
706 having regenerated segments but had stalled growth. One particular 60s amputee never
707 grew beyond 16 segments, eventually died and upon investigation had several large
708 oocytes in the regenerated segments. As gametes mature they are known to signal
709 negative feedback to suppress growth and regeneration, so the worms can initiate
710 sexual maturation. The failure of regeneration in some of the 60s amputees could be
711 due to the hormonal state differences and these signals from maturing gametes present
712 in the original segments (see below for detailed discussion on the hormones).

713 Finally, injury can lead to delays in developmental transitions in some organisms. In
714 *Drosophila*, injury to the imaginal disc during the regenerative competent larval phase
715 results in a developmental delay to accommodate regeneration (Jaszczak and Halme,
716 2016; Karanja et al., 2022). In annelids, several works suggested that injury affects
717 reproduction (Gibbs, 1968; Hill et al., 1982; Rennolds and Bely, 2023; Zajac, 1985). In
718 *Scoloplos*, Gibbs collected wild worms and observed that many of the worms were
719 injured and regenerating, or had regenerated at some point. Some worms that
720 appeared to have several regeneration cycles were delayed in their production of
721 gametes, and/or produced fewer cocoons as a result. Therefore injury negatively
722 affected fecundity/reproduction in this species (which is not semelparous). In *Polydora*,
723 Zajac made amputations to cause injury (but did not remove segments that contained
724 the gametes) and showed that injury negatively affected fecundity (measured in terms
725 of number of eggs or brood size). In addition, posteriorly amputated worms took
726 approximately twice as long to the production of the first brood and produced less
727 broods compared to the controls. In our experiments, when we compared control
728 unamputated siblings with the amputated groups, we found that amputation and
729 regeneration did not cause a delay to reach sexual maturation in younger groups (40s,
730 50s) although the older group (60s) was delayed to maturation compared to their
731 controls. This lack of delay in the younger groups was surprising because amputees
732 need to recover dozens of lost segments and regenerate germ cells, but this setback
733 does not come with any significant delay to maturation. One possibility that may explain
734 this observation is faster growth (segment addition) after regeneration. This
735 phenomenon has been observed in *P. dumerilii* before where segment addition rate is
736 highly increased in worms that have recently regenerated (Gazave et al., 2013). Indeed
737 our growth curves also support this: we observe that segment addition rates spiked
738 following amputation, remained high until ~30 DPA (regeneration is completed between
739 5-10 DPA), and eventually appeared to plateau and approach control levels by 63 DPA.

740 Therefore, we see an overall increased growth rate in all amputated groups compared
741 with the control siblings.

Hormonal control of growth and regeneration

743 It is possible complex hormonal interactions and changes explain some of the
744 observations outlined above. The changes in the brain's hormonal composition has
745 been studied extensively in *P. dumerilii* and other Nereididae, and the hormonal states
746 are known to change with growth, age, and during injury (Andreatta et al., 2020; Clark,
747 1969; Clark and Scully, 1964; Golding, 1974, 1967a, 1967b; Hauenschild, 1974, 1966;
748 Hofmann, 1976; Lawrence and Soame, 2009; Olive and Clark, 1978; Scully, 1964). The
749 sesquiterpenoid methylfarnesoate (MF) was recently identified as the 'brain hormone' in
750 *P. dumerilii* (Schenk et al., 2016). MF is known to promote growth and regeneration,
751 inhibit sexual maturation, and mediate developmental transitions in *P. dumerilii* and
752 other annelids (Álvarez-Campos et al., 2023; Biggers and Laufer, 1999, 1996;
753 Hauenschild, 1974, 1966; Hofmann, 1976; Lawrence and Soame, 2009). Brain
754 transplantation experiments have demonstrated that the brain hormone activity
755 generally decreases with age (Hofmann, 1976; Scully, 1964), though the exact
756 trajectory of hormone levels over the worm's lifetime remains unclear. The decreasing
757 levels of MF with age could explain the higher mortality and abnormal regeneration
758 observed in 60s amputees. Other transplantation experiments suggested that brain
759 hormone activity increases in response to amputation (Golding, 1967a), which is
760 thought to be one explanation for faster growth and segment addition rates in recently-
761 regenerated worms. However, no direct measurements of MF have been carried out in
762 these contexts yet, so it remains to be shown whether these observations directly
763 correlate with changes in MF hormone levels.

764 Another possible hormonal factor is thought to exert a negative feedback on the MF
765 activity, triggering sexual metamorphosis and causing the loss of regenerative
766 competency in *P. dumerilii* and other Nereididae (Andries, 2001; Porchet and Cardon,
767 1976; Schenk et al., 2016). The identity of this compound is still unknown but several
768 lines of evidence suggest that this 'feedback substance' is produced by the maturing
769 gametes, eventually inhibiting the MF activity altogether, leading to sexual maturation.
770 Transplantation of oocytes, lysates from the maturing gametes, or injection of coelomic
771 contents were shown to exert negative feedback on the activity of the brain hormone
772 (Andries, 2001; Durchon, 1952; Hofmann, 1975; Porchet, 1967; Porchet and Cardon,
773 1976; Porchet and Durchon, 1968). In *Nereis diversicolor*, worms with oocytes larger
774 than 140 μm show diminished posterior regeneration and worms with oocytes
775 exceeding 180 μm can not regenerate at all (Golding, 1967b).

776 The opposing effects of the brain hormone and the feedback substance could explain
777 some of our results, which may illustrate the trade-offs between germline and somatic
778 regeneration under this hormonal regulatory system. In our experiments, while 60s
779 amputees were capable of full germline regeneration and matured earlier than younger
780 amputees, they also showed higher rates of abnormal regeneration and mortality, with
781 one early wave of deaths by 7 DPA (the completion of posterior regeneration) and
782 several more deaths scattered over the rest of the experiment. Our amputations after
783 segment 8 remove most of the gonial clusters but do not remove the anterior cluster or
784 any gonial clusters remaining in the 8 head segments. The 60s group typically has
785 gametes closer to maturation, and a dispersed anterior cluster which is effectively
786 turning into gonial clusters going into gametogenesis. In contrast, in most 40s samples,
787 gonial clusters were not progressed enough to identify whether they were becoming
788 oocytes or sperm. Therefore the 60s group could presumably have gonial clusters
789 remaining in the 8 original segments with a high negative feedback effect on the brain
790 hormone activity. It is possible that large oocytes, in particular, could interfere with
791 regeneration, possibly through MF inhibition. This could contribute particularly to the
792 mortality that occurs in worms that have successfully added segments before arresting
793 growth and then dying, as observed in one sample that we fixed on the threshold of
794 death, and found oocytes that appeared to have mature size and morphology. It is
795 important to note that several other factors, including mechanical failures of wound
796 closure, could also contribute to increased mortality. Future studies will be required to
797 quantify MF levels following amputation at different time points in different segment size
798 groups to assess the relationship to segment addition rates and mortality.

