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Mating and Male pheromone kill *Caenorhabditis* males through distinct mechanisms

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Running Title: Pheromone killing is mediated by male germline and neurons

36 **Summary**

37

38 Differences in longevity between sexes is a mysterious yet general phenomenon
39 across great evolutionary distances. To test the roles of responses to environmental
40 cues and sexual behaviors in longevity regulation, we examined *Caenorhabditis* male
41 lifespan under solitary, grouped, and mated conditions. We find that neurons and the
42 germline are required for male pheromone-dependent male death. Hermaphrodites
43 with a masculinized nervous system secrete male pheromone and are susceptible to
44 male pheromone killing. Male pheromone-mediated killing is unique to
45 androdioecious *Caenorhabditis*, and may reduce the number of males in
46 hermaphroditic populations; neither males nor females of gonochoristic species are
47 susceptible to male pheromone killing. By contrast, mating-induced death, which is
48 characterized by germline-dependent shrinking, glycogen loss, and ectopic
49 vitellogenin expression, utilizes distinct molecular pathways and is shared between
50 the sexes and across species. The study of sex- and species-specific regulation of
51 aging reveals deeply conserved mechanisms of longevity and population structure
52 regulation.

53

54 Introduction

55
56
57 Males and females differ in many aspects of biology, including longevity. Sex differences in
58 lifespan are common in animals across great evolutionary distances (Austad and Fischer
59 2016). For example, women live longer than men in almost every country (WHO 2015).
60 Moreover, interventions in longevity also display sex-specific patterns in mice (Austad and
61 Fischer 2016). However, the underlying mechanisms of sex differences in longevity and
62 responses to aging interventions, and the degree of evolutionary conservation of these
63 mechanisms, are still largely unknown.

64
65 Interactions between the sexes influence an individual's longevity (Fowler and Partridge
66 1989; Gems and Riddle 1996; Partridge and Farquhar 1981; Van Voorhies 1992). Sex-
67 specific longevity patterns also exist in *Caenorhabditis elegans*: males live significantly
68 shorter when maintained in groups, whereas the longevity of hermaphrodites is not influenced
69 by population density (Gems and Riddle 2000). What causes this sex-specific longevity
70 pattern remains mysterious. Although worm studies have contributed significantly to our
71 general understanding of longevity mechanisms, how *C. elegans* male lifespan is regulated is
72 poorly understood, because nearly all lifespan experiments are performed using only
73 hermaphrodites. Analysis of *Caenorhabditis* males' longevity not only allows us to test
74 whether known longevity mechanisms are conserved between the sexes, but also provides
75 an opportunity to reveal novel longevity mechanisms. The lifespan of *Caenorhabditis* females
76 is shortened after mating through receipt of male sperm and seminal fluid (Shi and Murphy
77 2014), and separately by exposure to male pheromone (Maures et al. 2014). However,
78 previous studies reported contradictory results on the influence of mating on male lifespan
79 (Gems and Riddle 1996; Van Voorhies 1992). Thus, whether and how male lifespan is
80 affected by prolonged exposure and interactions with females, as well as the effect of
81 pheromone on male lifespan, is unknown.

82
83 In their natural environments, animals must not only find food, but also avoid competitors,
84 identify appropriate partners, and mate; the effects of these behaviors on lifespan are not well

85 understood. Some of these behaviors are mediated by ascaroside-based pheromones
86 (Ludewig and Schroeder 2013). *Caenorhabditis* females secrete pheromones that attract
87 males (Chasnov et al. 2007), while *C. elegans* hermaphrodites modify their pheromone profile
88 according to their sperm status, becoming more attractive to males once they have used up
89 their own sperm (Garcia et al. 2007; Kleemann and Basolo 2007; Morsci et al. 2011). Male
90 ascaroside pheromones can directly affect the reproductive system of hermaphrodites, aiding
91 recovery from heat stress and delaying the loss of hermaphrodite germline stem cells
92 (Aprison and Ruvinsky 2015, 2016).

93

94 The diversity of *Caenorhabditis* species allows us to evaluate male lifespan regulation from
95 an evolutionary perspective. The *Caenorhabditis* genus consists of both androdioecious
96 (male and hermaphrodite) and gonochoristic (male and female) species. In androdioecious
97 species such as *C. elegans*, the population is dominated by hermaphrodites, which reproduce
98 by self-fertilization. Males are usually very rare (less than 0.2% for the standard lab strain N2)
99 and are produced through spontaneous X chromosome nondisjunction (Chasnov and Chow
100 2002; Hodgkin 1983). Under stressful conditions, more oocytes undergo chromosome non-
101 disjunction; thus androdioecious species may periodically experience male population
102 explosions (Chasnov and Chow 2002; Hodgkin 1983). The existence of males in
103 androdioecious species reduces inbreeding and facilitates adaptation to changing
104 environments (Anderson et al. 2010). By contrast, gonochoristic species such as *C. remanei*
105 (50% male, 50% female) require mating for reproduction. How males in androdioecious and
106 gonochoristic species cope with these different mating situations remains poorly understood.
107 Moreover, the utility of killing females by exposure to male pheromone in gonochoristic
108 populations (Maures et al. 2014) is not obvious.

109

110 Here we have found that male-specific population density-dependent death in *C. elegans* is
111 due to the perception of male pheromone as a toxin; that is, while male pheromone itself is
112 not a general poison to all worms, its perception by *C. elegans* males leads to death and to
113 male-specific reproductive decline. *C. elegans* hermaphrodites, while still susceptible, are
114 less sensitive to this toxic aspect of male pheromone. Masculinization of the hermaphrodite

nervous system not only increases their sensitivity to male pheromone, but also is sufficient to induce male density-dependent death in these hermaphrodites, suggesting that neurons are key for the male pheromone killing mechanism. We found that the germline of the recipient male is also required for male pheromone-mediated death. This phenomenon is also present in two other independently-evolved androdioecious *Caenorhabditis* species, suggesting a role for male pheromone killing of males in otherwise hermaphroditic species; by contrast, neither males nor females of three gonochoristic *Caenorhabditis* species succumb to male pheromone killing. Mating-dependent death and germline-dependent shrinking, by contrast, are shared between all sexes and *Caenorhabditis* species, suggesting deep conservation. Our work highlights the importance of understanding the shared vs. sex- and species-specific mechanisms that regulate lifespan.

Results

Male-specific density-dependent lifespan decrease is largely due to male pheromone

When *C. elegans* males are housed together, they live shorter than solitary males, and the death rate increases with the number of males in a dose-dependent manner (Gems and Riddle 2000). *C. elegans* male lifespan is very sensitive to male density: just two males together significantly reduced each individual's lifespan, and grouping eight males decreases lifespan by more than 35%, whereas the lifespan of *C. elegans* hermaphrodites is not affected by population density (Fig. 1A, B). It was shown previously that *C. elegans* hermaphrodites can be killed by pheromone secreted by grouped *C. elegans* males (Maures et al. 2014). We wondered whether the population density-dependent lifespan decrease of grouped *C. elegans* males is also due to male pheromone toxicity. To study the role of pheromone, we tested the survival of grouped *daf-22* (ascaroside pheromone-production deficient (Golden and Riddle 1985)) males. Eight grouped *daf-22(m130)* males lived almost as long as solitary wild-type males, suggesting that male pheromone kills grouped wild-type males (Fig. 1C; Fig. 1 – figure supplement 1A).

145 **Males are more sensitive to male pheromone toxicity than are hermaphrodites**

146 The fact that adding just one other male significantly decreases male lifespan (Fig. 1A)
147 suggests that *C. elegans* males are very sensitive to male pheromone. To compare the
148 sensitivity to male pheromone between the sexes, we decreased the number of males from
149 30-150 as was previously used in male-conditioned plates (Maures et al. 2014) to only 8 per
150 plate (Fig. 1 – figure supplement 1B), and examined the lifespans of *daf-22* males and
151 hermaphrodites. This low dosage of male pheromone had no effect on hermaphrodites, but
152 significantly reduced *C. elegans* male lifespan (Fig. 1D, E), suggesting that males are more
153 sensitive than hermaphrodites to male pheromone toxicity. In fact, we found that exposure to
154 pheromone secreted by just one male was sufficient to significantly reduce the lifespan of *C.*
155 *elegans* males (Fig. 1F).

156

157 **Male pheromone toxicity requires the germline**

158 To identify the transcriptional effects of male pheromone treatment on males, we performed
159 expression analysis of *daf-22* (pheromone-less) males exposed to male pheromone for six
160 days of adulthood. To avoid any germline-dependent effects of male interactions, we added
161 the DNA replication inhibitor 5-fluorodeoxyuridine (FUdR) (Fig. 1G) to the plates, which
162 inhibits germline proliferation (Shi and Murphy 2014). (Adult treatment with the DNA
163 replication inhibitor 5-fluorodeoxyuridine (FUdR) has little effect on lifespan and meiosis at
164 low dosage (50 μ M (Luo et al. 2009)), but rapidly blocks germline proliferation in mated
165 hermaphrodites (Shi and Murphy 2014); FUdR and other germline-blocking approaches are
166 commonly used in expression analyses to avoid confounds (Reinke et al. 2000; Shaw et al.
167 2007; Maures et al. 2014).) To our surprise, this comparison revealed no differences in gene
168 expression (Fig. 1 – figure supplement 1C), even at a high % False Discovery Rate (FDR),
169 suggesting that blocking male germline proliferation prevents male pheromone's effects on
170 gene expression. Indeed, we found that there was no lifespan difference when *daf-22(m130)*
171 males were subjected to a high level of exogenous wild-type male pheromone (30 males per
172 plate for conditioning) in the presence of FUdR, mimicking the microarray conditions (Fig. 1G).
173 Furthermore, no population density-dependent lifespan decrease was observed when
174 germline-deficient *glp-1(e2141)* males were grouped (Fig. 1H). Similarly, exposing grouped

175 *glp-1* hermaphrodites to very high levels of male pheromone (60 wild-type males per plate for
176 conditioning) also failed to shorten lifespan or to induce significant transcriptional changes
177 (Fig. 1 – figure supplement 1D, E). Therefore, our results suggest that germline activity in the
178 recipient is required for male pheromone-mediated death in both sexes.

179
180 Interestingly, we also found that the loss of the germline itself also affects the production of
181 male pheromone: males on plates conditioned by germline-less *glp-1* males lived longer than
182 those on plates conditioned by wild-type males (Fig. 1 – figure supplement 1F), suggesting
183 that there is communication between the status of germline and the production of male
184 pheromone. However, males on plates conditioned by germline-less males still live shorter
185 than the controls, suggesting that the germline cannot be the site of pheromone production,
186 but rather may modulate pheromone levels or quality.

187

188

189 **Neuronal masculinization of hermaphrodites is sufficient to induce male-like** 190 **pheromone production and sensitivity in hermaphrodites**

191 The worm perceives environmental cues through its nervous system (Bargmann 2006). To
192 test whether the nervous system influences worms' sensitivity to male pheromone, we utilized
193 a strain of *C. elegans* hermaphrodite in which the neurons have been masculinized (EG4389:
194 *him-5(e1490) V; lin-15(n765ts)X; oxEx860[P(rab-3)::fem-3(wt)::mCherry(worm)::unc-54, pkd-*
195 *2::gfp(S65C), lin-15(+)]*, a gift from the Jorgensen Lab (White et al. 2007)). Solitary
196 neuronally-masculinized hermaphrodites died earlier when exposed to a low level of male
197 pheromone (8 males per plate for conditioning; Fig. 2A), whereas normal hermaphrodites
198 were insensitive to male pheromone at this concentration (Fig. 1D), suggesting that sex-
199 specific neuronal properties are responsible for male and hermaphrodite's different
200 sensitivities to male pheromone's toxicity.

201

202 Neuronal masculinization also changed the composition of pheromone secreted by the
203 hermaphrodites: *C. elegans* males are normally attracted to pheromones secreted by old (self
204 sperm-depleted) hermaphrodites (Morsci et al. 2011; Leighton et al. 2014; Kleemann and

205 Basolo 2007), but males were not attracted to pheromones secreted by aged neuronally-
206 masculinized hermaphrodites (Fig. 2B). More surprisingly, when these masculinized
207 hermaphrodites were grouped, they lived shorter than the solitary controls (Fig. 2C),
208 suggesting that neuronal masculinization of the hermaphrodites is sufficient to induce the
209 production of male-like pheromone in these hermaphrodites, and that neurons are key for
210 male pheromone-mediated death. Inhibiting germline proliferation in these grouped
211 masculinized hermaphrodites by FUdR rescued lifespan (Fig. 2D), which again supports the
212 conclusion that the germline, whether male or female, is required for male pheromone-
213 mediated killing.

214

215

216 ***C. elegans* males shrink and die after mating**

217 We previously found that mating greatly shortens hermaphrodite lifespan (Shi and Murphy
218 2014), but the effect of mating on male lifespan is not yet known. To distinguish mating
219 effects from the toxic effect of male pheromone, we measured the lifespans of solitary males
220 and single males paired with a single hermaphrodite for 6 days from Day 1 to Day 6 of
221 adulthood. (We used *fog-2(q71)* mutants, as *fog-2* males are equivalent to wild-type (N2)
222 males, while *fog-2* hermaphrodites are self-spermless (Schedl and Kimble 1988), enabling
223 identification of successful mating.) Mating decreased male lifespan ~35% compared with
224 unmated solitary males (Fig. 3A, Supplementary file 1), similar to the decrease in
225 hermaphrodite lifespan caused by mating (Shi and Murphy 2014). Males die faster when
226 paired with a hermaphrodite for a longer period: mating with a hermaphrodite for one day did
227 not significantly affect the lifespan of the male, while 2-3 days' mating shortened male
228 lifespan by 15%, 4-5 days' mating reduced their lifespan by 25%, and mating for 6 days
229 reduced lifespan more than 35% (Fig. 3B). By contrast, the number of hermaphrodites paired
230 with the single male during mating had little effect (Fig. 3C). The time at which mating occurs
231 within the reproductive period is also not critical for males' post-mating lifespan decrease;
232 given the same mating duration, males mated with hermaphrodites for the first three days of
233 adulthood had a similar lifespan decrease as those mated with hermaphrodites during days 6-
234 8 of adulthood (Fig. 3D).

235

236 As we previously observed in hermaphrodites (Shi and Murphy 2014), males shrank after 6
237 days of mating; by Day 7, the mated males were 10% smaller than the unmated solitary
238 males control (Fig. 3E, F, Supplementary file 2). No such shrinking was apparent when males
239 were treated with male pheromone (Fig. 3G), suggesting that mating and male pheromone
240 act through different pathways.

241

242 **Male post-mating shrinking and death depend on the germline**

243 We wondered whether pheromone is required for mating-induced death in males; however,
244 wild-type males still died early post-mating when paired with a *daf-22* hermaphrodite for 6
245 days (Fig. 4A). Likewise, *daf-22* mutant males lived shorter after 6 days' mating with *fog-2*
246 hermaphrodites (Fig. 4B), indicating that the post-mating lifespan decrease in our single-
247 worm pair lifespan assay is due to mating itself rather than to the presence of pheromone
248 from either sex.

249

250 Elevated germline proliferation is one of the major causes of hermaphrodites' early death
251 after mating (Shi and Murphy 2014). This killing mechanism appears to be conserved in
252 males: when treated with 50 μ M FUdR during the three-day mating period, males no longer
253 died earlier (Fig. 4C). FUdR treatment also eliminated male post-mating lifespan decrease in
254 our 6 day mating regime (Fig. 4 – figure supplement 1A). The absence of the germline also
255 prevented mating-induced shrinking: germline-less *glp-1(e2141)* males experienced neither
256 shrinking nor lifespan decrease after mating (Fig. 4D,E, Fig. 4 – figure supplement 1B). These
257 results suggest that germline-mediated post-mating death and shrinking is conserved
258 between *C. elegans* sexes.

259

260 **Ectopic expression of vitellogenin contributes to male post-mating death**

261 To identify molecular mechanisms that contribute to post-mating death in males, we
262 performed genome-wide transcriptional analyses of mated and unmated males: we paired a
263 single male with a hermaphrodite for 3.5 days of mating (150 pairs per biological replicate),
264 then picked the males individually from the hermaphrodites on Day 4 for microarray analysis

265 (Fig. 4 – figure supplement 2A). As a control, we collected the same number of age-matched
266 solitary males. Only 14 genes were significantly up-regulated and 41 were significantly down-
267 regulated (FDR=0%; Fig. 4F; Supplementary file 3.). Genes whose expression decreased in
268 mated males include extracellular proteins (*scl-11*, *scl-12*, *zig-4*) and predicted lipase-related
269 hydrolases (*lips-11*, *lips-12*, *lips-13*) that may participate in fat metabolism. As we previously
270 found in hermaphrodites (Shi and Murphy 2014), mating decreases fat storage in males (Fig.
271 5B).

272
273 Surprisingly, vitellogenins (*vit-4*, *vit-3*, *vit-5*, *vit-6*, *vit-2*), which encode yolk protein precursors
274 made in the female/hermaphrodite intestine for transport into developing oocytes (Kimble and
275 Sharrock 1983) and as such are not normally expressed in males (Figure 4G, left), were the
276 top up-regulated genes in mated males, expressed on average 19 times higher in mated
277 males than in solitary unmated males (Supplementary file 3). Overproduction of vitellogenins
278 has been shown to be deleterious for hermaphrodites: vitellogenins accumulate in the head
279 and body of older hermaphrodites (Garigan et al. 2002); long-lived insulin signaling mutants
280 repress *vit* gene expression (Murphy et al. 2003; Seah et al. 2016); overexpression of
281 vitellogenin reduces the lifespan of long-lived mutants (Seah et al. 2016); and knockdown of
282 the *vit* genes in wild-type hermaphrodites extends lifespan (Murphy et al. 2003; Seah et al.
283 2016). Mating induced ectopic expression of VIT-2::GFP in the anterior intestine of males,
284 confirming our gene expression data. Such expression induction was germline function-
285 dependent, as FUdR treatment of males blocked VIT-2::GFP expression in mated males (Fig.
286 4G, Fig. 4 – figure supplement 2D).

287
288 In addition to the increase in *vit* gene expression, the binding motif for UNC-62, a master
289 transcriptional regulator of *vit* genes in hermaphrodites (Van Nostrand et al. 2013), emerged
290 from unbiased motif analysis of the up-regulated gene set (Fig. 4 – figure supplement 2C).
291 Using RNAi, we found that knocking down *unc-62* was sufficient to rescue the lifespan
292 decrease in mated males (Fig. 4H). Thus, the mis-expression of vitellogenins upon mating
293 contributes to post-mating death in males. The DAE (DAF-16 Associated Element) motif is
294 present in most *vit* gene promoters, which are also Class 2 DAF-16 genes (Murphy et al.

295 2003). The DAE is bound by PQM-1, a transcription factor that is involved in the regulation of
296 multiple processes, including development, stress response, metabolism, and longevity
297 (Tepper et al. 2013; Downen et al. 2016). We found that mated *pqm-1(ok485)* deletion males
298 lived as long as unmated controls (Fig. 4I), suggesting that PQM-1 is also required for post-
299 mating male death.

300

301

302 **Mating-induced death and pheromone-induced killing use distinct molecular** 303 **mechanisms**

304 To determine whether pheromone-dependent killing, which like mating-induced death
305 requires the germline, utilizes the same downstream mechanisms to kill males, we performed
306 genome-wide transcriptional analysis of worms under conditions where they are exposed to
307 high levels of male pheromone and have short lifespans (Fig. 1G, 2C): 1) grouped *daf-22*
308 males on plates conditioned by wild-type males vs. grouped *daf-22* males on control plates,
309 and 2) grouped vs. solitary neuronally-masculinized hermaphrodites (Supplementary file 4).
310 Three main Gene Ontology groups emerged from these comparisons: innate immunity and
311 defense responses, metal ion/cadmium response, and glycoprotein metabolism. Notably, this
312 pattern of gene expression was very different from that of mated males (Fig. 5A, Fig. 5 –
313 figure supplement 1; Pearson correlation = -0.0594 for the whole transcriptome). Specifically,
314 upregulation of vitellogenin genes was not a signature of male pheromone-treated animals
315 (Fig. 5 – figure supplement 1C), also supporting the notion that the two pathways act through
316 distinct molecular mechanisms. While fat is reduced in males after mating (Fig. 5B), MCP
317 treatment caused no significant changes in fat metabolism gene expression (Fig. 5 – figure
318 supplement 1A) or Oil Red O staining (Fig. 5C). We showed previously that osmotic stress
319 resistance correlates well with shrinking in mated hermaphrodites, whereas fat loss does not
320 account for such shrinking (Shi and Murphy 2014). Likewise, we found that mated wild-type
321 worms lost about 30% of their glycogen stores post-mating in a germline-dependent manner
322 (Fig. 5D). The mating-induced glycogen storage decrease and subsequent shrinking is
323 conserved between sexes (Fig. 5 – figure supplement 2), while glycogen stores are not
324 affected by male pheromone (Fig. 5E).

325
326 Previously identified gene expression patterns of longevity pathways did not appear in our
327 analysis (Tepper et al. 2013; Lakhina et al. 2015), suggesting a novel pathway for lifespan
328 shortening in the presence of male pheromone. However, comparison of the list of genes
329 significantly up-regulated upon MCP treatment of *daf-22* males (Supplementary file 5) to
330 previously published arrays of male pheromone-treated hermaphrodites (Maures, et al. 2014)
331 yielded a significant overlap (p-value=3.05E-06), including *ins-11*; *ins-11* mutants are partially
332 resistant to death by male pheromone (Maures et al. 2014). These results suggest that some
333 mechanisms of male-pheromone-induced death are shared between the sexes, but may act
334 independently of known longevity pathways.

335
336 Unbiased motif analysis revealed that DAE (DAF-16 Associated Element) motif was also
337 enriched in these male pheromone-treated conditions (Fig. 5 – figure supplement 3). We
338 found that *pqm-1(ok485)* null mutant males were not short-lived when treated with male
339 pheromone (8 males per plate for conditioning; Fig. 5F), suggesting that male pheromone
340 killing, like mating-induced death, is mediated at least in part by PQM-1.

341
342 Both male pheromone-induced killing and mating-induced death require the germline; to
343 examine possible mechanisms of germline effects, we performed DAPI staining of males in
344 pheromone-treated and mated conditions. In a fraction of Day 4 unmated solitary males, the
345 transition zone (marked by crescent shaped nuclei) disappeared (Fig. 5G, Fig. 5 – figure
346 supplement 4), and the meiotic region expanded to the distal arm, evidenced by the presence
347 of sperm before U-shaped turn (see Fig. 5 – figure supplement 4), indicating the loss of
348 mitotic proliferating cells in the germline. Shrinking of the transition zone was observed
349 significantly less in mated animals, but no significant differences were apparent in MCP-
350 treated males (Fig. 5G). Therefore, our results suggest that mating causes an increase in the
351 number of mitotically proliferating cells, whereas an unknown signal from the germline, rather
352 than an obvious morphological change, may be responsible for the lifespan shortening effects
353 of male pheromone.

354

355 **Mating-induced male death is evolutionarily conserved and unavoidable**

356 Previously, we found that *C. remanei* females, like *C. elegans* hermaphrodites, shrink and die
357 faster after mating (Shi and Murphy 2014), suggesting that these mechanisms are
358 evolutionarily conserved in females. We found that *C. remanei* males also lived significantly
359 shorter after mating with a female *C. remanei* for 6 days (Fig. 6A). While female death
360 requires successful cross-progeny production (Shi and Murphy 2014), *C. elegans* males died
361 early when mated with a *C. remanei* female for 6 days (Fig. 6B), even though no cross-
362 progeny result from this mating. This result, together with the germline dependence of mating-
363 induced death, suggests the process of mating and up-regulation of male germline activity is
364 sufficient to induce death, regardless of the species of the recipient female.

365

366 **Gonochoristic species are immune to male pheromone toxicity**

367 Because *C. elegans* is an androdioecious (males and hermaphrodites) species, we wondered
368 whether male pheromone-mediated killing also occurs in a true 50/50 male/female
369 (gonochoristic) species, where one would expect the level of male pheromone to be high
370 under normal conditions. When exposed to low levels of *C. remanei* male pheromone (8
371 males per plate for conditioning), neither *C. remanei* males nor females were short-lived (Fig.
372 6C, D). At a higher concentration (30 males per plate for conditioning), multiple trials of *C.*
373 *remanei* females and males on male-conditioned plates failed to reveal any sensitivity to
374 either *C. remanei* or *C. elegans* male pheromone (Fig. 6E, F), in contrast to previous reports
375 in which males and females were grouped (Maures et al. 2014); this result suggests that the
376 lifespan shortening in the latter study was caused by mating rather than by male pheromone.

377

378 Interestingly, we found that male pheromone toxicity can act across species: *C. elegans*
379 hermaphrodites died early when exposed to *C. remanei* male pheromone (Fig. 6G),
380 suggesting that the difference in sensitivity to male pheromone might stem from the
381 *perception* of pheromone as a toxin, rather than from toxicity of the pheromone itself. *C.*
382 *remanei* can sense pheromone, but uses it to distinguish potential partners and competitors
383 (Fig. 6H), rather than to kill males. The differential sensitivity to male-pheromone-induced
384 killing between *C. elegans* males and hermaphrodites also suggests that the latter might only

385 be experienced as an off-target effect under extremely high male pheromone conditions (Fig.
386 6I).

387

388 To determine whether the differences in male-pheromone-induced killing are a more general
389 phenomenon, we examined the effect of male pheromone on other androdioecious and
390 gonochoristic species. Like *C. remanei* males, two other gonochoristic species, *C. brenneri*
391 and *C. nigoni*, are also immune to male pheromone killing (Fig. 7A, B). By contrast, males
392 from two evolutionarily distant androdioecious species, *C. briggsae* and *C. tropicalis*, died
393 significantly earlier when exposed to male pheromone (Fig. 7C, D), just as *C. elegans* males
394 do. These results strongly indicate that male pheromone-dependent killing is shared by males
395 of androdioecious species.

396

397 **Male pheromone reduces male offspring**

398 The fact that male pheromone selectively kills males of multiple, independently-evolved
399 androdioecious species suggests a role for male pheromone killing in these populations, but
400 the lifespan effects we observed to this point are largely post-reproductive, arguing against
401 any reproductive selection under these conditions. However, those experiments were
402 designed specifically to probe adult phenotypes rather than development or mating, by only
403 applying pheromone to adult worms. In order to better mimic conditions in which worms would
404 be exposed to male pheromone their entire lives, we placed *fog-2* eggs on male pheromone
405 conditioned plates, and measured developmental rates, lifespan, mating rates, and brood size.
406 Other than a slight difference at 42 hrs that disappeared by 48 hrs, we observed no significant
407 effects of MCP on the developmental rates of either males or hermaphrodites (Fig. 8 – figure
408 supplement 1). By contrast, male pheromone conditioning from egg onward caused a severe
409 (36%) shortening of lifespan (Fig. 8A, Fig. 8 – figure supplement 2A). Moreover, male
410 pheromone significantly decreased male fertility (Fig. 8B, Fig. 8 – figure supplement 2B).
411 (Note that male pheromone-induced male fertility decrease is distinct from defects in male
412 mating that arise with age (Chatterjee et al. 2013); we observed similar male fertility decline
413 with age in control animals, but the males treated with male pheromone from egg onward
414 exhibited an additional fertility decline compared with age-matched control males.) Finally,

male pheromone treatment decreases the number of progeny produced by those animals who do successfully mate (Fig. 8C). By contrast, male pheromone treatment did not affect the brood size of self-fertilized hermaphrodites (Fig. 8D). These results suggest that exposure to male pheromone during early life specifically reduces male fertility.

Discussion

***Caenorhabditis* males die early from two independent phenomena: male pheromone-mediated killing and mating-induced death**

Here we found that male pheromone killing is the major cause of population density-dependent lifespan decrease in *C. elegans* males, and is only utilized by androdioecious *Caenorhabditis* males. By contrast, all sexes of *Caenorhabditis* species succumb to mating-induced death, while both sexes of gonochoristic *Caenorhabditis* species are immune to male pheromone toxicity. The toxicity of male pheromone may explain the contradictory results from previous publications in which grouped males were used as the control in testing whether mating affects the lifespan of *Caenorhabditis* males (Gems and Riddle 1996; Van Voorhies 1992). Masculinization of neurons in hermaphrodites not only increases their sensitivity to male pheromone, but also is sufficient to induce the production of male-like toxic pheromone, suggesting that neurons play two major and distinct roles in this type of killing. The germline and the DAE-dependent transcription factor PQM-1 are required for both mating-induced and male pheromone-mediated death, but the downstream expression changes upon mating and pheromone treatment are distinct; only mating induces *vitellogenin* gene expression in males and causes shrinking. Thus, we have discovered two distinct mechanisms that accelerate aging in *Caenorhabditis* males.

Germline activation induces deleterious changes that kill all sexes in *Caenorhabditis* species

C. elegans males and hermaphrodites share many post-mating changes. As we found previously for mated females and hermaphrodites (Shi and Murphy 2014), *Caenorhabditis* males also experience germline-dependent shrinking, glycogen loss, and death after mating. Germline up-regulation also leads to ectopic expression of vitellogenins, which contributes to

the post-mating lifespan decrease in males. Previously, these yolk protein precursors were only reported to be expressed in hermaphrodites, where vitellogenin proteins are taken up by oocytes; vitellogenin production in males does not have an obvious purpose. Mating also induces significant overexpression of *vit* genes in hermaphrodites (DePina et al. 2011), indicating that vitellogenin expression is closely coupled with mating-induced germline up-regulation in both sexes. Such coupling may be strong enough to overcome the normal repression of male vitellogenin expression. Germline-dependent body shrinking, glycogen loss, and ectopic vitellogenin expression contribute to male post-mating death, which is conserved between the sexes. The striking similarity of germline-dependent post-mating changes in *Caenorhabditis* males and females suggests that this mechanism is largely conserved between sexes, and may represent an unavoidable cost of reproduction as a result of mating.