799 **Developmental thresholds**

800 Organisms have mechanisms to assess growth and nutritional state to make resource
801 allocation decisions towards more growth or sexual metamorphosis and reproduction
802 (Henry and Hart, 2005; Hyun, 2018; Lui and Baron, 2011; Olive and Clark, 1978). For
803 example, holometabolous insects need to reach a threshold body weight for
804 metamorphosis (Tennessen and Thummel, 2011). Studies of polychaete reproductive
805 physiology and endocrinology indicate that in some species maturity, oogenesis, and/or
806 spawning are arrested or delayed until regeneration is complete following tissue loss,
807 usually anterior segments which produce hormones controlling these facets of
808 polychaete reproduction (Olive and Clark, 1978). Similarly in *P. dumerilii*, our previous
809 studies demonstrated a threshold of about 35 segments when small germline clusters
810 start populating the body cavity (germline expansion) and that if worms are amputated
811 before germline expansion, they must still reach ~35 segments to undergo germline
812 expansion. However, in the current study, we observe that once worms have reached
813 and passed the germline expansion stage, they do not need to grow back to 35
814 segments to start producing gonial clusters after regeneration. For example, by 23 DPA,

22

815 worms have numerous gonial clusters even though most of them have not reached the
816 ~35 segment threshold: 40s amputees that have started regenerating gonial clusters
817 range between 20-30 segments, and 60s amputees that have started regenerating
818 gonial clusters range between 26-37 segments. These results suggest that once worms
819 have crossed the ~35-segment milestone, they no longer need to reach 35 segments to
820 produce gonial clusters again.

821 **Potential sources of regenerated gonial clusters and future directions**

822 Many annelids express germline/multipotency genes such as *vasa*, *piwi*, *nanos* in
823 tissues that are not strictly germline (Gazave et al., 2013; Kostyuchenko and Smirnova,
824 2023; Özpolat and Bely, 2016, 2015; Planques et al., 2018). For example, in *P.*
825 *dumerilii*, *vasa* is expressed not only in the gonial tissues, but also in the posterior
826 growth zone, and in the regeneration blastema. It is still unclear if these tissues are able
827 to give rise to both somatic and germ cells. In this study, we also found *vasa* expression
828 to persist days and weeks after regeneration is completed in a broad pattern that
829 extends beyond the growth zone into many segments. This expression appears very
830 different from expression patterns observed in worms that were amputated in stages
831 before gonial clusters form (e.g. 10s or 20s worms) (Gazave et al., 2013; Kuehn et al.,
832 2021; Planques et al., 2018). These worms have individual or groups of *vasa*-
833 expressing cells that do not look like either the singlet oocytes, typical gonial clusters, or
834 the posterior growth zone expression that is typically confined to the segment addition
835 zone and a few newly-produced segments. Therefore these cells could be a potential
836 source of regenerated cell types, including the gonial clusters. Future studies with
837 genetic lineage tracing will be able to directly address this open question.

838 In addition, due to the unique biology and morphology of *P. dumerilii*, where germline
839 clusters float freely throughout the coelom, we could not determine whether the clusters
840 that we observed in the newly regenerated segments, especially at the earlier
841 regeneration time points, were themselves regenerated *in situ*, or were a product of the
842 original gonial clusters that drifted into the regenerated region from the original
843 segments, or both. It is important to note that especially in 60s group, gonial clusters are
844 generally progressed in their gametogenesis, and if new gonial clusters are made from
845 these existing gonial clusters, how do progressed gonial clusters at such distinct states
846 of gametogenesis give rise to new gonial clusters will be an interesting area of inquiry. It
847 is possible some gonial clusters regress back to an earlier more undifferentiated stage
848 to undergo mitosis. In addition, if the new gonial clusters only regenerate from the
849 original anterior cluster and/or gonial clusters, it will be interesting to investigate whether
850 these sources can be depleted upon repeated amputation-regeneration cycles. In
851 previous studies we observed that the anterior cluster eventually disperses in control
852 worms, and in this study, we also show that it disperses in 60s amputees. The worms

23

853   with the dispersed anterior cluster phenotype may respond to the repeated amputation-

854   regeneration challenge differently. Future studies focusing on genetic lineage tracing, or

855   live cell tracing using tissue-specific expression of fluorescent markers will address

856   these open questions.

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865 **FIGURES**

866

867 **FIGURE 1**

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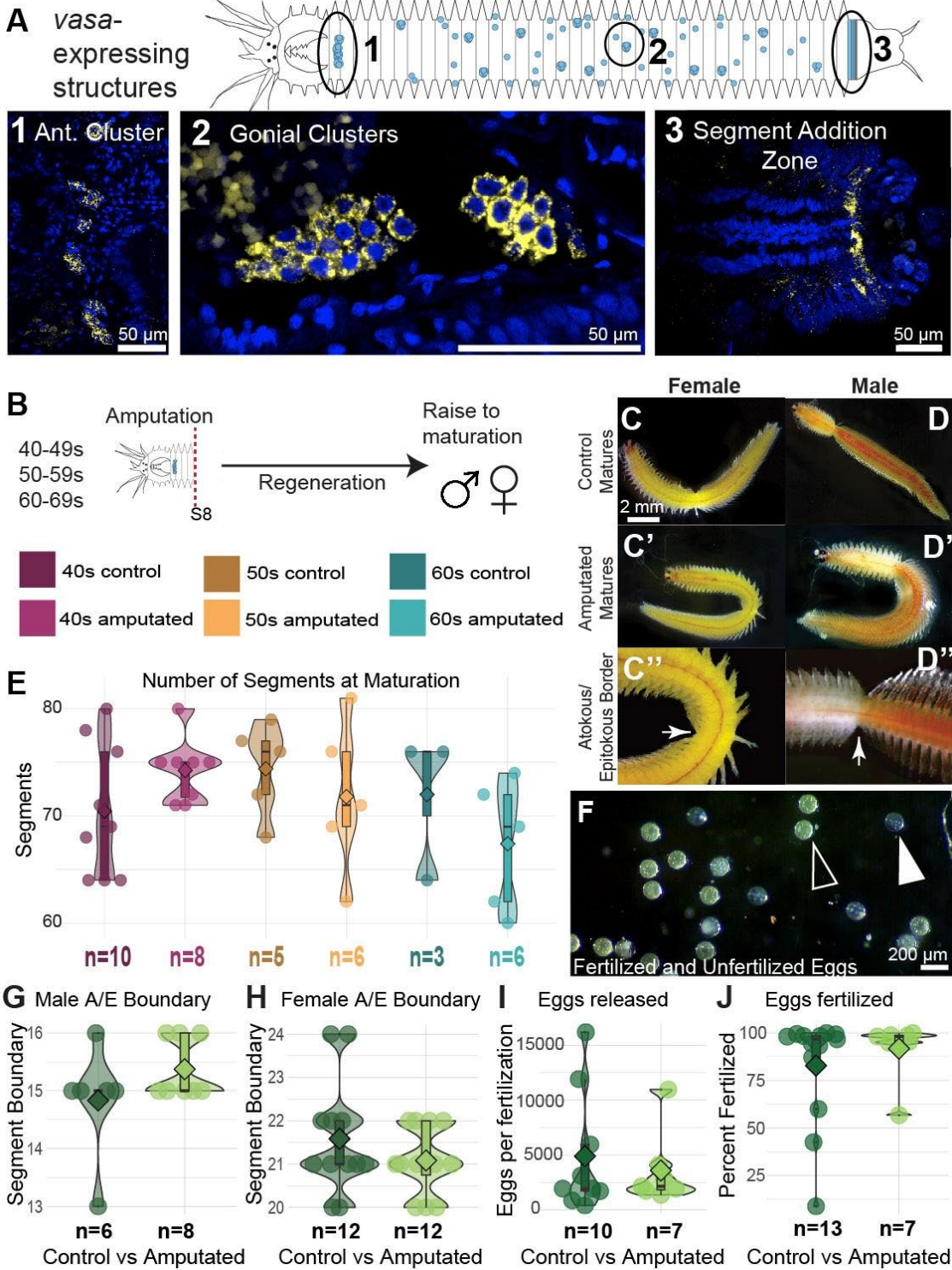
Figure 1: Amputated worms have morphologically and functionally normal maturation after germline regeneration.