Germline-dependent lifespan shortening appears to be conserved across species over large evolutionary distances, as it occurs in all *Caenorhabditis* species we tested. Male post-mating death is also conserved beyond the *Caenorhabditis* genus, as *Drosophila* males die earlier after mating, as well (Partridge and Farquhar 1981). It was previously noted that the lifespan of Korean eunuchs was significantly longer than the lifespan of non-castrated men with similar socio-economic status (Min et al. 2012), analogous to the long lifespan of germline-less *C. elegans* (Hsin and Kenyon 1999) and *Drosophila* (Flatt et al. 2008), while the significantly (35%) shortened lifespan of Chinese emperors who were noted to be particularly promiscuous might be an example of the opposite effect on the germline (Shi et al. 2015), suggesting that some aspects of germline-dependent male post-mating death may be conserved across great evolutionary distances.

Male pheromone-induced killing may be a strategy to selectively reduce the male population

C. elegans are subject to killing by male pheromone, while *C. remanei* are not. Our cross-species results suggest that *C. remanei* male pheromone is perceived as a toxin by *C. elegans*, but *C. remanei* are immune to both *elegans* and *remanei* pheromone (Fig. 6C-F).

475 The preponderance of males in a 50:50 population, as in the case of *C. remanei*, makes the
476 use of pheromone as a toxin less likely, as it would cause too much off-target death to be
477 useful for sperm competition purposes. The toxic effect of pheromone may not be due to the
478 pheromone itself, but rather to a perception of pheromone as a toxin, with a greater effect in
479 males than in hermaphrodites. Hermaphrodite death at high male pheromone concentration
480 (Maures et al. 2014)—which might happen rarely in nature – might simply be collateral
481 damage, as hermaphrodites are far less sensitive than males to male pheromone (Figure 6I)
482 toxin. The lack of significant changes observed in the developmental rates of either males or
483 hermaphrodites, or on the brood size of hermaphrodites, indicates that the primary effect of
484 male pheromone might be on male reproductive capacity.

485

486 *Caenorhabditis* species might utilize pheromones in such different ways due to their different
487 modes of reproduction. Androdioecious species males do not appear to use pheromones
488 efficiently as chemical messengers to facilitate mating, since they are less able to distinguish
489 hermaphrodites' pheromone from other species' female or male pheromone; in fact, *C.*
490 *elegans* males are slightly attracted to their own male pheromone, in part explaining their
491 clumping (Chasnov et al. 2007) (Fig. 6H), despite the fact that male pheromone is very toxic
492 to *C. elegans* males. In the androdioecious species such as *C. elegans*, males are normally
493 rare (0.2%), so the chance that any worm he encounters will be an appropriate mating partner
494 is very high; thus, there may be less selection pressure to evolve pheromones as chemical
495 messengers to identify mates. By contrast, *C. remanei* uses pheromone to distinguish males
496 from females, an important requirement for mating in 50:50 mixed populations. *C. remanei*
497 males are slightly repelled by male pheromone (Chasnov et al. 2007) (Fig. 6H), but are
498 extremely attracted to *C. remanei* female pheromone, while *C. remanei*, as well as both
499 males and females from other gonochoristic species, are immune to male pheromone toxicity
500 (Fig. 6C, D). Thus, gonochoristic species use pheromones primarily as chemical cues to
501 identify mates, rather than to kill males.

502

503 The fact that male pheromone toxicity is present in three distantly-related and separately
504 evolved hermaphroditic *Caenorhabditis* species (Cho et al. 2004; Kiontke et al. 2004; Kiontke

et al. 2011) suggests an important role for male pheromone killing. Periodic explosions of male populations in androdioecious species (e.g., under stressful conditions) allow outcrossing and ensure genetic diversity (Anderson et al. 2010). After this beneficial period, however, males are more costly to maintain, and there may be pressure to return to a primarily hermaphroditic population (Fig. 8E). It is notable that because *C. elegans* males are XO, rather than XY, males may have no selfish drive to maintain their own chromosomes. Using male pheromone as a dose-dependent toxin may be an effective way to cull the male population and ensure that the species returns to the self-reproduction mode when the stressful condition has passed, aiding the return to hermaphroditism. Because a high fraction of males can only be produced by mating (mating produces 50% males, while male production rates from hermaphroditic selfing is 0.2% (Chasnov and Chow 2002; Hodgkin 1983)), the combination of decreased mating efficacy and decreased progeny production might be expected to specifically affect the number of males produced each generation (Fig. 8 - figure supplement 3). Male pheromone alone could effectively drive the population back to a primarily hermaphroditic state after several generations (Figure 8E). Previous experiments showed that the average time for males to disappear in N2 strain is 12-20 days (i.e. 4-7 generations) (Wegewitz et al. 2008). The discrepancy between our modeling (~15 generations) and the previous experimental result may indicate that multiple factors, including increased hermaphroditic progeny production and decreased mating rates (Wegewitz et al. 2008), decreased copulation performance in aging males (Chatterjee et al. 2013), and hermaphrodites' response to males (Garcia et al. 2007; Kleemann and Basolo 2007; Morsci et al. 2011)) could act in tandem with pheromone-dependent killing of males to cull the male population and thus promote a return to hermaphroditism. Male-specific culling occurs in species such as *Drosophila bifasciata*, in which *Wolbachia* infection leads to the killing of male embryos, suggesting that sex ratio can be controlled through male-killing (Stevens et al. 2001). Mathematical modeling shows that selection in *C. elegans* favors low populations of males (Stewart and Phillips 2002), and our model provides a mechanism for how this might be achieved.

535 In summary, germline-dependent early death after mating is conserved between sexes and
536 perhaps even across great evolutionary distances, and is likely due to an unavoidable cost
537 of mating, the result of mated animals ramping up germline proliferation and subsequently
538 exhausting their own resources as quickly as possible to produce the next generation of
539 progeny. The differential use of pheromones as toxins or chemical messengers by males in
540 androdioecious and gonochoristic species, respectively, demonstrates that they adopt
541 different strategies to compete, mate, and maintain optimal sex ratios.
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545 REFERENCES
546
547 WHO 2015 data:
548 http://www.who.int/gho/publications/world_health_statistics/2016/Annex_B/en/
549
550 Anderson JL, Morran LT, Phillips PC (2010) Outcrossing and the maintenance of males within
551 *C. elegans* populations. *The Journal of heredity* 101 Suppl 1:S62-74.
552 doi:10.1093/jhered/esq003

553 Aprison EZ, Ruvinsky I (2015) Sex Pheromones of *C. elegans* Males Prime the Female
554 Reproductive System and Ameliorate the Effects of Heat Stress. *PLoS genetics* 11
555 (12):e1005729. doi:10.1371/journal.pgen.1005729

556 Aprison EZ, Ruvinsky I (2016) Sexually Antagonistic Male Signals Manipulate Germline and
557 Soma of *C. elegans* Hermaphrodites. *Current biology : CB*. doi:10.1016/j.cub.2016.08.024

558 Austad SN, Fischer KE (2016) Sex Differences in Lifespan. *Cell metabolism* 23 (6):1022-1033.
559 doi:10.1016/j.cmet.2016.05.019

560 Bargmann CI (2006) Chemosensation in *C. elegans*. *WormBook : the online review of C*
561 *elegans* biology:1-29. doi:10.1895/wormbook.1.123.1

562 Chasnov JR, Chow KL (2002) Why are there males in the hermaphroditic species
563 *Caenorhabditis elegans*? *Genetics* 160 (3):983-994

564 Chasnov JR, So WK, Chan CM, Chow KL (2007) The species, sex, and stage specificity of a
565 *Caenorhabditis* sex pheromone. *Proceedings of the National Academy of Sciences of the*
566 *United States of America* 104 (16):6730-6735. doi:10.1073/pnas.0608050104

567 Chatterjee I, Ibanez-Ventoso C, Vijay P, Singaravelu G, Baldi C, Bair J, Ng S, Smolyanskaya
568 A, Driscoll M, Singson A (2013) Dramatic fertility decline in aging *C. elegans* males is
569 associated with mating execution deficits rather than diminished sperm quality. *Experimental*
570 *gerontology* 48 (11):1156-1166. doi:10.1016/j.exger.2013.07.014

571 DePina AS, Iser WB, Park SS, Maudsley S, Wilson MA, Wolkow CA (2011) Regulation of
572 *Caenorhabditis elegans* vitellogenesis by DAF-2/IIS through separable transcriptional and
573 posttranscriptional mechanisms. *BMC physiology* 11:11. doi:10.1186/1472-6793-11-11

574 Downen RH, Breen PC, Tullius T, Conery AL, Ruvkun G (2016) A microRNA program in the *C.*
575 *elegans* hypodermis couples to intestinal mTORC2/PQM-1 signaling to modulate fat transport.
576 *Genes & development* 30 (13):1515-1528. doi:10.1101/gad.283895.116

577 Flatt T, Min KJ, D'Alterio C, Villa-Cuesta E, Cumbers J, Lehmann R, Jones DL, Tatar M
578 (2008) *Drosophila* germ-line modulation of insulin signaling and lifespan. *Proceedings of the*
579 *National Academy of Sciences of the United States of America* 105 (17):6368-6373.
580 doi:10.1073/pnas.0709128105

581 Fowler K, Partridge L (1989) A Cost of Mating in Female Fruit-Flies. *Nature* 338 (6218):760-
582 761. doi:10.1038/338760a0

583 Frazier HN, 3rd, Roth MB (2009) Adaptive sugar provisioning controls survival of *C. elegans*
584 embryos in adverse environments. *Current biology : CB* 19 (10):859-863.
585 doi:10.1016/j.cub.2009.03.066

586 Garcia LR, LeBoeuf B, Koo P (2007) Diversity in mating behavior of hermaphroditic and male-
587 female *Caenorhabditis* nematodes. *Genetics* 175 (4):1761-1771.
588 doi:10.1534/genetics.106.068304

589 Garigan D, Hsu AL, Fraser AG, Kamath RS, Ahringer J, Kenyon C (2002) Genetic analysis of
590 tissue aging in *Caenorhabditis elegans*: a role for heat-shock factor and bacterial proliferation.
591 *Genetics* 161 (3):1101-1112

592 Gems D, Riddle DL (1996) Longevity in *Caenorhabditis elegans* reduced by mating but not
593 gamete production. *Nature* 379 (6567):723-725. doi:10.1038/379723a0

594 Gems D, Riddle DL (2000) Genetic, behavioral and environmental determinants of male
595 longevity in *Caenorhabditis elegans*. *Genetics* 154 (4):1597-1610

596 Golden JW, Riddle DL (1985) A gene affecting production of the *Caenorhabditis elegans*
597 dauer-inducing pheromone. *Molecular & general genetics* : MGG 198 (3):534-536

598 Hodgkin J (1983) Male Phenotypes and Mating Efficiency in *CAENORHABDITIS ELEGANS*.
599 *Genetics* 103 (1):43-64

600 Hsin H, Kenyon C (1999) Signals from the reproductive system regulate the lifespan of *C.*
601 *elegans*. *Nature* 399 (6734):362-366. doi:10.1038/20694

602 Kimble J, Sharrock WJ (1983) Tissue-specific synthesis of yolk proteins in *Caenorhabditis*
603 *elegans*. *Developmental biology* 96 (1):189-196

604 Kleemann GA, Basolo AL (2007) Facultative decrease in mating resistance in hermaphroditic
605 *Caenorhabditis elegans* with self-sperm depletion. *Anim Behav* 74:1339-1347.
606 doi:10.1016/j.anbehav.2007.02.031

607 Lakhina V, Arey RN, Kaletsky R, Kauffman A, Stein G, Keyes W, Xu D, Murphy CT (2015)
608 Genome-wide functional analysis of CREB/long-term memory-dependent transcription
609 reveals distinct basal and memory gene expression programs. *Neuron* 85 (2):330-345.
610 doi:10.1016/j.neuron.2014.12.029

611 Leighton DH, Choe A, Wu SY, Sternberg PW (2014) Communication between oocytes and
612 somatic cells regulates volatile pheromone production in *Caenorhabditis elegans*.
613 *Proceedings of the National Academy of Sciences of the United States of America* 111
614 (50):17905-17910. doi:10.1073/pnas.1420439111

615 Ludwig AH, Schroeder FC (2013) Ascaroside signaling in *C. elegans*. *WormBook* : the
616 online review of *C. elegans* biology:1-22. doi:10.1895/wormbook.1.155.1

617 Luo S, Kleemann GA, Ashraf JM, Shaw WM, Murphy CT (2010) TGF-beta and insulin
618 signaling regulate reproductive aging via oocyte and germline quality maintenance. *Cell* 143
619 (2):299-312. doi:10.1016/j.cell.2010.09.013

620 Luo S, Shaw WM, Ashraf J, Murphy CT (2009) TGF-beta Sma/Mab signaling mutations
621 uncouple reproductive aging from somatic aging. *PLoS genetics* 5 (12):e1000789.
622 doi:10.1371/journal.pgen.1000789

623 Maures TJ, Booth LN, Benayoun BA, Izrayelit Y, Schroeder FC, Brunet A (2014) Males
624 shorten the life span of *C. elegans* hermaphrodites via secreted compounds. *Science* 343
625 (6170):541-544. doi:10.1126/science.1244160

626 Min KJ, Lee CK, Park HN (2012) The lifespan of Korean eunuchs. *Current biology* : CB 22
627 (18):R792-793. doi:10.1016/j.cub.2012.06.036

628 Morsci NS, Haas LA, Barr MM (2011) Sperm status regulates sexual attraction in
629 *Caenorhabditis elegans*. *Genetics* 189 (4):1341-1346. doi:10.1534/genetics.111.133603

630 Murphy CT, McCarroll SA, Bargmann CI, Fraser A, Kamath RS, Ahringer J, Li H, Kenyon C
631 (2003) Genes that act downstream of DAF-16 to influence the lifespan of *Caenorhabditis*
632 *elegans*. *Nature* 424 (6946):277-283. doi:10.1038/nature01789

633 O'Rourke EJ, Soukas AA, Carr CE, Ruvkun G (2009) *C. elegans* major fats are stored in
634 vesicles distinct from lysosome-related organelles. *Cell metabolism* 10 (5):430-435.
635 doi:10.1016/j.cmet.2009.10.002

636 Partridge L, Farquhar M (1981) Sexual-Activity Reduces Lifespan of Male Fruitflies. *Nature*
637 294 (5841):580-582. doi:10.1038/294580a0

638 Reinke V, Smith HE, Nance J, Wang J, Van Doren C, Begley R, Jones SJ, Davis EB, Scherer
639 S, Ward S, Kim SK (2000) A global profile of germline gene expression in *C. elegans*.
640 *Molecular cell* 6 (3):605-616

641 Schedl T, Kimble J (1988) *fog-2*, a germ-line-specific sex determination gene required for
642 hermaphrodite spermatogenesis in *Caenorhabditis elegans*. *Genetics* 119 (1):43-61

643 Seah NE, de Magalhaes Filho CD, Petrashen AP, Henderson HR, Laguer J, Gonzalez J,
644 Dillin A, Hansen M, Lapierre LR (2016) Autophagy-mediated longevity is modulated by
645 lipoprotein biogenesis. *Autophagy* 12 (2):261-272. doi:10.1080/15548627.2015.1127464

646 Shaw WM, Luo S, Landis J, Ashraf J, Murphy CT (2007) The *C. elegans* TGF-beta Dauer
647 pathway regulates longevity via insulin signaling. *Current biology : CB* 17 (19):1635-1645.
648 doi:10.1016/j.cub.2007.08.058

649 Shi C, Murphy CT (2014) Mating induces shrinking and death in *Caenorhabditis* mothers.
650 *Science* 343 (6170):536-540. doi:10.1126/science.1242958

651 Shi C, Runnels AM, Murphy CT (2015) Mating-induced Male Death and Pheromone Toxin-
652 regulated Androstasis. *bioRxiv*. doi:10.1101/034181

653 Stevens L, Giordano R, Fialho RF (2001) Male-killing, nematode infections, bacteriophage
654 infection, and virulence of cytoplasmic bacteria in the genus *Wolbachia*. *Annu Rev Ecol Syst*
655 32:519-545. doi:10.1146/Annurev.Ecolsys.32.081501.114132

656 Stewart AD, Phillips PC (2002) Selection and maintenance of androdioecy in *Caenorhabditis*
657 *elegans*. *Genetics* 160 (3):975-982

658 Tepper RG, Ashraf J, Kaletsky R, Kleemann G, Murphy CT, Bussemaker HJ (2013) PQM-1
659 complements DAF-16 as a key transcriptional regulator of DAF-2-mediated development and
660 longevity. *Cell* 154 (3):676-690. doi:10.1016/j.cell.2013.07.006

661 Tusher VG, Tibshirani R, Chu G (2001) Significance analysis of microarrays applied to the
662 ionizing radiation response. *Proceedings of the National Academy of Sciences of the United*
663 *States of America* 98 (9):5116-5121. doi:10.1073/pnas.091062498

664 van Helden J (2003) Regulatory sequence analysis tools. *Nucleic Acids Res* 31 (13):3593-
665 3596

666 Van Nostrand EL, Sanchez-Blanco A, Wu B, Nguyen A, Kim SK (2013) Roles of the
667 developmental regulator *unc-62*/Homothorax in limiting longevity in *Caenorhabditis elegans*.
668 *PLoS genetics* 9 (2):e1003325. doi:10.1371/journal.pgen.1003325

669 Van Voorhies WA (1992) Production of sperm reduces nematode lifespan. *Nature* 360
670 (6403):456-458. doi:10.1038/360456a0

671 Wahlby C, Conery AL, Bray MA, Kametsky L, Larkins-Ford J, Sokolnicki KL, Veneskey M,
672 Michaels K, Carpenter AE, O'Rourke EJ (2014) High- and low-throughput scoring of fat mass
673 and body fat distribution in *C. elegans*. *Methods (San Diego, Calif)* 68 (3):492-499.
674 doi:10.1016/j.jymeth.2014.04.017

675 Wegewitz V, Schulenburg H, Streit A (2008) Experimental insight into the proximate causes
676 of male persistence variation among two strains of the androdioecious *Caenorhabditis*
677 *elegans* (Nematoda). BMC ecology 8:12. doi:10.1186/1472-6785-8-12

678 White JQ, Nicholas TJ, Gritton J, Truong L, Davidson ER, Jorgensen EM (2007) The sensory
679 circuitry for sexual attraction in *C. elegans* males. Current biology : CB 17 (21):1847-1857.
680 doi:10.1016/j.cub.2007.09.011
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696

697 **Figure Legends:**

698

699 **Abbreviations and nomenclature in the paper:**

700 **C. e.:** *C. elegans*

701 **C. r.:** *C. remanei*

702 **1m1h_6d:** “h” = hermaphrodite, “f” = female, “m” = male. The number before h or m = the
703 number of worms on the same 35mm plate. “6d” = mating for 6 days.

704 **MCP(xm):** male-conditioned plates, conditioned by “x” number of wild-type males.

705 * p<0.05; **p<0.01; ***p<0.001; n.s. = no significance.

706

707 **Figure 1. Male pheromone leads to early death in *C. elegans* males**

708 (A) Lifespans of grouped *fog-2(q71)* males. We used *fog-2(q71)* mutants, as *fog-2* males
709 are equivalent to wild-type (N2) males (Schedl and Kimble 1988). Solitary males:
710 12.0 ± 0.4 days, n=40; two males: 10.6 ± 0.4 days, n=40, p=0.0397; four males: $9.9 \pm$
711 0.4 days, n=60, p=0.0012; eight males: 7.7 ± 0.2 days, n=80, p<0.0001. For all the
712 lifespan assays performed in this study, Kaplan-Meier analysis with log-rank (Mantel-
713 Cox) test was used to determine statistical significance. All the lifespan results are
714 included in Supplementary file 1.

715 (B) Lifespans of solitary N2 hermaphrodites: 12.3 ± 0.4 days, n=40; grouped N2
716 hermaphrodites: 12.0 ± 0.3 days, n=58, p=0.6436.

717 (C) Grouped *daf-22(m130)* males have a similar lifespan to solitary wild-type *fog-2* males.
718 (*daf-22(m130)* mutants are ascaroside pheromone-production deficient.) Solitary *fog-2*
719 males: 13.8 ± 0.7 days, n=35; eight *fog-2* males: 9.8 ± 0.5 days, n=48, p<0.0001;
720 eight *daf-22(m130)* males: 14.7 ± 0.7 days, n=48, p=0.4039. Details about male-
721 conditioned plates lifespan assays are included in Methods and Fig. 1 – supplement
722 1B.

723 (D) Lifespans of solitary *C. elegans daf-22(m130)* hermaphrodites on plates conditioned
724 by eight *fog-2* males. Solitary *daf-22* hermaphrodites on control plates: 14.2 ± 0.6
725 days, n=35; solitary *daf-22* hermaphrodites on male-conditioned plates: 14.8 ± 0.8
726 days, n=35, p=0.4356.

727 (E) Lifespans of solitary *C. elegans daf-22(m130)* males on plates conditioned by eight
728 *fog-2* males. *daf-22(m130)* mutants are ascaroside pheromone-production deficient.
729 Therefore, the effect of male pheromone is due to the male pheromone secreted by
730 wild-type males when conditioning the plates. Solitary *daf-22* males on control plates:
731 19.7 ± 0.5 days, n=34; solitary *daf-22* males on male-conditioned plates: 13.1 ± 0.4
732 days, n=33, p<0.0001.

733 (F) *daf-22(m130)* male lifespans on plates conditioned by wild-type *fog-2* males. Solitary
734 *daf-22(m130)*: 23.0 ± 0.9 days, n=30; *daf-22(m130)* on plates conditioned by one *fog-2*
735 male: 17.3 ± 0.7 days, n=29, p<0.0001; *daf-22(m130)* on plates conditioned by
736 eight *fog-2* male: 16.1 ± 0.6 days, n=30, p<0.0001.

- (G) MCP-induced shorter lifespan of grouped *daf-22(m130)* males is inhibited in the presence of 50 μ M FUDR. Grouped *daf-22* males on NGM: 12.7 ± 0.3 days, n=150; grouped *daf-22* males on MCP: 11.0 ± 0.2 days, n=150, $p < 0.0001$; grouped *daf-22* males on NGM with FUDR: 14.9 ± 0.2 days, n=150, grouped *daf-22* males on MCP with FUDR: 15.3 ± 0.2 days, n=150 $p = 0.2964$.
- (H) Lifespans of grouped *glp-1(e2141)* males. Solitary males: 12.7 ± 0.8 days, n=44; eight males: 13.3 ± 0.8 days, n=56, $p = 0.699$.

Figure 1 – supplement 1. Male pheromone-mediated toxicity requires germline

- (A) Grouped *daf-22(m130)* males live slightly shorter than the solitary control (left). Solitary *daf-22* males: 21.7 ± 1.2 days, n=32; eight *daf-22* males: 18.8 ± 1.0 days, n=38, $p = 0.0394$. Lifespans are not different between solitary and grouped *daf-22(m130)* in the presence of FUDR (right). Solitary *daf-22(m130)*: 15.3 ± 0.3 days, n=35; eight *daf-22(m130)*: 14.7 ± 0.3 days, n=48, $p = 0.2117$.
- (B) Schematic illustration of how lifespan assays on male-conditioned plates were performed. Detailed description is included in Methods.
- (C) SAM plot of *daf-22(m130)* males \pm MCP(30m) for 6 days in the presence of 50 μ M FUDR. Only 10 genes' expression significantly changed, even at high FDR (17%).
- (D) SAM plot of *glp-1(e2141)* hermaphrodites \pm MCP(60m) for 6 days in the presence of 50 μ M FUDR. Only 3 genes had significant changes in expression, even at high FDR (14%).
- (E) Lifespans of grouped N2 and *glp-1* hermaphrodites on plates conditioned by 60 wild-type *fog-2* males. Grouped N2 control: 14.7 ± 0.5 days, n=107; grouped N2 hermaphrodites on MCP: 10.6 ± 0.3 days, n=98, $p < 0.0001$; Grouped *glp-1(e2141)* control: 16.9 ± 0.5 days, n=96; grouped *glp-1(e2141)* on MCP: 15.9 ± 0.6 days, n=97, $p = 0.8933$. Lifespan assay of *glp-1* hermaphrodites was performed at 25 $^{\circ}$ C in the first week of adulthood and at room temperature afterwards.
- (F) Germline also affects the production of male pheromone. Lifespans of solitary *daf-22(m130)* males control: 15.8 ± 0.9 days, n=25; solitary *daf-22(m130)* males on plates conditioned by 8 wild-type males: 11.8 ± 0.5 days, n=25, $p = 0.0002$; solitary *daf-22(m130)* males on plates conditioned by 8 germline-less *glp-1* males: 13.4 ± 0.6 days, n=25, $p = 0.0339$ (compared to the control), $p = 0.0472$ (compared to MCP(8 wt m)).

772 **Figure 2. Neuronal masculinization of *C. elegans* hermaphrodites**

- 773 (A) Neuronal masculinization of *C. elegans* hermaphrodites increases their sensitivity to
774 male pheromone toxicity. Lifespans of solitary masculinized hermaphrodites: $12.3 \pm$
775 0.3 days, $n=96$; solitary masculinized hermaphrodites on plates conditioned by 8
776 males: 9.6 ± 0.3 days, $n=56$, $p<0.0001$.
- 777 (B) Supernatant solutions from Day 5 *C. elegans* hermaphrodites and masculinized
778 hermaphrodites and Day 1 *fog-2* males were used to do the chemotaxis assay. See
779 Methods for detailed description. Chemotaxis Index (C.I.) of wild-type hermaphrodites'
780 supernatant: 0.65 ± 0.10 , C.I. of masculinized hermaphrodites' supernatant: $0.03 \pm$
781 0.03 ; $p=0.0261$, unpaired t-test.
- 782 (C) Neuronal masculinization is sufficient to induce male-like population-density-
783 dependent lifespan decrease in hermaphrodites. Lifespans of solitary N2
784 hermaphrodites: 12.3 ± 0.4 days, $n=40$; grouped N2 hermaphrodites (about 30
785 worms per 35mm plate): 12.0 ± 0.3 days, $n=58$, $p=0.6436$. Solitary masculinized
786 hermaphrodites: 12.4 ± 0.5 days, $n=40$; grouped masculinized hermaphrodites (30
787 per plate): 10.4 ± 0.3 days, $n=60$, $p=0.0015$.
- 788 (D) FUDR rescue the lifespans of grouped masculinized hermaphrodites. Lifespans of
789 grouped masculinized hermaphrodites: 9.8 ± 0.2 days, $n=111$, grouped masculinized
790 hermaphrodites (30 per plate) in the presence of $50 \mu\text{M}$ FUDR: 12.2 ± 0.3 days,
791 $n=119$, $p<0.0001$.
792
793

794 **Figure 3. *C. elegans* males shrink and die early after mating**

- 795 (A) Lifespans of unmated solitary and mated *fog-2(q71)* males. Solitary males: 13.1 ± 0.6
 796 days, $n=50$; mated males: 8.3 ± 0.4 days, $n=34$, $p<0.0001$. Each male was paired
 797 with a *fog-2(q71)* hermaphrodite on a single 35mm plate during Day 1-6 of adulthood.
 798 Unless noted, all the hermaphrodites used are *fog-2(q71)*.
- 799 (B) Male post-mating lifespan decrease is mating duration-dependent: Unmated solitary
 800 males: 10.9 ± 0.6 days, $n=35$; one male and one hermaphrodite mating on Day 1 of
 801 adulthood: 11.4 ± 0.6 days, $n=31$, $p=0.3697$; mating from Day 1-2: 9.0 ± 0.6 days,
 802 $n=30$, $p=0.0325$; mating from Day 1-3: 9.1 ± 0.6 days, $n=34$, $p=0.0452$; mating from
 803 Day 1-4: 7.9 ± 0.5 days, $n=32$, $p=0.0002$; mating from Day 1-5: 8.3 ± 0.4 days, $n=34$,
 804 $p=0.0006$; mating from Day 1-6: 6.8 ± 0.3 days, $n=33$, $p<0.0001$.
- 805 (C) Lifespans of one male paired with different number of hermaphrodites during Day 1-3
 806 of adulthood: solitary unmated males: 13.8 ± 0.7 days, $n=35$; one male with one
 807 hermaphrodite: 10.8 ± 0.6 days, $n=32$, $p=0.0175$; one male with two hermaphrodites:
 808 11.6 ± 0.9 days, $n=33$, $p=0.1435$; one male with three hermaphrodites: 10.6 ± 0.8
 809 days, $n=34$, $p=0.0147$.
- 810 (D) Lifespans of one male paired with three hermaphrodites for 3 days but at different
 811 time of adulthood. Solitary unmated males: 13.8 ± 0.7 days, $n=35$; mating during Day
 812 1-3 of adulthood: 10.6 ± 0.8 days, $n=34$, $p=0.0147$; mating during Day 6-8 of
 813 adulthood: 10.8 ± 0.6 days, $n=37$, $p=0.0022$.
- 814 (E) Length of unmated and mated *fog-2* males: t-test, $**p<0.01$, $***p<0.001$.
- 815 (F) Representative pictures of the same unmated solitary male and male paired with one
 816 hermaphrodite from Day 1-Day 6 of adulthood.
- 817 (G) Male pheromone does not induce body shrinking. Length of solitary *fog-2* males on
 818 plates conditioned by 8 wild-type males.