A) Example worm showing the *vasa* expressing structures (yellow). An anteriorly-located band of germ cells (1) is thought to be the source of the gonial clusters (2) which spread across the worm's body and float in the coelomic fluid. DAPI stain in blue. Scale bars 50 µm. B) Experimental schematic. Worms were collected into three groups of 10-segment ranges: 40-49 segments, 50-59 segments, and 60-69 segments. These ranges span the developmental period between germline expansion (~35 segments) and sexual metamorphosis (~70 segments). Worms were amputated after the 8th pair of chaetae and raised to maturation alongside unamputated controls. C) Control mature female. Scale bar is 2 mm for C-D'. C') Amputated and regenerated mature female. C'') Female atokous-epitokous border, indicated by white arrow. D) Control mature male. D') Amputated and regenerated mature male. D'') Male atokous-epitokous border, indicated by white arrow. E) Number of segments at maturation. In univariate models, there was no difference among the 3 segment group (p=0.3115), between the control and amputated groups (p=0.9127) or between males and females (p=0.2257). F) Example photo showing how fertilized (solid arrowhead) and unfertilized eggs (outlined arrowheads) can be visually differentiated by color and opacity by 2 hours post fertilization. Scale bar 200 µm. G-H) The location of the male and female atokous/epitokous border did not differ significantly between control and amputated matures (Wilcoxon signed rank test on paired samples, p=0.285 for males, p=0.464 for females). I) The number of eggs released upon spawning did not differ significantly between control and amputated mature females (Wilcoxon, p=0.812). J) Percentage of eggs fertilized did not differ significantly between control and amputated matures (Wilcoxon, p=0.751).
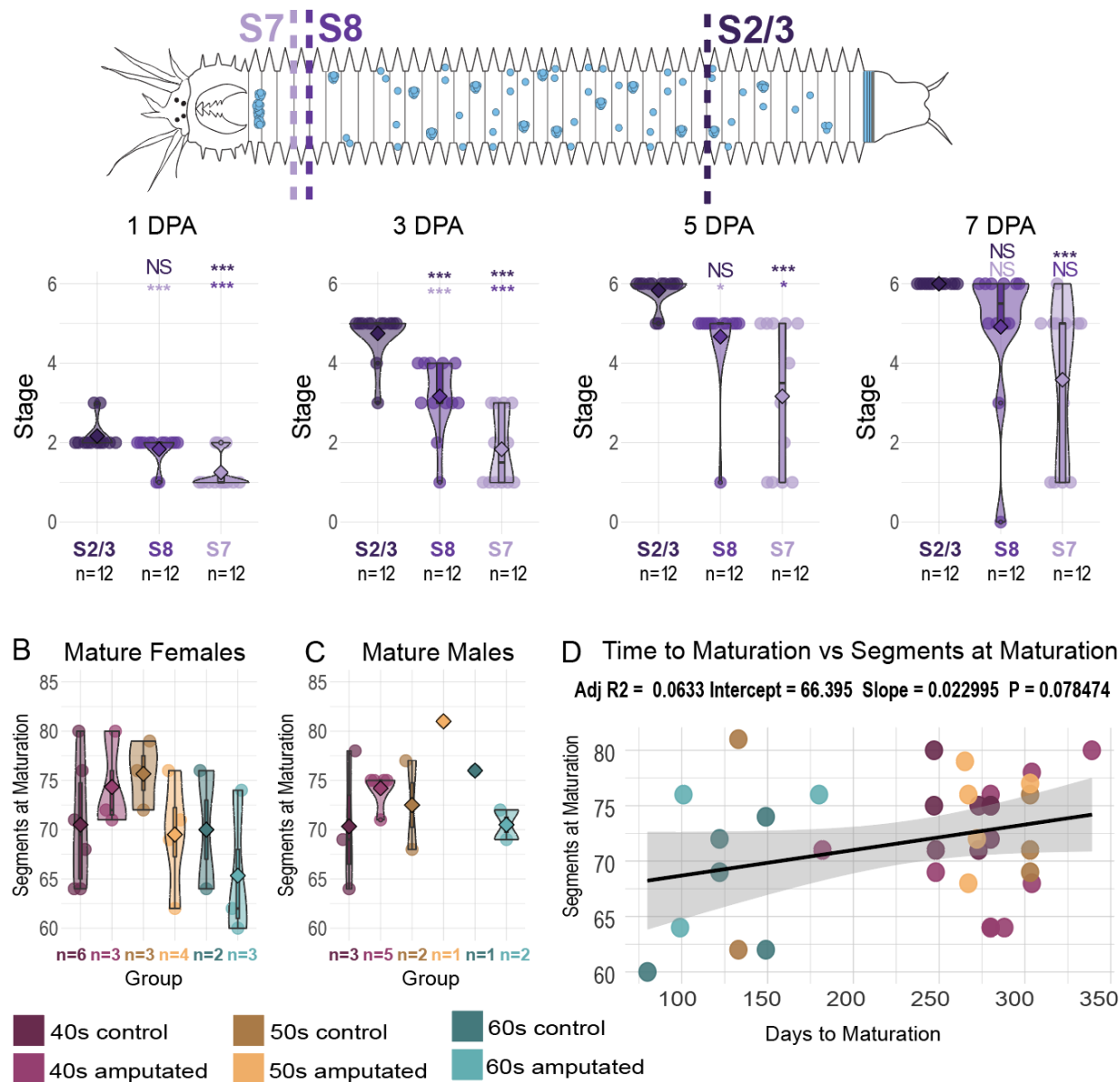
897 **FIGURE S1**

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900 **Figure S1:**
901 **Worms cut at different locations show variable progression of posterior**
902 **regeneration.** Amputations after the 7th pair of chaetae, the 8th pair of chaetae, or
903 approximately 2/3rds down the length of the body (removing ~1/3rd) were compared.
904 The stage of regeneration was recorded for each group at A) 1 day post amputation
905 (DPA), B) 3 DPA, C) 5 DPA, and D) 7 DPA. By 7 DPA, worms amputated after the 8th
906 pair of chaetae did not significantly differ from worms with ~1/3rd of segments removed

27

907   (t test, p=0.058). Therefore the 8th segment was chosen as the amputation plane to

908   maximize segment removal for subsequent experiments.

909   E,F) The number of segments at maturation showed no significant differences between

910   groups when separated by sex.

911   G) When all groups are plotted by days to maturation against the number of segments

912   at maturation, there is not a statistically significant linear correlation (p=0.08).

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915 **FIGURE 2**

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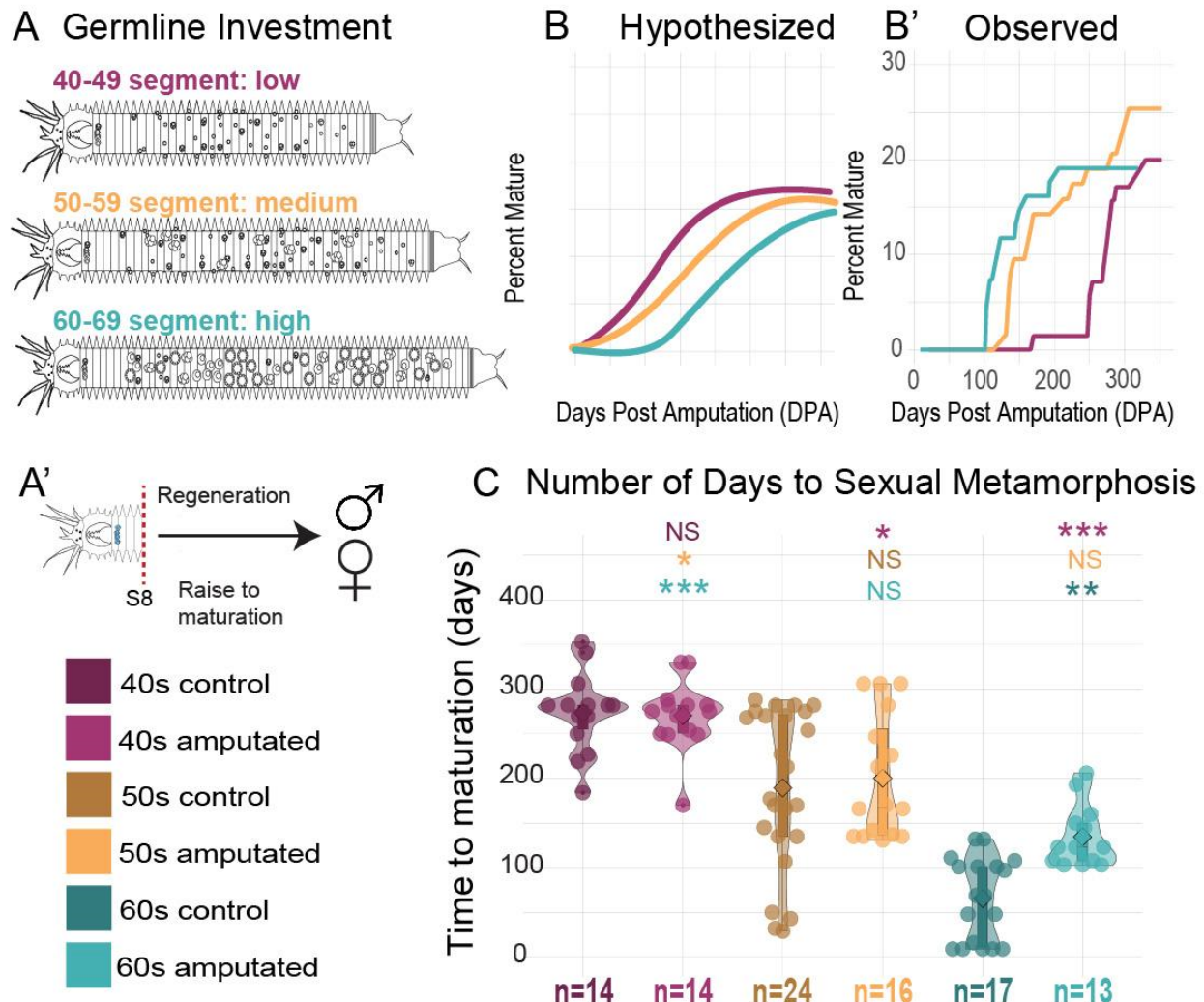
Figure 2: Developmentally "older" regenerates mature earlier than "younger" regenerates.

A-A') Experimental schematic. Worms of three 10-segment ranges spanning low, medium, and high germline investment (A) were amputated after the 8th pair of chaetae and raised to maturation alongside unamputated controls (A').

B-B') Hypothetical vs observed results. We hypothesized that older worms would take longer to maturation, however we observed the opposite result.

C) Violin plots showing the number of days to reach maturation per group (controls and amputated). 60s amputees matured the earliest at an average of 134 days to reach sexual metamorphosis, followed by 50s amputees at an average of 200 days, and 40s amputees at an average of 270 days. Time to maturation differed significantly between 60s amputees and 40s amputees (p<0.001). While 50s amputees did not differ significantly from 40s or 60s amputees, this group was in between the other two groups,

29

931 showing a gradual shift. Asterisks indicate the level of significance of planned pairwise
932 comparisons (* p<0.05, ** p<0.01, *** p<0.001, NS if p>=0.05).
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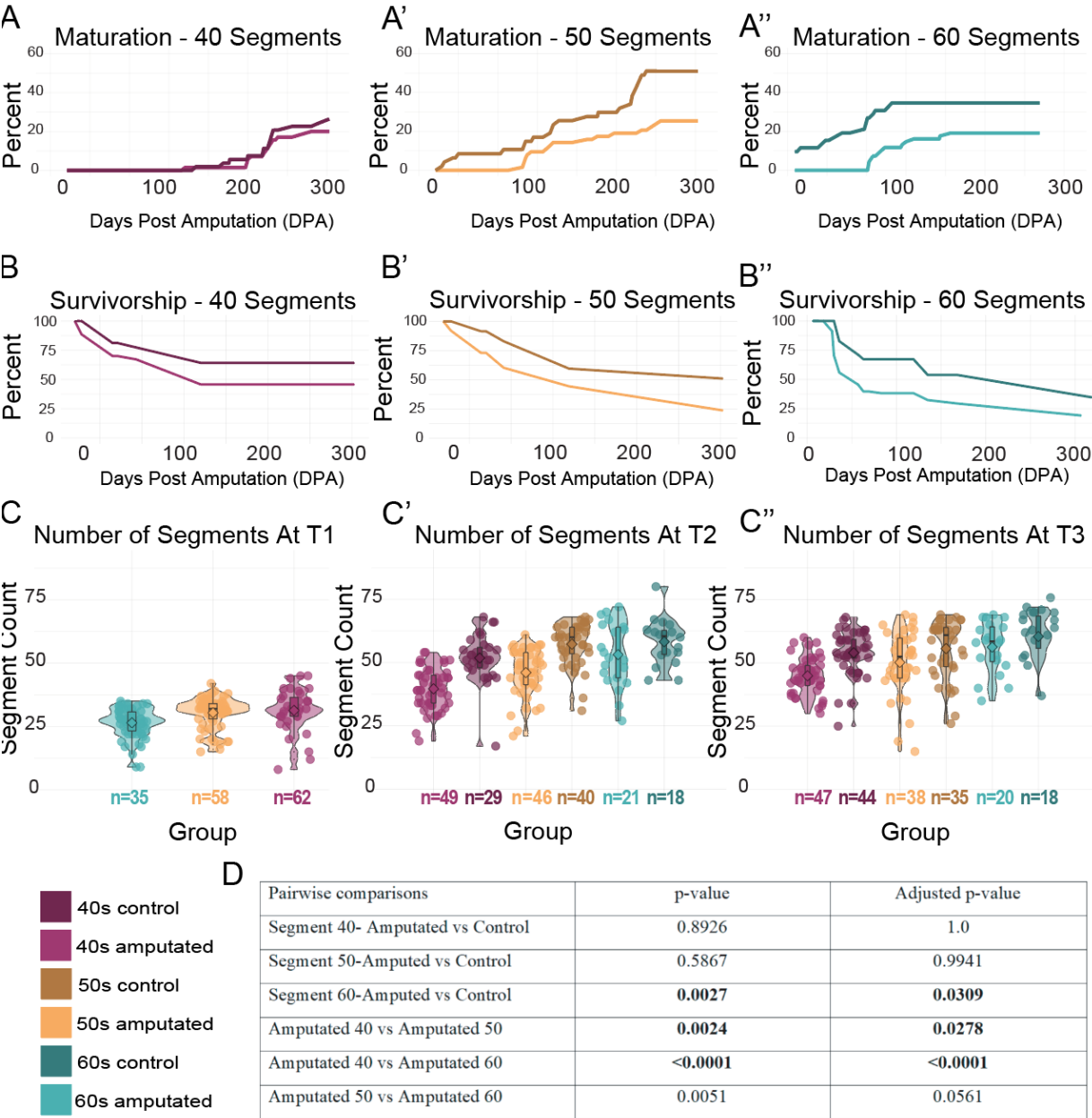
FIGURE S2

Figure S2

A-A") Plots show maturation over time for three segment groups. B-B") Plots show
survivorship over time for three segment groups. Color codes for all groups are
indicated in the lower left.