819

820

821 **Figure 4. Mating-induced death is germline-dependent**

- 822 (A) Lifespans of *fog-2* males mated with *daf-22(m130)* hermaphrodites. Unmated solitary
823 *fog-2* males: 12.1 ± 0.6 days, n=32; mated males: 9.0 ± 0.4 days, n=29, $p=0.0001$. In
824 the mated group, one *fog-2(q71)* male was paired with one *daf-22(m130)*
825 hermaphrodite from Day 1- Day 6 of adulthood.
- 826 (B) Lifespans of unmated and mated *daf-22(m130)* males. Unmated solitary *daf-*
827 *22(m130)* males: 13.8 ± 0.6 days, n=40; mated *daf-22(m130)* males: 7.4 ± 0.4 days,
828 n=34, $p<0.0001$. In the mated group, one *daf-22(m130)* male was paired with one
829 *fog-2(q71)* hermaphrodite from Day 1- Day 6 of adulthood.
- 830 (C) FUdR can rescue male post-mating early death. Unmated solitary males: 10.5 ± 0.5
831 days, n=35; one male with three hermaphrodites for three days: 6.4 ± 0.3 days, n=31,
832 $p<0.0001$; one male with three hermaphrodites for three days but in the presence of
833 50 μ M FUdR during the three days' mating: 10.2 ± 0.4 days, n=36, $p=0.7086$
834 (compared with unmated solitary group).
- 835 (D) Lifespans of unmated and mated *glp-1(e2141)* males: unmated solitary *glp-1* males:
836 8.0 ± 0.4 days, n=40; mated *glp-1* males: 7.2 ± 0.4 days, n=40, $p=0.3178$. The assay
837 was performed at 26 °C, in mated group, one *glp-1* male was paired with one *fog-2*
838 hermaphrodite from Day 1-6.
- 839 (E) Length of mated and unmated *glp-1(e2141)* males. (The same population as in Fig.
840 4D.)
- 841 (F) Expression heatmap of genes whose expression is significantly changed in mated
842 males based on SAM analysis.
- 843 (G) Ectopic expression of VIT-2::GFP in mated males is germline-dependent. 5 days'
844 mating, pictures were taken on Day 6 of adulthood. Representative images are
845 shown above the quantification of VIT-2::GFP expression [maximum \pm SE (error
846 bars)], a.u., arbitrary units. ***, $p<0.001$, t-test.
- 847 (H) *unc-62* RNAi suppresses male post-mating early death. Unmated solitary male on
848 L4440: 12.6 ± 0.7 days, n=25; mated males on L4440: 8.8 ± 0.5 days, n=33,
849 $p=0.0001$. Unmated males on *unc-62* RNAi: 11.9 ± 0.8 days, n=25; mated males on
850 *unc-62* RNAi: 10.6 ± 0.5 days, n=34, $p=0.1249$ (compared to unmated males on *unc-*
851 *62* RNAi).
- 852 (I) *pqm-1(ok485)* mated males have similar lifespans as unmated controls. Unmated
853 solitary *pqm-1(ok485)* males: 11.9 ± 0.5 days, n=25; mated *pqm-1(ok485)* males:
854 11.0 ± 0.6 days, n=29, $p=0.2782$. In the mated group, one *pqm-1(ok485)* male was
855 paired with one *fog-2(q71)* hermaphrodite from Day 1- Day 6 of adulthood.

856
857
858 **Figure 4 – supplement 1. Mating-induced lifespan decrease is germline-dependent**

859 (A) Germline proliferation blocking with FUdR can rescue male post-mating early death.
 860 Unmated solitary males: 13.8 ± 0.7 days, $n=35$; one male with one hermaphrodite for
 861 six days: 10.3 ± 0.6 days, $n=31$, $p=0.0006$; solitary male in the presence of $50 \mu\text{M}$
 862 FUdR: 13.9 ± 0.4 days, $n=35$, $p=0.4079$ (compared to unmated solitary group). One
 863 male mated with one hermaphrodites for 6 days in the presence of FUdR: 13.6 ± 0.5
 864 days, $n=34$, $p=0.3992$ compared to unmated solitary group.
 865 (B) Lifespans of unmated and mated *glp-1(e2141)* males: unmated solitary *glp-1* males:
 866 11.1 ± 1.0 days, $n=27$; mated *glp-1* males: 11.1 ± 0.5 days, $n=43$, $p=0.9149$. The
 867 assay was performed at 25°C ; in mated group, one *glp-1* male was paired with one
 868 *fog-2* hermaphrodite from Day 1-6.

869
 870 **Figure 4 – supplement 2. Microarray analysis of mated males**

871 (A) Clustered heat map of whole transcriptome expression comparison of mated vs
 872 unmated males. Individual males were paired with one hermaphrodite for 3.5 days
 873 and collected on Day 4 for microarrays.
 874 (B) Enriched GO terms for significantly up- and down-regulated genes in mated males.
 875 (C) Enriched motifs in promoter region (1kb upstream of TSS) of significantly up- and
 876 down-regulated genes using (RSAT) Regulatory Sequence Analysis Tools
 877 (www.rsat.eu).
 878 (D) VIT-2::GFP expression in males increases significantly after mating. Upper: DIC and
 879 GFP images; Lower: GFP intensity quantitation, left: $\text{max} \pm \text{SE}$ (error bars); right:
 880 $\text{mean} \pm \text{SE}$ (error bars), a.u., arbitrary units. **, $p<0.01$, t-test.

882

883 **Figure 5. Mating-induced and male pheromone-induced death are distinct**

- 884 (A) Transcriptional profiles of mated males and MCP-treated males are different.
 885 Heatmap cluster of mated males (left) and MCP-treated vs untreated grouped *daf-22*
 886 males (right); Pearson correlation = -0.27. The cluster only contains genes with
 887 significant changes in mated males by SAM, 0% FDR.
- 888 (B) Mating induces significant fat loss in males. Representative pictures of Oil red O
 889 staining are shown above the quantitation. Males lost about 20% of their fat after
 890 mating on Day 4, $p < 0.001$. Error bars represent SE.
- 891 (C) Male pheromone exposure fails to induce fat loss in males. Four days' MCP
 892 treatment. Representative pictures of Oil red O staining are shown above the
 893 quantitation. Unconditioned control males are framed by black lines, and MCP-
 894 treated males are framed by green lines.
- 895 (D) Glycogen staining of mated and unmated males. Left: mated *fog-2* (wt) males lost
 896 over 30% glycogen after 5 days' mating; *** $p < 0.001$. Right: mated *glp-1* males did
 897 not lose glycogen after mating. The staining intensity was normalized to unmated
 898 males of each genotype. Representative pictures are shown above the quantitation.
 899 Unmated males are framed by dashed lines, and mated males are framed by solid
 900 lines.
- 901 (E) No glycogen loss after male pheromone exposure. Four days' MCP treatment.
 902 Representative pictures of iodine staining are shown above the quantitation.
 903 Unconditioned control males are framed by black lines, and MCP-treated males are
 904 framed by green lines. (In D-E, error bars represent SD.)
- 905 (F) Loss of the DAE-dependent transcription factor PQM-1 suppresses male pheromone-
 906 induced death. Lifespans of control solitary *pqm-1(ok485)* males: 15.6 ± 0.6 days,
 907 $n=25$; solitary *pqm-1(ok485)* males on plates conditioned by 8 males: 14.4 ± 0.6 days,
 908 $n=25$, $p=0.1627$.
- 909 (G) DAPI staining of Day 6 males' germlines (left). Right: categorical quantification of
 910 germline morphology: mating causes more obvious change in male germline
 911 morphology than male pheromone does. TZ: transition zone; U: U-shaped turn of
 912 male germline. See Fig. 5 – supplement 4 for details.

914 **Figure 5 – supplement 1. Transcriptional profiles of mated and male pheromone-**
 915 **induced males are distinct**

- 916 (A) Heatmap of (1) mated males (genes with significant changes by SAM, 0% FDR) and
 917 (2) MCP treated vs untreated grouped *daf-22* males. Top: Pearson correlation.
- 918 (B) Heatmap of (1) mated males and (2) MCP treated vs untreated grouped *daf-22* males
 919 (genes with significant changes by SAM, 1% FDR). Top: Pearson correlation.
- 920 (C) Vit genes are not up-regulated in males exposed to male pheromone.

921

922 **Figure 5 – supplement 2. Glycogen staining of mated vs unmated hermaphrodites and**
923 **males**

924 Left: representative pictures of iodine staining of worms. Unmated worms are framed
925 by dashed lines, whereas mated worms are framed by solid lines. In the first picture,
926 mated and unmated *fog-2* hermaphrodites were mixed together, with red arrows
927 pointing to mated *fog-2* hermaphrodites. Worms were mated from Day 1 – Day 5 and
928 were imaged on Day 5.

929 Right: quantitation of iodine staining. The intensity of mated worms was normalized to
930 unmated control of the same genotype. Mated *fog-2* hermaphrodites have only 30%
931 of the glycogen levels of unmated *fog-2* hermaphrodites of the same age ($p < 0.0001$).
932 Mated *glp-1* hermaphrodites have 99% glycogen compared to unmated *glp-1*
933 hermaphrodites control ($p = 0.6070$). Mated *fog-2* males have 64% of the glycogen
934 level of unmated *fog-2* males of the same age ($p < 0.0001$). Mated *glp-1* males have
935 101% glycogen compared to unmated *glp-1* males control ($p = 0.7107$). Error bars
936 represent SD. ***, $p < 0.0001$, t-test.

937

938 **Figure 5 – supplement 3. Enriched motifs of male pheromone-induced transcriptional**
939 **changes**

940 Enriched motifs associated with significantly up- and down-regulated genes predicted
941 by RSAT (Regulatory Sequence Analysis Tools) in grouped vs. single neuronal
942 masculinized hermaphrodites (left) and MCP treated vs untreated grouped *daf-22*
943 males.

944

945 **Figure 5 – supplement 4. Germline of mated and MCP-treated males**

946 (A) Representative micrographs of each category: left: male germline with clear transition
947 zone (yellow arrow indicates transition zone marked by crescent shaped nuclei);
948 middle: male germline with no apparent transition zone; right: male germline with
949 mature sperm appearing before U shaped turn.

950 (B) Mating (one male one hermaphrodites for 4 days) or treating solitary males with male
951 pheromone (4 days on MCP(8m)) does not change the number of proliferating nuclei
952 in the male germline. The number of nuclei in mitotic proliferating zone (only counting
953 germlines with clear transition zone) is similar in all three conditions.

954

955

956 **Figure 6. Mating-induced death is evolutionarily conserved, whereas male pheromone-**
 957 **induced death is not.**

- 958 (A) Mated *C. remanei* males also live shorter. Unmated solitary *C. remanei* males: $31.4 \pm$
 959 1.7 days, $n=72$; mated *C. remanei* males: 15.7 ± 1.2 days, $n=28$, $p<0.0001$. In mated
 960 group: one *C. remanei* male was paired with one *C. remanei* female from Day 1-Day
 961 6 of adulthood.
- 962 (B) Lifespans of *C. elegans* males mated with *C. elegans* hermaphrodites and *C.*
 963 *remanei* females. Unmated solitary *C. elegans* males: 10.2 ± 0.6 days, $n=35$; *C.*
 964 *elegans* males mated with *C. elegans* hermaphrodites: 7.4 ± 0.4 days, $n=35$,
 965 $p=0.0001$; *C. elegans* males mated with *C. remanei* females: 7.4 ± 0.4 days, $n=35$,
 966 $p=0.0003$. In mated groups, one *C. elegans* male was paired with either one *C.*
 967 *elegans* hermaphrodite or one *C. remanei* female from Day 1-6 of adulthood.
- 968 (C) Lifespans of solitary *C. remanei* males on plates conditioned by eight *C. remanei*
 969 males. Solitary *C. remanei* males on control plates: 35.8 ± 2.0 days, $n=34$; solitary *C.*
 970 *remanei* males on male-conditioned plates: 37.8 ± 1.2 days, $n=34$, $p=0.8501$.
- 971 (D) Lifespans of solitary *C. remanei* females on plates conditioned by eight *C. remanei*
 972 males. Solitary *C. remanei* females on control plates: 27.6 ± 2.2 days, $n=24$; solitary
 973 *C. remanei* females on male-conditioned plates: 27.0 ± 2.5 days, $n=30$, $p=0.8306$.
- 974 (E) Lifespans of grouped *C. remanei* females on plates conditioned by 30 males. *C.*
 975 *remanei* females on control plates: 15.8 ± 0.9 days, $n=60$; *C. remanei* females on
 976 plates conditioned by *C. remanei* males: 19.5 ± 1.3 days, $n=30$, $p=0.0636$; *C.*
 977 *remanei* females on plates conditioned by *C. elegans fog-2* males: 18.5 ± 0.9 days,
 978 $n=60$, $p=0.1770$.
- 979 (F) Lifespans of solitary *C. remanei* males on plates conditioned by eight *C. elegans*
 980 males. Solitary *C. remanei* males on control plates: 36.0 ± 1.2 days, $n=40$; solitary *C.*
 981 *remanei* males on *C. elegans* male-conditioned plates: 36.0 ± 1.2 days, $n=38$,
 982 $p=0.8217$.
- 983 (G) Lifespans of grouped *C. elegans fog-2* hermaphrodites on plates conditioned with 30
 984 males. *fog-2* hermaphrodites control: 14.4 ± 0.8 days, $n=90$. *fog-2* hermaphrodites on
 985 plates conditioned by *fog-2* males: 10.9 ± 0.6 days, $n=60$, $p=0.0004$; *fog-2*
 986 hermaphrodites on plates conditioned by *C. remanei* males: 11.9 ± 0.5 days, $n=90$,
 987 $p=0.0042$.
- 988 (H) Chemotaxis of *C. elegans* (left, blue) and *C. remanei* (right, red) to supernatants from
 989 *C. elegans* males, *C. remanei* males, *C. elegans* N2 hermaphrodites, and *C. remanei*
 990 females. See Methods for detailed description. *C. e.* males to supernatant of *C. r.*
 991 females: Chemotaxis Index (CI) is 0.46 ± 0.11 (mean \pm SEM, $n=12$ [plates]); *C. e.*
 992 males to supernatant of *C. e.* hermaphrodites: CI = 0.14 ± 0.13 ($n=10$); *C. e.* males to
 993 supernatant of *C. e.* males: CI = 0.17 ± 0.17 ($n=12$); *C. e.* males to supernatant of *C.*
 994 *r.* males: CI = 0.11 ± 0.12 ($n=11$); *C. r.* males to supernatant of *C. r.* females: CI =
 995 0.85 ± 0.04 ($n=12$); *C. r.* males to supernatant of *C. e.* hermaphrodites: CI = $0.04 \pm$

996 0.08 (n=12); *C. r.* males to supernatant of *C. e.* males: CI = 0.04 ± 0.15 (n=12); *C. r.*
997 males to supernatant of *C. r.* males: CI = -0.09 ± 0.16 (n=12).
998 (l) Sensitivity to male pheromone-induced lifespan reduction. *C. elegans* males are the
999 most sensitive to male pheromone-induced killing, whereas both *C. remanei* sexes
1000 are immune to this effect.
1001
1002

1003 **Figure 7. Gonochoristic species are immune to male pheromone killing;**
1004 **androdioecious species are susceptible.**

1005 (A) Lifespans of solitary *C. brenneri* males on plates conditioned by 8 *C. brenneri* males.
1006 Solitary *C. brenneri* males control: 18.1 ± 0.8 days, n=33; solitary *C. brenneri* males
1007 on MCP: 17.8 ± 1.1 days, n=32, p=0.9915.