942   C-C'') The number of segments at T1 (28 days post amputation (DPA)). Only amputated

943   groups represented in this count. C') The number of segments at T2, 63 DPA. C'') The

944   number of segments at T3, 84 DPA. Both amputated and control groups are shown in

945   C' and C''. D) P values for pairwise comparisons of time to maturation before and after

946   Tukey-Kramer adjustment. Statistically significant values in bold (p>=0.05).

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949   **FIGURE 3**
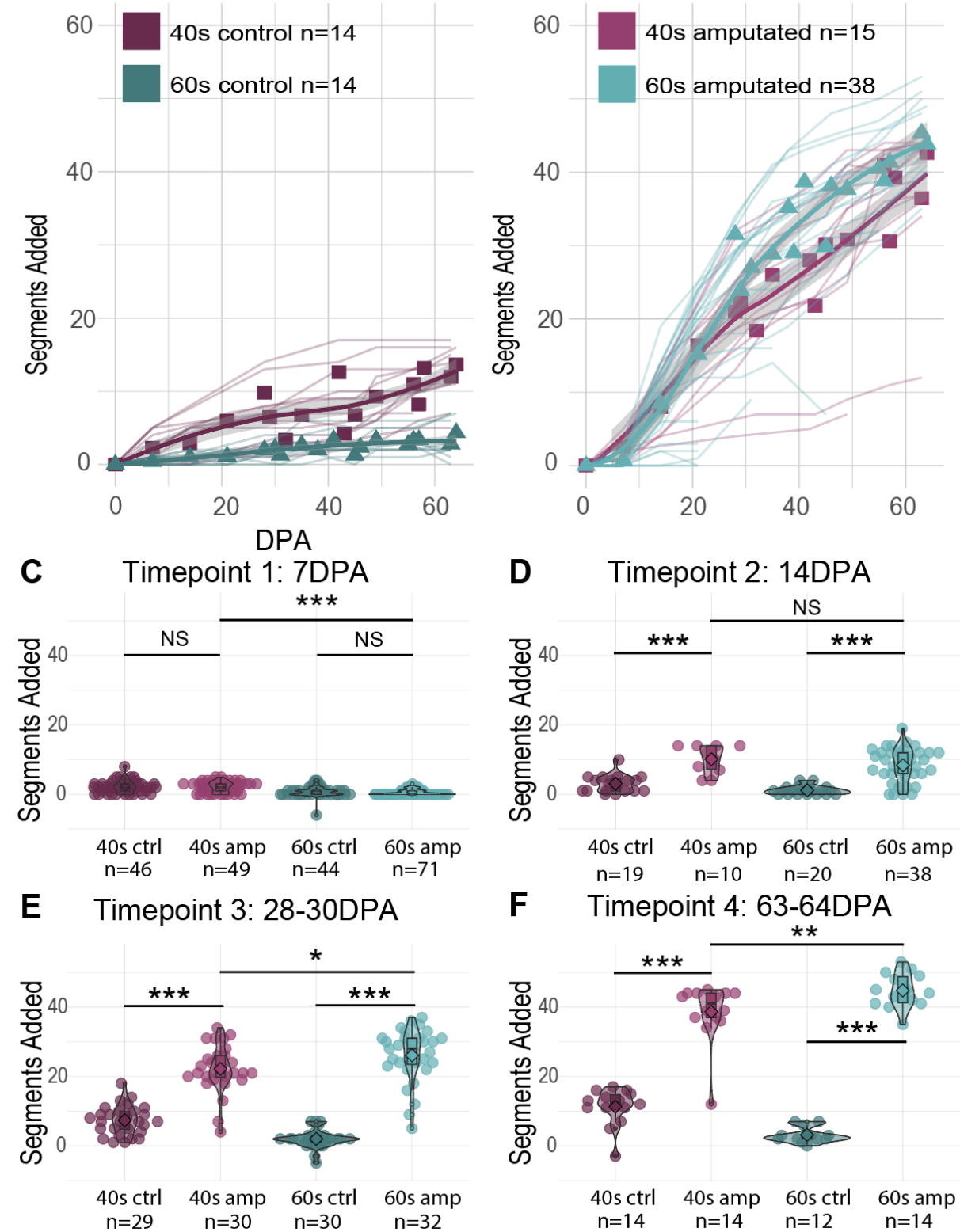
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951   **Figure 3: Older (60S) and younger (40S) worms differ in long-term regenerative**
952   **growth trajectories.**

953 A-B) Line graphs show the number of segments added since the beginning of the
954 experiment in days post amputation (DPA) for control and amputated groups. Individual
955 growth trajectories are depicted as thin semi-transparent lines. Triangles represent the
956 means at each point for which data is available (days when segments were counted). A
957 locally weighted regression line is drawn in bold with a 90% confidence region in
958 transparent gray. (Note that graphs in A-B show a detailed subset of data from plots in
959 C-F. In A-B, the segments of individual worms were counted up to 64 DPA. Plots in C-F
960 include segment counts from similarly treated worms at different timepoints (however
961 not all these samples were raised to 64 DPA therefore numbers of samples change)).
962 C-F) Violin plots show the distribution of the number of segments added per group at 4
963 time points spanning the duration of the experiment, revealing changes in the pace of
964 segment addition among and between groups. Individuals are represented by semi-
965 transparent dots, and violins with box plots superimposed illustrate the distribution
966 within each group. A diamond shows the group mean. A multivariate ANOVA was
967 performed assessing the impact of segment group (40-49 segments or 60-69
968 segments), amputation (amputated or control), and interaction between these terms. T
969 tests were performed with Benjamini-Hochberg correction for multiple comparisons.
970 Asterisks indicate the level of significance of pairwise comparisons (t tests; * $p<0.05$, **
971 $p<0.01$, *** $p<0.001$, NS if $p>=0.05$). At 7 DPA, 40s amputees have added more
972 segments than 60s amputees ($p=2.40E-06$). By 14 DPA, amputees no longer
973 significantly differ from each other ($p=0.20$). By 28 DPA, 60s amputees have added
974 more segments than 40s amputees ($p=0.01058$), which persists as growth appears to
975 plateau approaching 63 DPA ($p=0.0072$). One 40s control sample and three 60s control
976 samples auto-amputated 29 days after the beginning of the experiment and were
977 removed from analysis.
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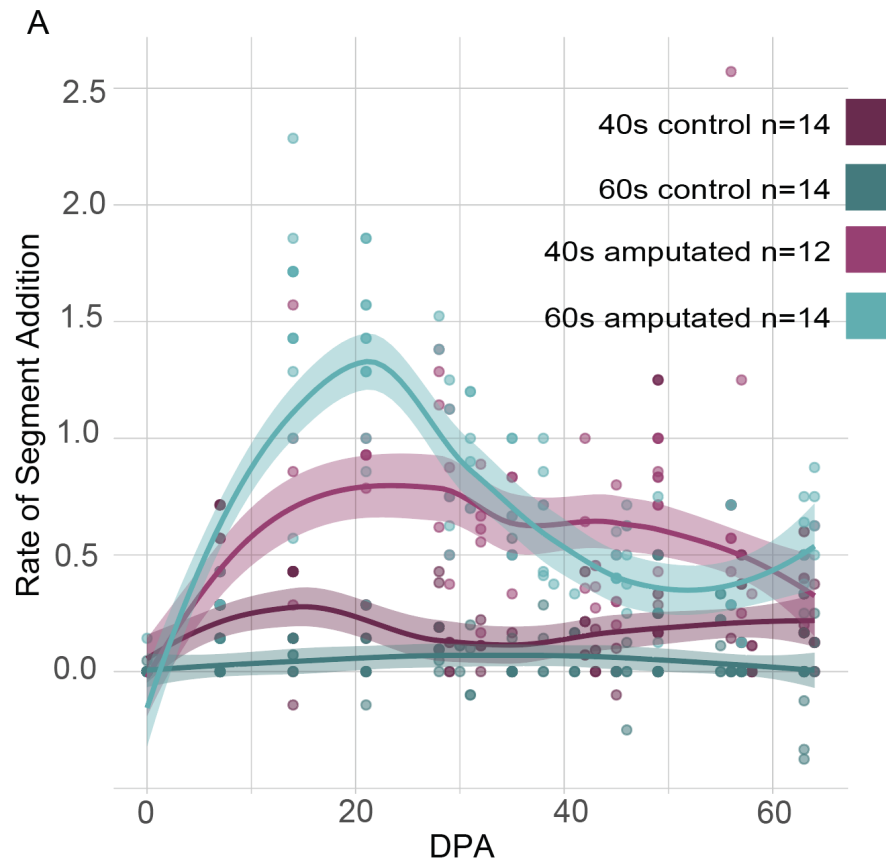
982   **FIGURE S3**

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984   **Figure S3**

985   A) Worms were counted approximately once per week. Segment addition rate was
986   calculated as the number of segments added divided by the number of days since the
987   last measurement. Only worms that survived until the final measurement time point at
988   63 DPA were included in this analysis, since worms that die slow down and then stop
989   adding segments in advance of their death and the elevated mortality rate of 60s
990   amputees would obscure trends in segment addition rate. A few individual worms have
991   negative growth rates; this is likely due to +/-1 variation in segment counting and occurs
992   exclusively in controls with slow to no segment addition, so +/-1 results in a negative
993   rate.

994 **FIGURE 4**

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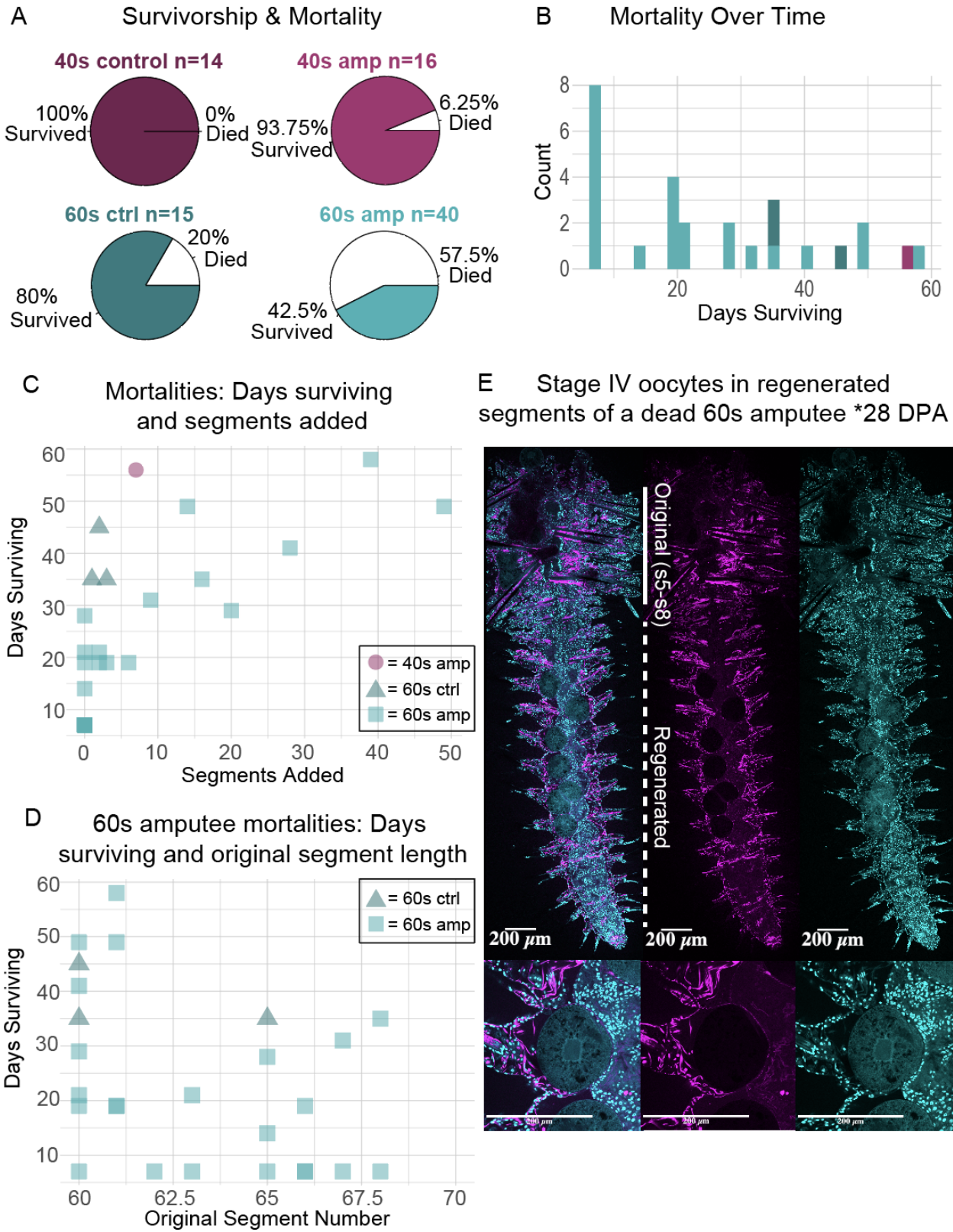
997 **Figure 4**: **Age-specific mortality, regeneration defects and delays**

998 A) Pie charts showing the percentage of survivorship and mortality for each group at the

999 end of 63 DPA. Survivorship is represented by color and mortality is represented by

1000 white space.

1001 B) Stacked histogram of mortality in each group over time. 60s amputees have elevated

1002 mortality compared to all other groups.

1003 C) A scatterplot illustrating the number of days surviving and the original number of

1004 segments for all worms that died 0-64 DPA.

1005 D) A scatterplot illustrating the number of days surviving and the number of segments

1006 added for all 60s worms that died 0-64 DPA.