1008 (B) Lifespans of solitary *C. nigoni* males on plates conditioned by 8 *C. nigoni* males.
1009 Solitary *C. nigoni* males control: 15.3 ± 0.4 days, n=32; solitary *C. nigoni* males on
1010 MCP: 15.2 ± 0.6 days, n=40, p=0.7443.

1011 (C) Lifespans of solitary *C. briggsae* males on plates conditioned by 8 *C. briggsae* males.
1012 Solitary *C. briggsae* males control: 13.7 ± 0.8 days, n=38; solitary *C. briggsae* males
1013 on MCP: 10.3 ± 0.3 days, n=54, p=0.0192.

1014 (D) Lifespans of solitary *C. tropicalis* males on plates conditioned by 8 *C. tropicalis* males.
1015 Solitary *C. tropicalis* males control: 17.7 ± 0.8 days, n=40; solitary *C. tropicalis* males
1016 on MCP: 12.2 ± 1.0 days, n=60, p=0.0002.

1017 (E) Model of the effects of mating and male pheromone on androdioecious and
1018 gonochoristic female and males. *C. elegans* hermaphrodites (upper left); *C. remanei*
1019 females (upper right); *C. elegans* males (lower left); *C. remanei* males (lower right).
1020
1021

Figure 8. Male pheromone reduces male offspring

- (A) Male pheromone conditioning from egg onward causes a more severe lifespan shortening. (Pooled results from two independent assays. See Figure 8 – supplement 2 for results of separate lifespan assays, which were also significant.) Solitary *C. elegans fog-2* males control: 18.5 ± 0.6 days, $n=75$; solitary *C. elegans fog-2* males on MCP from early adulthood onward (aMCP): 13.9 ± 0.4 days, $n=79$, $p<0.0001$ (compared to the control); solitary *C. elegans fog-2* males on MCP from egg onward (eMCP): 11.4 ± 0.4 days, $n=80$, $p<0.0001$ (compared to the control), $p<0.0001$ (compared to aMCP).
- (B) Male pheromone decreases *fog-2* male fertility on Day 6. Male treatment started from egg onward. Each male was paired with one virgin *fog-2* hermaphrodite at indicated time for 24 hours. On Day 4 of adulthood, the percent of males who were able to fertilize Day 1 virgin *fog-2* hermaphrodites: three biological replicates: ctrl: $82 \pm 2\%$; MCP: $69 \pm 6\%$, $p=0.12$, unpaired t-test. By Day 6 of adulthood, the percent of males who were able to fertilize Day 1 virgin *fog-2* hermaphrodites (three biological replicates) had significantly decreased: ctrl: $69 \pm 4\%$; MCP: $29 \pm 4\%$, $p=0.0019$.
- (C) Male pheromone treatment decreases the number of progeny produced by those animals who do successfully mate. The difference appears by Day 5. See Figure 8 – supplement 3 for detailed numbers. *, $p<0.05$, unpaired t-test.
- (D) Brood size of self-fertilized N2 hermaphrodites is not affected by male pheromone. Ctrl: 238.2 ± 5 , $n=19$; MCP-treated: 240.5 ± 5 , $n=18$, $p=0.7571$, unpaired t-test.
- (E) Theoretical calculation of male pheromone's effect on male population control (not considering any other contributing factors). See Discussion and Figure 8 – supplement 3 for more details.

Figure 8 – supplement 1. Male pheromone does not affect developmental rates

Eggs from *fog-2* worms were bleached onto control ($n=359$) or MCP ($n=257$) plates. The number of eggs, L1, L2, L3, and L4 worms was counted at 18, 24, 42, and 48 hours post-bleach. We observed a slight deceleration in development at L3 and no difference by L4, resulting in no significant change in total developmental rate upon treatment with MCP.

Figure 8 – supplement 2. Male pheromone treatment from egg onward severely affects both lifespan and cross-offspring production.

- (A) Male pheromone conditioning from egg onward causes a severe lifespan shortening (replicate 1). Solitary *C. elegans fog-2* males control: 17.4 ± 0.7 days, $n=34$; solitary

- 1059 *C. elegans fog-2* males on MCP from early adulthood onward (aMCP): 13.9 ± 0.7
 1060 days, $n=39$, $p<0.0001$ (compared to the control); solitary *C. elegans fog-2* males on
 1061 MCP from egg onward (eMCP): 11.1 ± 0.6 days, $n=40$, $p<0.0001$ (compared to the
 1062 control), $p=0.0021$ (compared to aMCP).
- 1063 (B) Male pheromone conditioning from egg onward causes a severe lifespan shortening
 1064 (replicate 2). Solitary *C. elegans fog-2* males control: 19.5 ± 0.9 days, $n=41$; solitary
 1065 *C. elegans fog-2* males on MCP from early adulthood onward (aMCP): 13.4 ± 0.5
 1066 days, $n=40$, $p<0.0001$ (compared to the control); solitary *C. elegans fog-2* males on
 1067 MCP from egg onward (eMCP): 11.8 ± 0.4 days, $n=40$, $p<0.0001$ (compared to the
 1068 control), $p=0.024$ (compared to aMCP).
- 1069 (C) Percentages of *fog-2* males who are able to successfully fertilize virgin *fog-2*
 1070 hermaphrodites in the presence and absence of MCP treatment. See Figure 8 –
 1071 supplement 3 for details.
- 1072 (D) Male pheromone treatment does not selectively kill one sex in offspring. The male
 1073 ratio is always ~50% (checked from the progeny produced by *fog-2* hermaphrodites
 1074 successfully fertilized by males in C).

1075

1076 **Figure 8 – supplement 3. Male pheromone treatment from egg onward reduces**
 1077 **offspring and might be a novel mechanism to cull male population in hermaphroditic**
 1078 **species.**

- 1079 (A) Percentage of *fog-2* males who were able to successfully fertilize virgin *fog-2*
 1080 hermaphrodites in the presence and absence of MCP treatment.
- 1081 (B) Progeny produced by those animals who do successfully mate.
- 1082 (C) Example of theoretical calculation of how male pheromone affects cross progeny
 1083 production in the next generation (simplified modeling, not considering any other
 1084 factors). In both control and eMCP conditions, the population starts with 60 males.
 1085 Assuming they have equal chance of mating from Day 1 – Day 6 of adulthood, the
 1086 data of percent fertile and number of progeny produced in A and B were used at
 1087 each time points, in the end, the males in eMCP group produce about 76.8% of the
 1088 total progeny produced by males in control group. This number was used when
 1089 plotting the curve in Fig. 8E, for generation X, percent males of control group is 50%,
 1090 percent males of eMCP group is $50\% \times (76.8\%)^X$.

1096 **Supplementary files:**

1097

1098 **Supplementary file 1:** Summary of all lifespan assays performed in this study.

1099

1100 **Supplementary file 2:** Summary of body size measurements.

1101

1102 **Supplementary file 3:** Significantly up- and down-regulated genes in mated males identified

1103 by Significance Analysis of Microarrays (FDR=0%).

1104

1105 **Supplementary file 4:** Significantly up- and down-regulated genes under male pheromone-

1106 induced conditions identified by Significance Analysis of Microarrays (FDR=1%).

1107 *Sheet 1:* grouped vs. solitary neuronally-masculinized hermaphrodites.

1108 *Sheet 2:* grouped *daf-22* males on plates conditioned by wild-type males vs. grouped *daf-22*

1109 males on control plates.

1110 *Sheet 3:* overlap between the two conditions.

1111

1112 **Supplementary file 5:** Comparison of the list of genes significantly up- and down-regulated

1113 upon MCP treatment of *daf-22* males to previously published arrays of male pheromone-

1114 treated hermaphrodites (Maures, et al. 2014).

1115 *Sheet 1:* comparison of up-regulated genes.

1116 *Sheet 2:* comparison of down-regulated genes.

1117

1118 **Materials and Methods**

1119 **Strains:**

1121 N2 (wild type)
1122 CB4108: *fog-2(q71)* V
1123 CB4037: *glp-1(e2141)* III
1124 DR476: *daf-22(m130)* II
1125 RB711: *pqm-1(ok485)* II
1126 RT130: pwls23 [vit-2::GFP]
1127 PB4641: *Caenorhabditis remanei*
1128 PB2801: *Caenorhabditis brenneri*
1129 AF16: *Caenorhabditis briggsae*
1130 JU1422: *Caenorhabditis nigoni*
1131 JU1373: *Caenorhabditis tropicalis*
1132 6699 EG4389: *him-5(e1490)* V; *lin-15(n765ts)*X; *oxEx860[P(rab-3)::fem-*
1133 *3(wt)::mCherry(worm)::unc-54, pkd-2::gfp(S65C), lin-15(+)]* (gift from the Jorgensen Lab)
1134

1135 **Individual male mating lifespan assays:**

1136 All lifespan assays were performed at room temperature (~20-21°C), except for *glp-1* male
1137 lifespan assays (performed at 25-26°C). 35mm NGM plates were used for all the experiments
1138 in this study. 20 µl of OP50 was dropped onto each plate to make a bacterial lawn of ~10 mm
1139 diameter. The next day, one synchronized late L4 male and one late L4
1140 hermaphrodite/female were transferred onto each 35 mm NGM plate. For experiments in Fig.
1141 3C, 3D, and 4C, multiple L4 hermaphrodites were transferred together with one male. One
1142 late L4 male of the same age and genotype was transferred onto the control plates. Except
1143 for Fig. 4A, *fog-2(q71)* hermaphrodites were used as the *C. elegans* hermaphrodites in the
1144 mating assay, because *fog-2* hermaphrodites do not have self sperm, thus allowing us to
1145 easily detect successful mating (i.e. eggs and progeny on the plates). In mated groups, **we**
1146 **only included males that were able to produce progeny in our analysis**. However, for the
1147 experiments regarding *glp-1* males, mating on FUDR, and the inter-species cross between *C.*
1148 *elegans* males and *C. remanei* females, we included all the males in the analysis. Worms
1149 were transferred onto new plates every other day. If the hermaphrodites were lost or bagged,
1150 new unmated Day 1 *fog-2* hermaphrodites were added as replacements. Males and
1151 hermaphrodites/females were kept together for 6 days (unless noted otherwise in the text);
1152 afterwards only males were transferred on to newly seeded plates every 2-3 days. For RNAi
1153 experiments in Fig. 5A, synchronized eggs were transferred onto NGM plates with RNAi
1154 bacteria, late L4 males were transferred and paired with *fog-2* L4 hermaphrodites onto NGM
1155 plates seeded with OP50 (to eliminate the possible effect on mating efficiency for different
1156 RNAi treatments). Two days later, males and hermaphrodites were transferred onto fresh
1157 plates seeded with corresponding RNAi bacteria and males were maintained on RNAi

1158 bacteria thereafter. 30-50 worms were included in each group of individual worm lifespan
1159 assays. The sample size was similar to previous published study of individual hermaphrodite
1160 lifespan assays. When lifespan assays were completed, Kaplan-Meier analysis with log-rank
1161 (Mantel-Cox) method was performed to compare the lifespans of different groups. The
1162 summary of all lifespan experiments is included in Supplementary file 1.

1163

1164 **Grouped males:**

1165 35mm NGM plates were used for all the experiments in this study. 20 μ l of OP50 was
1166 dropped onto each plate to make a bacterial lawn of ~10 mm diameter. The next day, eight
1167 synchronized late L4 males were transferred onto each plate. (Two or four males per plate for
1168 experiment in Fig. 1A.) One late L4 male of the same age and genotype was transferred onto
1169 the control plates. Males were transferred onto fresh plates every two days, when the males
1170 were lost or dead, males from other plates were transferred together to make the size of the
1171 group stable.

1172

1173 **Male-conditioned plates (MCP) setup:**

1174 Male-conditioned plates for lifespan assays were prepared similar to the previous description
1175 (Maures et al. 2014). Briefly, 60 μ l of OP50 was dropped onto each 35mm NGM plate to
1176 make a bacterial lawn of ~25 mm diameter. Young Day 1 wild-type males (*fog-2* males) were
1177 transferred onto each plate. Two days later, they were removed and worms for lifespan
1178 assays were immediately transferred onto these male-conditioned plates (MCP). These male-
1179 conditioned plates were prepared throughout the course of the lifespan assays (Fig. 1 – figure
1180 supplement 1B) to ensure fresh MCP plates were available. The number of wild-type males
1181 used for conditioning is stated in the text and labeled in the figures. In Fig. 1 – figure
1182 supplement 1F, *glp-1* mutant males as well as the wild-type males were used for conditioning
1183 at 25 °C for 2 days. For MCP treatment from egg onward, 30 Day 1 wild-type males were
1184 used to condition plates for 2-3 days. Males were removed and about 15 Day 1 mated *fog-2*
1185 hermaphrodites were picked onto these MCP plates for 4-5 hours, allowing them to lay 60-80
1186 eggs. Two days later, L4 males were individually transferred onto MCP plates (conditioned by
1187 8 males, as previously described) for the lifespan assays.

1188

1189 **Body size measurement:**

1190 Images of live males on 35mm plates were taken daily for the first week of adulthood with a
1191 Nikon SMZ1500 microscope. Image J was used to analyze the body size of the worms. The
1192 middle line of each worm was delineated using the segmented line tool and the total length
1193 was documented as the body length of the worm. T-test was performed to compare the body
1194 size differences between groups of males in the same day. See Supplementary file 2 for
1195 summary.

1196

1197 **FUdR experiment:**

1198 FUDR was added to the NGM media to the final concentration of 50 μ M. Late L4 males and
1199 hermaphrodites were transferred onto NGM+FUDR plates seeded with OP50. Worms were
1200 transferred every two days, and were kept on FUDR plates for different period of time (3 days,
1201 6 days or lifetime as indicated by text).

1202

1203 **DAPI staining and analysis of male germline:**

1204 Worms were stained according to Bio-protocol ([http://www.bio-](http://www.bio-protocol.org/wenzhang.aspx?id=77)
1205 [protocol.org/wenzhang.aspx?id=77](http://www.bio-protocol.org/wenzhang.aspx?id=77)) using VECTASHIELD HardSet Mounting Medium with
1206 DAPI from Vector Laboratories. Images were taken with a Nikon Ti. The mitotically
1207 proliferating germline region was determined by the crescent shape of DAPI-stained nuclei in
1208 the transition zone. Z-series of pictures were taken and the numbers of cells in the mitotically
1209 proliferating germline region were counted manually. We scored the germline morphology as
1210 '1' (clear transition zone marked by crescent shaped nuclei and sperm after U turn of the
1211 germline), '2' (no clear transition zone), and '3' (sperm appear before U turn of the germline).
1212 Nonparametric comparison between each treatment group was performed using Prism
1213 Graphpad. Mann Whitney test was used to determine the statistical significance.