1007 E) A fixed 60s amputee stained with DAPI (cyan) and phalloidin (magenta) to illustrate

1008 the presence of mature oocytes in the regenerated segments. This individual survived

1009 until 28 DPA, had regenerated successfully, and had added 16 segments by the time of

1010 death. Original segments are denoted by the solid white bar, and regenerated segments

1011 by a dashed white bar. All scale bars are 200 µm.

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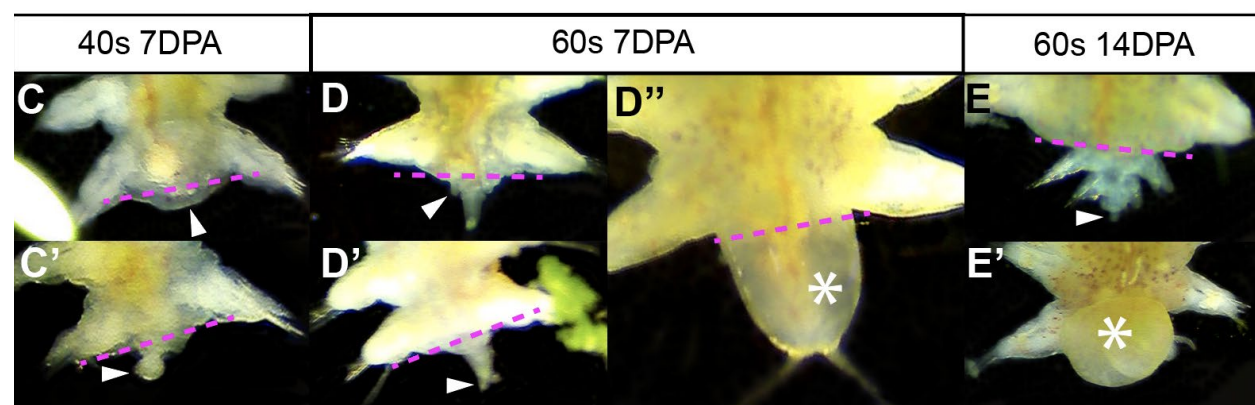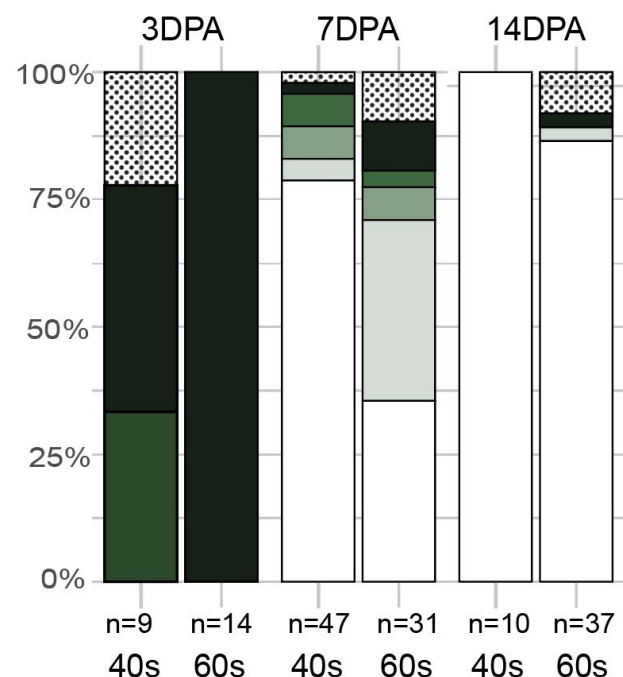1013

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1015   **FIGURE 5**

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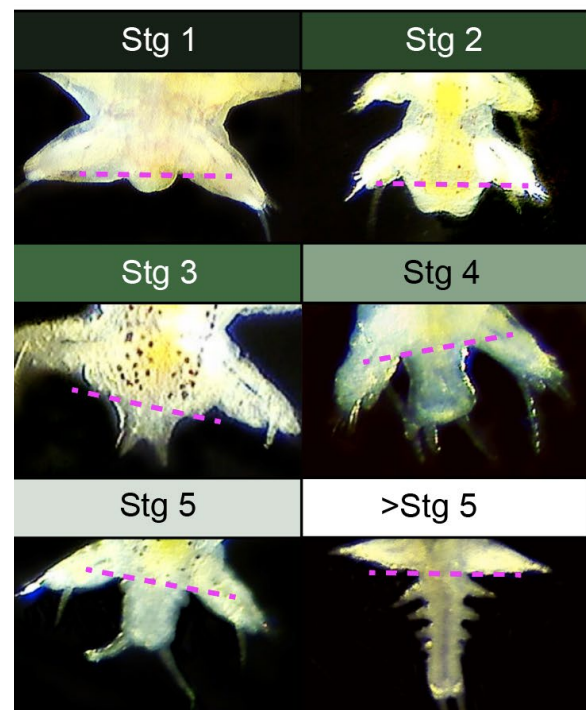
1017   **Figure 5: Older Amputees Have Higher Rates of Abnormalities and Delayed**
1018   **Regeneration.**
1019   A) The stages of normal posterior regeneration in 40s worms. The magenta dashed line
1020   indicates the amputation plane separating original tissue (above) and regenerated
1021   tissue (below). Stages 1-5 are based on previously published results (Planques et al.,
1022   2018): wound healing (Stg 1), formation of the blastema and anus (Stg 2), formation of
1023   anal cirri (Stg 3), elongation of the regenerated region and anal cirri (Stg 4), and the
1024   patterning of segment primordia (Stg 5). >Stg 5 indicates newly formed segments have
1025   visible chaetae, signaling a shift from posterior regeneration into posterior growth. Any
1026   morphological deviation was scored as abnormal.

37

1027   B) Bar graphs showing the proportion of each stage at time points 3-14 days post

1028   amputation (DPA), color coded by stages in A. Abnormal samples indicated with bars

1029   filled with dots. C-E') Examples of abnormal regeneration in the 40s and 60s worms

1030   from 7-14 DPA. The white arrowheads show morphological abnormalities, and white

1031   asterisks denote edema. C) Regeneration does not appear to have progressed past

1032   wound healing. C') A small abnormal outgrowth. D) An abnormally small regenerated

1033   pygidium that appears to come to a single point rather than symmetrical cirri, with two

1034   apparent segment primordia. D') Anal cirri have formed, but the regenerate is

1035   abnormally small. D'') The regenerated region is swollen and filled with fluid, though

1036   patterning appears otherwise correct. E) The pygidium is abnormally small and appears