1214

1215 **Oil Red O staining and quantification:**

1216 Oil Red O staining was adapted from the published protocol for staining of a small number of
1217 worms (Wahlby et al. 2014). About 20 worms per treatment were imaged with Nikon Ti. Oil
1218 Red O quantification was also performed as published (O'Rourke et al. 2009). In brief, the
1219 color images were split into RGB monochromatic images in Image J. The Oil-Red-O staining
1220 arbitrary unit (a.u.) was determined by mean gray value within the worm region by Image J
1221 (Intensity in the Green channel was used as the signal, adjusted by the intensity in the Red
1222 channel as the background). T-test analysis was performed to compare the fat staining of
1223 different groups of worms.

1224

1225 **Glycogen staining:**

1226 Glycogen staining was performed according to the published protocol (Frazier and Roth
1227 2009). Mating of males was set up as previously described. Right before staining, live males
1228 of the same group were picked into an M9 droplet with 1M sodium azide on a 3% agarose
1229 pad. Immediately after the liquid was dry, the pad was inverted over the opening of a 50g
1230 bottle of iodine crystal chips (Sigma) for 1 minute. After the color stained by iodine vapor on
1231 the pad disappeared (non-specific staining), the worms (about 20 worms per treatment) were
1232 immediately imaged by a Nikon microscope. Due to uncontrollable differences, it is hard to
1233 compare the staining performed at different times. Thus, worms from the groups of
1234 comparison were mounted onto the same pad (using a separate M9 droplet if there is no
1235 visible difference). Image J was used to compare the mean intensity of iodine staining after
1236 the background was subtracted. T-test was performed to compare the staining between
1237 different groups (on the same pad).

1238

1239 **GFP intensity quantification:**

1240 10-20 worms of each group were imaged by Nikon Ti. Image J was used to measure the

1241 mean and the maximum GFP intensity of the whole body area. T-test analysis was performed

1242 to compare the GFP intensity of different groups of worms.

1243

1244 **Mated males microarrays:**

1245 We paired a single male with a *fog-2* hermaphrodite for about 3.5 days of mating, then picked

1246 the males individually on Day 4 for microarray analysis. As a control, solitary males were

1247 collected at the same time. About 150 males (on 150 individual 35mm plates) were collected

1248 for each condition and replicate. Three biological replicates were performed. RNA was

1249 extracted by the heat-vortexing method. Two-color Agilent microarrays were used for

1250 expression analysis; detailed steps and analysis were performed as we previously reported

1251 (Luo et al. 2010).

1252

1253 ***daf-22* grouped males microarrays:**

1254 Synchronized late L4 *daf-22* males were picked on to 35mm plates (control and MCP). 30

1255 males per plate, 150 males in total were used for each biological replicate. Males were

1256 transferred on to freshly seeded plates or MCP plates every two days, and collected on Day 6

1257 for RNA extraction. Four biological replicates were performed.

1258

1259 **6699 EG4389 masculinized hermaphrodites grouped vs single microarrays:**

1260 Synchronized late L4 worms were picked onto 35mm plates. In the “grouped” condition, 30

1261 hermaphrodites were picked onto one plate, and ~120 worms were used for each replicate.

1262 Worms were transferred every two days to exclude progeny, and were collected on Day 6 for

1263 RNA extraction. Four biological replicates were performed.

1264

1265 **Microarray data accession links:**

1266 Microarray data can be found in PUMAdb (<http://puma.princeton.edu>).

1267 Mated males microarrays (3 biological replicates):

1268 https://puma.princeton.edu/cgi-bin/exptsets/review.pl?exptset_no=7244

1269 *daf-22* grouped males microarrays (4 biological replicates):

1270 https://puma.princeton.edu/cgi-bin/exptsets/review.pl?exptset_no=7246

1271 https://puma.princeton.edu/cgi-bin/exptsets/review.pl?exptset_no=7250

1272 *glp-1* hermaphrodites treated with MCP microarrays (4 biological replicates):

1273 https://puma.princeton.edu/cgi-bin/exptsets/review.pl?exptset_no=7251

1274 EG4389 masculinized hermaphrodites grouped vs single microarrays (4 biological replicates):

1275 https://puma.princeton.edu/cgi-bin/exptsets/review.pl?exptset_no=7245

1276

1277 **Analyses of Microarray data:**

1278 Significant differentially-expressed gene sets were identified using SAM (Tusher et al. 2001).
1279 Previously reported microarray results exploring the effect of males on hermaphrodites
1280 (Maures et al. 2014) were downloaded from NCBI and compared to our differentially
1281 expressed gene lists. Enriched motifs were found using RSAT (van Helden 2003).
1282

1283 **Pheromone chemotaxis assay:**

1284 This assay (Fig. 6H) was modified from a previous assay (Chasnov et al. 2007). 10 Day 1
1285 virgin *C. remanei* or *C. elegans* hermaphrodites were placed in 100 µl of M9 buffer at room
1286 temperature overnight with shaking. 100 males of either *C. elegans* or *C. remanei* were
1287 placed in 100 µl of M9. The supernatant solutions were then used for the pheromone
1288 chemotaxis assay. 60 mm NGM plates (no food) were used for the chemotaxis assay. Two
1289 destination spots (supernatant and M9 control) were separated by about 45 mm; the distance
1290 from the origin spot to either destination spot is 30mm. Two 1µl drops of 1M sodium azide
1291 were first applied to the destination spots. When dry, a drop of 1 µl M9 or supernatant was
1292 separately added onto the destination spots. Then, over 10 young adult (Day 2) males were
1293 placed at the origin spot, transferring as little bacteria as possible. After 60 minutes, the
1294 paralyzed male worms were scored based on their location. The chemotaxis index was
1295 calculated as: (#worms at supernatant destination - #worms at control destination)/(#total
1296 worms - #worms at origin). The chemotaxis assay in Fig. 2B was also modified from
1297 established protocol (Chasnov et al. 2007). 10 Day 5 hermaphrodites of either N2 or 6699
1298 EG4389 were put in 100 µl of M9 buffer at room temperature overnight with shaking. Two
1299 destination spots were 3mm apart. The origin spot was in the middle. 20 Day 1 *fog-2* males
1300 were used in each assay; two replicates were performed.

1301

1302

1303 **Male fertility assay:**

1304

1305 Males from the control plates and MCP plates were individually paired with one virgin Day 1
1306 *fog-2* hermaphrodite at various time points on a seeded 35mm NGM plate for 24 hours. About
1307 20 pairs were set up for each group in each biological replicate. The percent fertile was
1308 calculated from the number of plates with eggs/progeny divided by the total number of plates
1309 set up for this group. Each mated hermaphrodite was numbered and was transferred
1310 individually onto a new seeded NGM plate every day to count the total progeny/male number.

1311

1312

1313 **Materials and Methods References:**

1314

1315 Chasnov, J.R., So, W.K., Chan, C.M., and Chow, K.L. (2007). The species, sex, and stage
1316 specificity of a *Caenorhabditis* sex pheromone. *Proceedings of the National Academy of*
1317 *Sciences of the United States of America* 104, 6730-6735.

1318 Frazier, H.N., 3rd, and Roth, M.B. (2009). Adaptive sugar provisioning controls survival of *C.*
1319 *elegans* embryos in adverse environments. *Current biology* : CB 19, 859-863.

1320 Luo, S., Kleemann, G.A., Ashraf, J.M., Shaw, W.M., and Murphy, C.T. (2010). TGF-beta and
1321 insulin signaling regulate reproductive aging via oocyte and germline quality maintenance.
1322 *Cell* 143, 299-312.

1323 Maures, T.J., Booth, L.N., Benayoun, B.A., Izrayelit, Y., Schroeder, F.C., and Brunet, A.
1324 (2014). Males shorten the life span of *C. elegans* hermaphrodites via secreted compounds.
1325 *Science* 343, 541-544.

1326 O'Rourke, E.J., Soukas, A.A., Carr, C.E., and Ruvkun, G. (2009). *C. elegans* major fats are
1327 stored in vesicles distinct from lysosome-related organelles. *Cell metabolism* 10, 430-435.

1328 Tusher, V.G., Tibshirani, R., and Chu, G. (2001). Significance analysis of microarrays applied
1329 to the ionizing radiation response. *Proc Natl Acad Sci U S A* 98, 5116-5121.

1330 van Helden, J. (2003). Regulatory sequence analysis tools. *Nucleic Acids Res* 31, 3593-3596.

1331 Wahlby, C., Conery, A.L., Bray, M.A., Kamentsky, L., Larkins-Ford, J., Sokolnicki, K.L.,
1332 Veneskey, M., Michaels, K., Carpenter, A.E., and O'Rourke, E.J. (2014). High- and low-
1333 throughput scoring of fat mass and body fat distribution in *C. elegans*. *Methods (San Diego,*
1334 *Calif)* 68, 492-499.
1335
1336