1037   to come to a single point. E') The area around the wound site is swollen and filled with
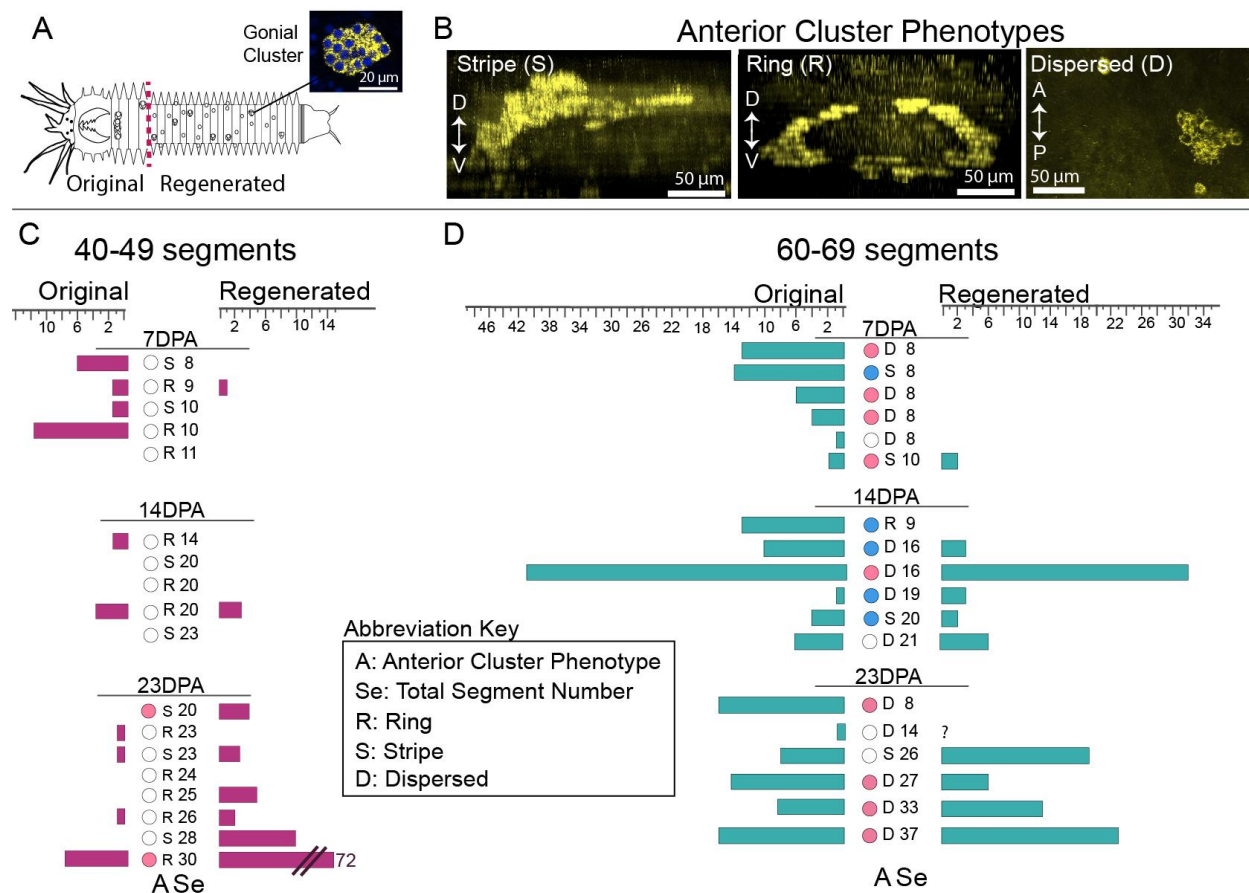
1038   fluid.

Figure 6: Gonial cluster regeneration across time in the 40s and 60s groups
A) Cartoon showing the regions compared in the graphs. Dashed line indicates the amputation plane. B) Examples of anterior cluster phenotypes visualized via *vasa in situ* HCR (yellow). Note that the first two show dorsal-ventral cross section, where the anterior cluster is either in a stripe layout dorsal to the gut, or it forms a ring that extends dorso-ventrally around the gut. A third phenotype is called dispersed, when a clear pattern is lacking and only smaller clusters of *vasa*-expressing clusters are found in this region. C-D) Graphs showing the number of gonial clusters observed in the 40s or 60s regenerates across time points 7, 14, 23 DPA. Only discreet gonial clusters, and singlet oocytes were included in these counts (see Fig. 7G for examples) . The circles indicate whether observed clusters have started sexual differentiation (blue/pink for M/F, empty for undifferentiated or NA, if there are no clusters observed). The "A" column indicates the state of the anterior cluster: "D" for dispersed, "R" for ring, "S" for stripe. The column "Se" reports the total number of segments in each sample. Samples are arranged from shortest to longest in terms of segment number.
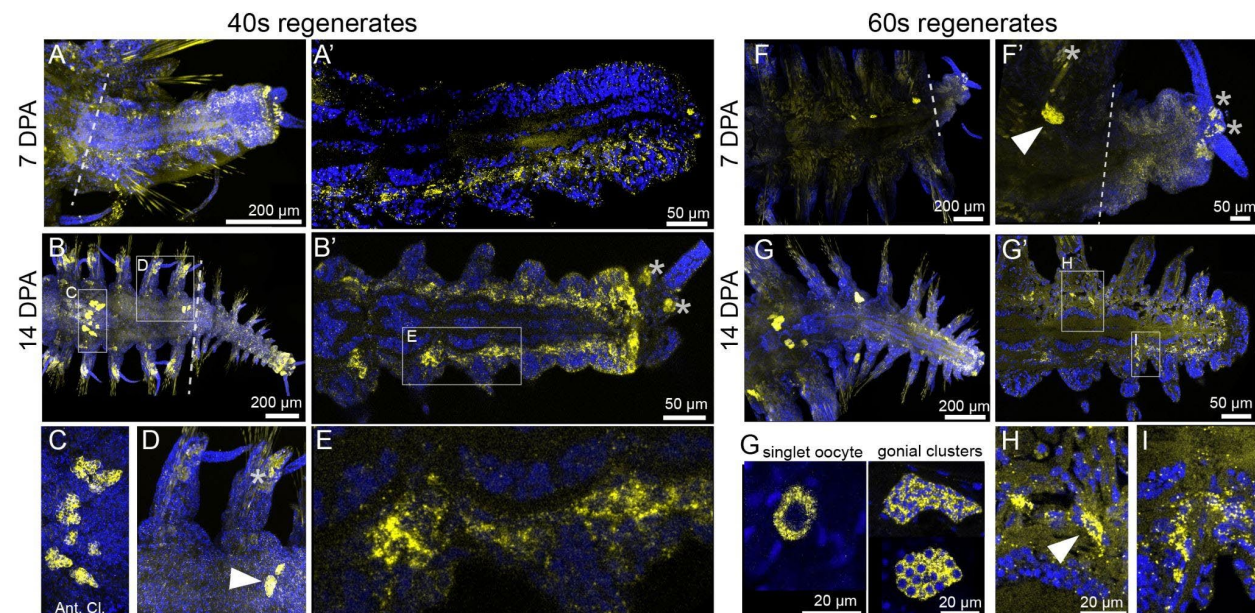
1057 **FIGURE 7**

1060 **Figure 7: *in situ* HCR for *vasa* expression in 40s and 60s regenerates**

1061 Examples of samples from Figure 6 shown. At 7 DPA (A-A') and 14 DPA (B-B') in 40s
1062 worms, there is a broad *vasa* expression (yellow) that looks distinct from gonial clusters
1063 (arrowheads in D and F'). E) Zoomed version of the broad expression in the 40s
1064 amputee. This broad expression is also present in 60s regenerates but more prominent
1065 at 14 DPA (G-G'). H and I show zoomed versions of the *vasa*-expressing cells that are
1066 found during regeneration. Compared with the singlet oocytes or gonial clusters, these
1067 cells appear less organized, have smaller nuclei, and are generally located in the region
1068 between the gut and the epidermis. Zoomed version of the anterior cluster (example of
1069 "stripe") is shown in C. Blue color is DAPI nuclear stain. Dashed line indicates the
1070 amputation plane. Stars indicate non-specific signal that is commonly found in chaetal
1071 sacs, chaetae and the pygidium (B', D, F').

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