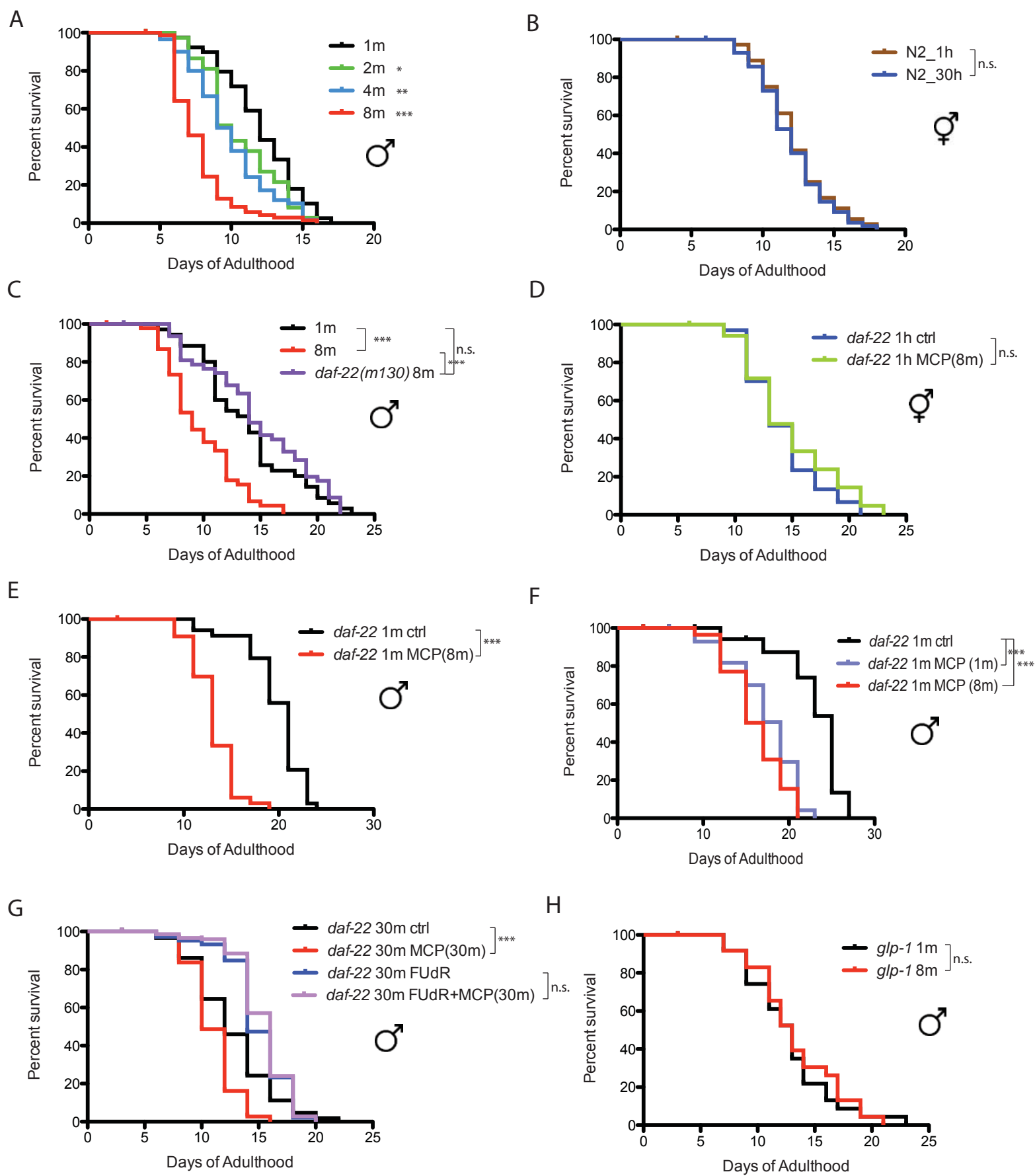


Figure 1

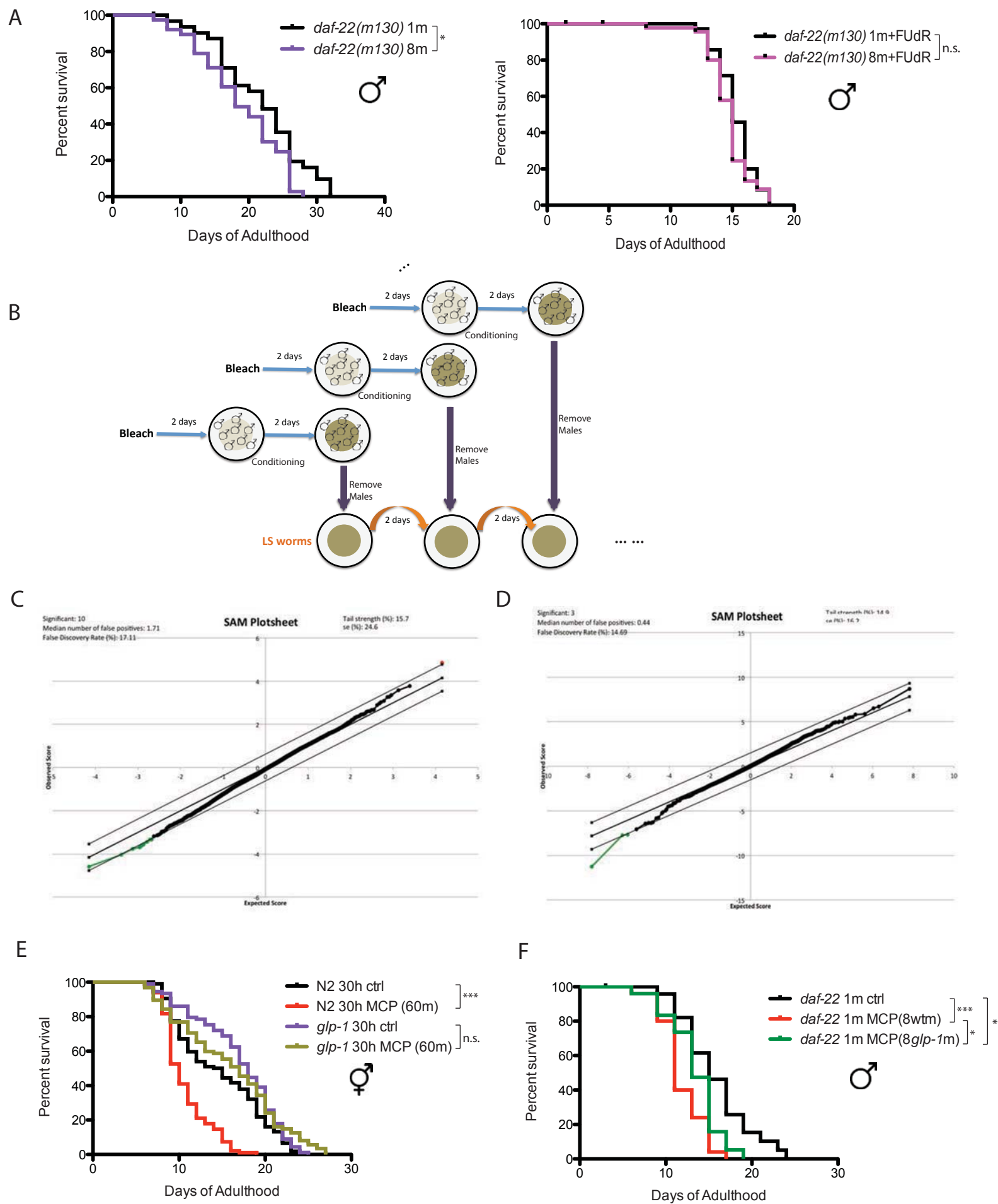


Figure 1 - figure supplement 1

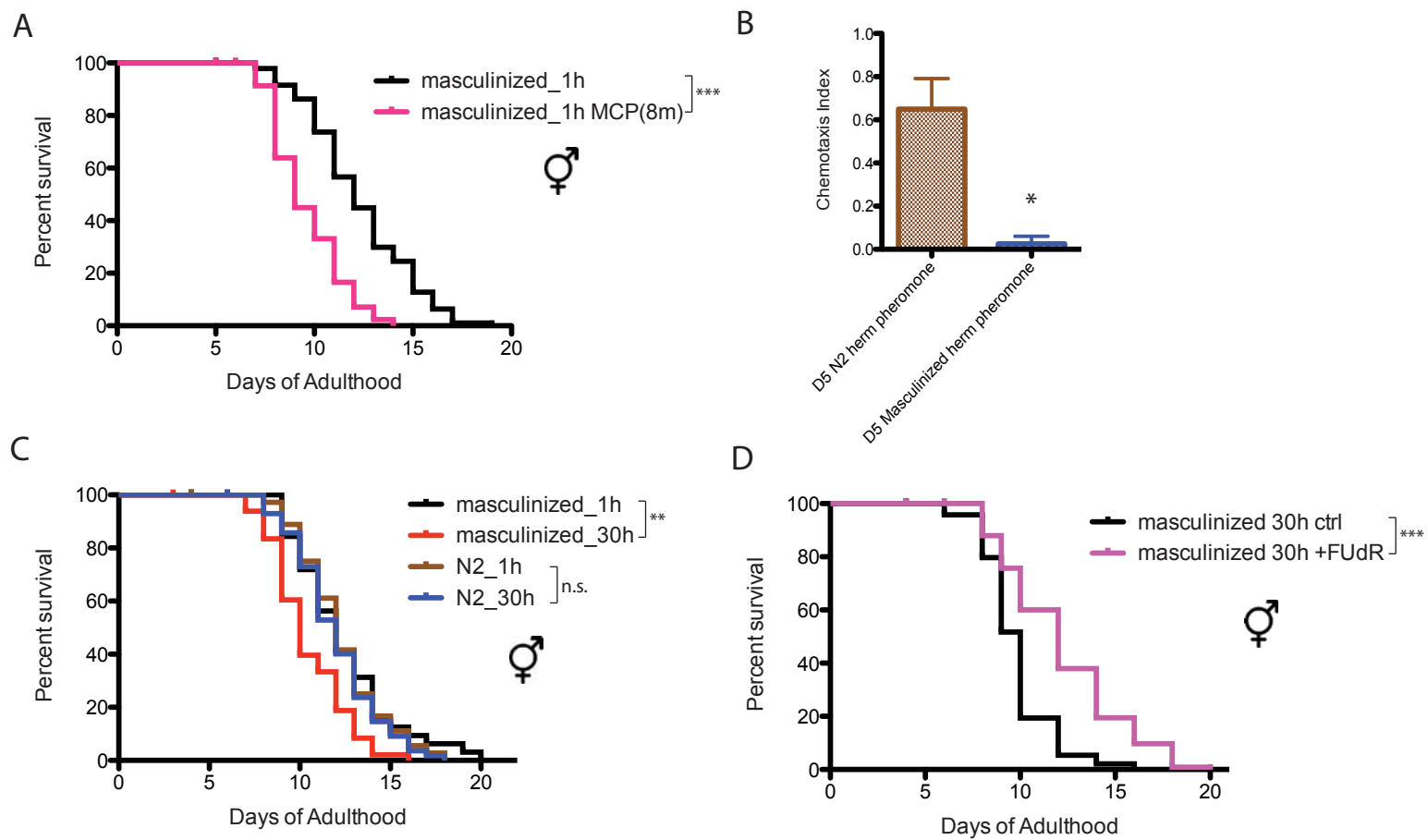


Figure 2

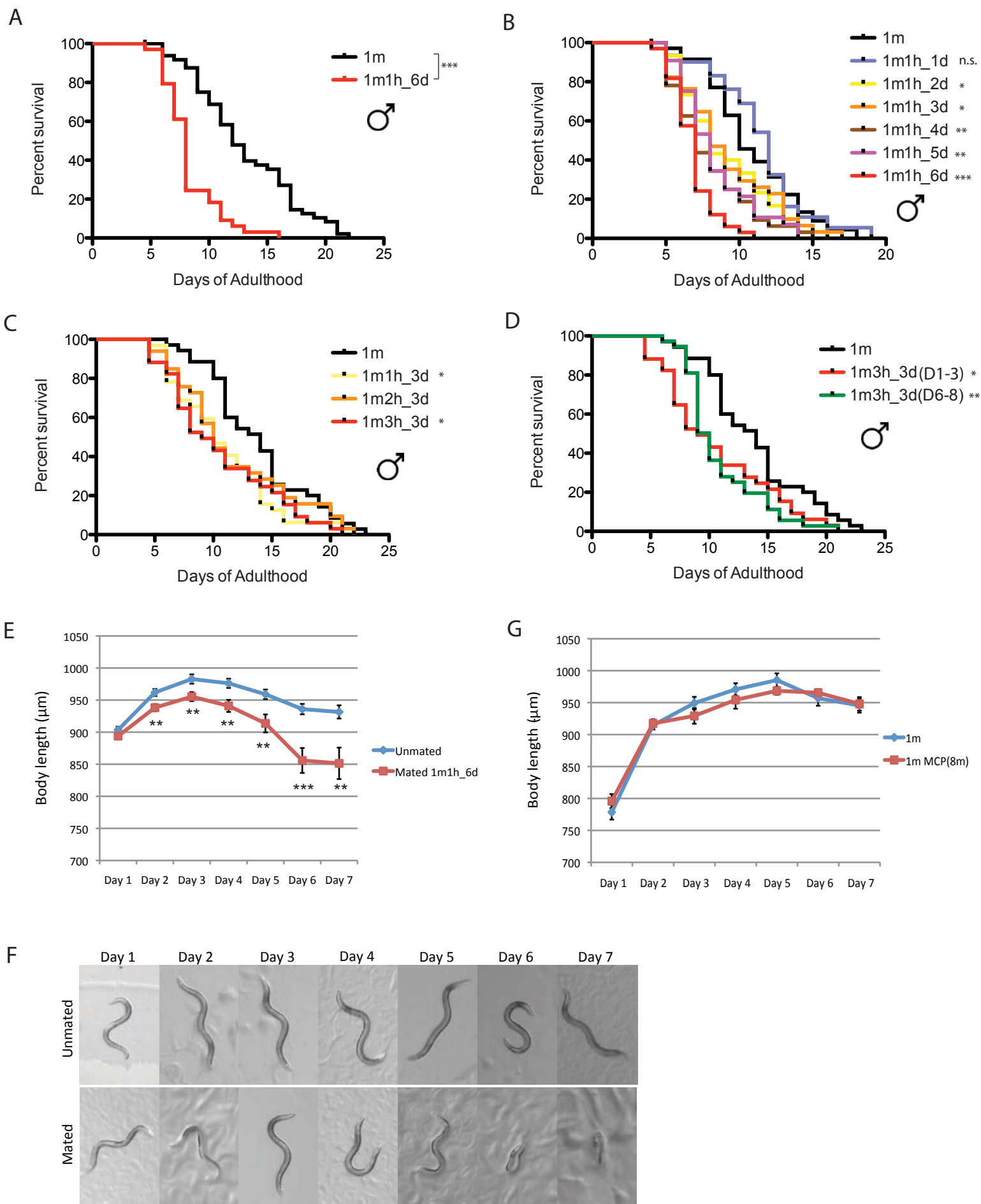


Figure 3

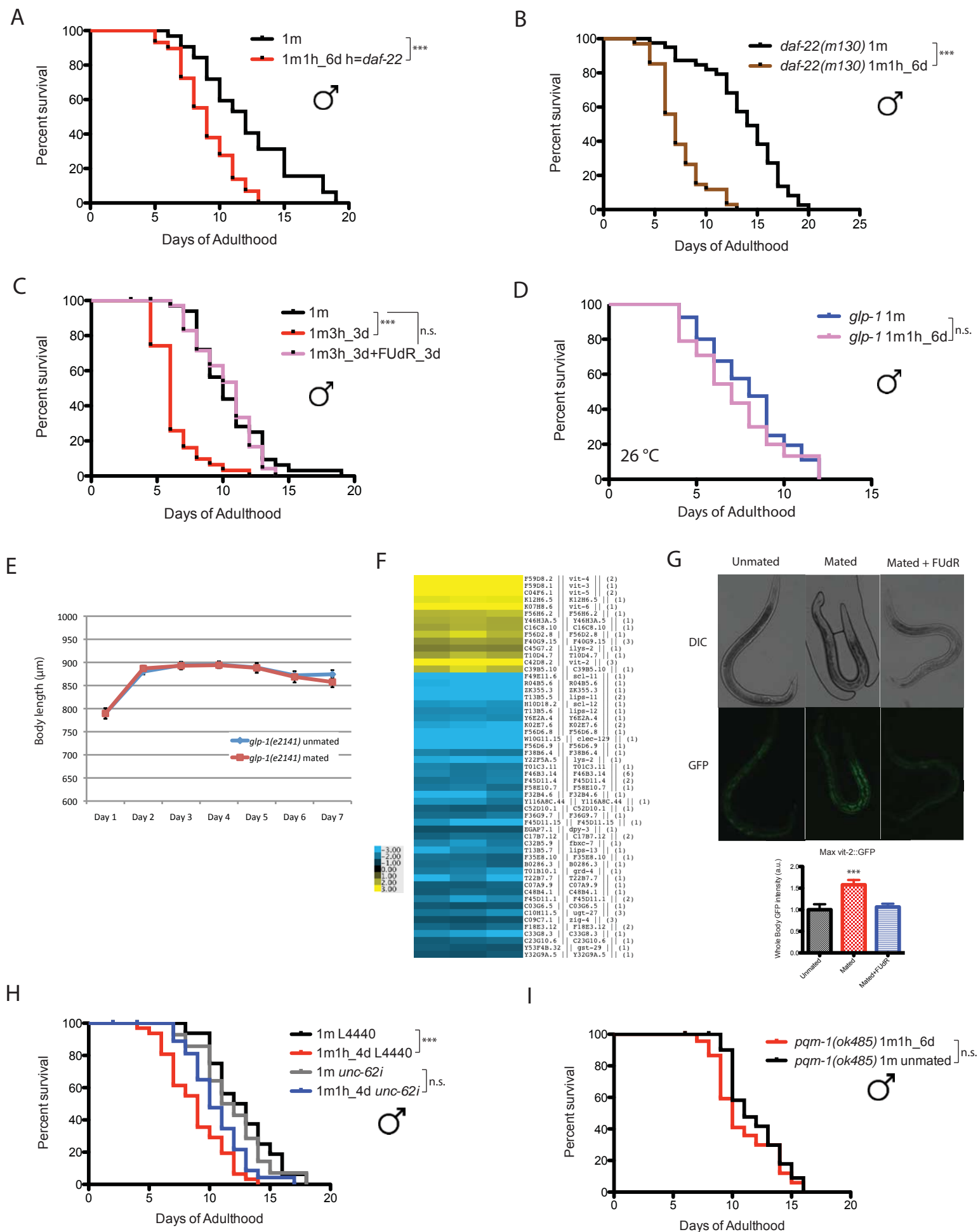
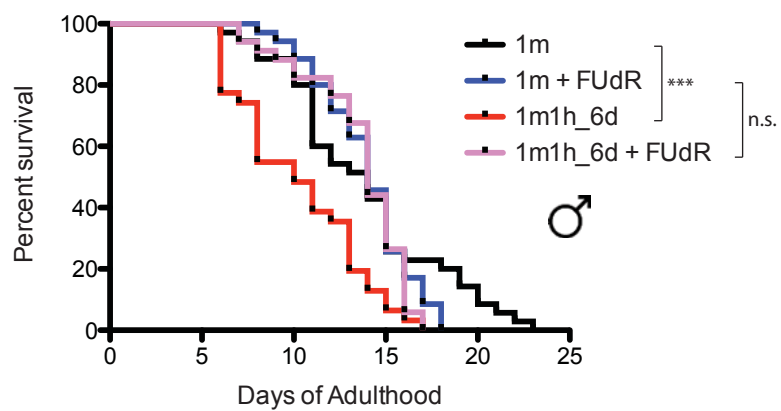


Figure 4

A



B

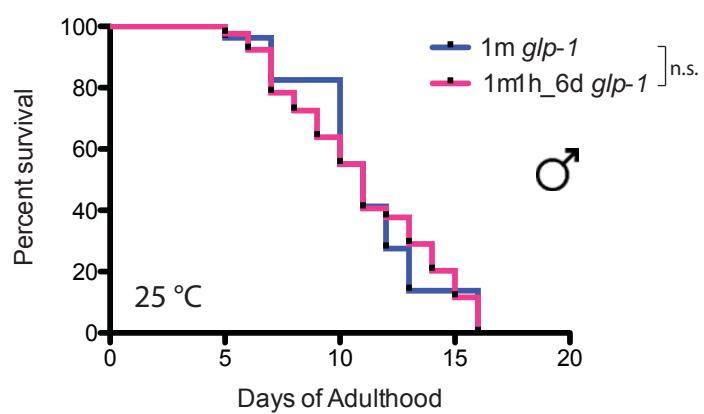


Figure 4 - figure supplement 1

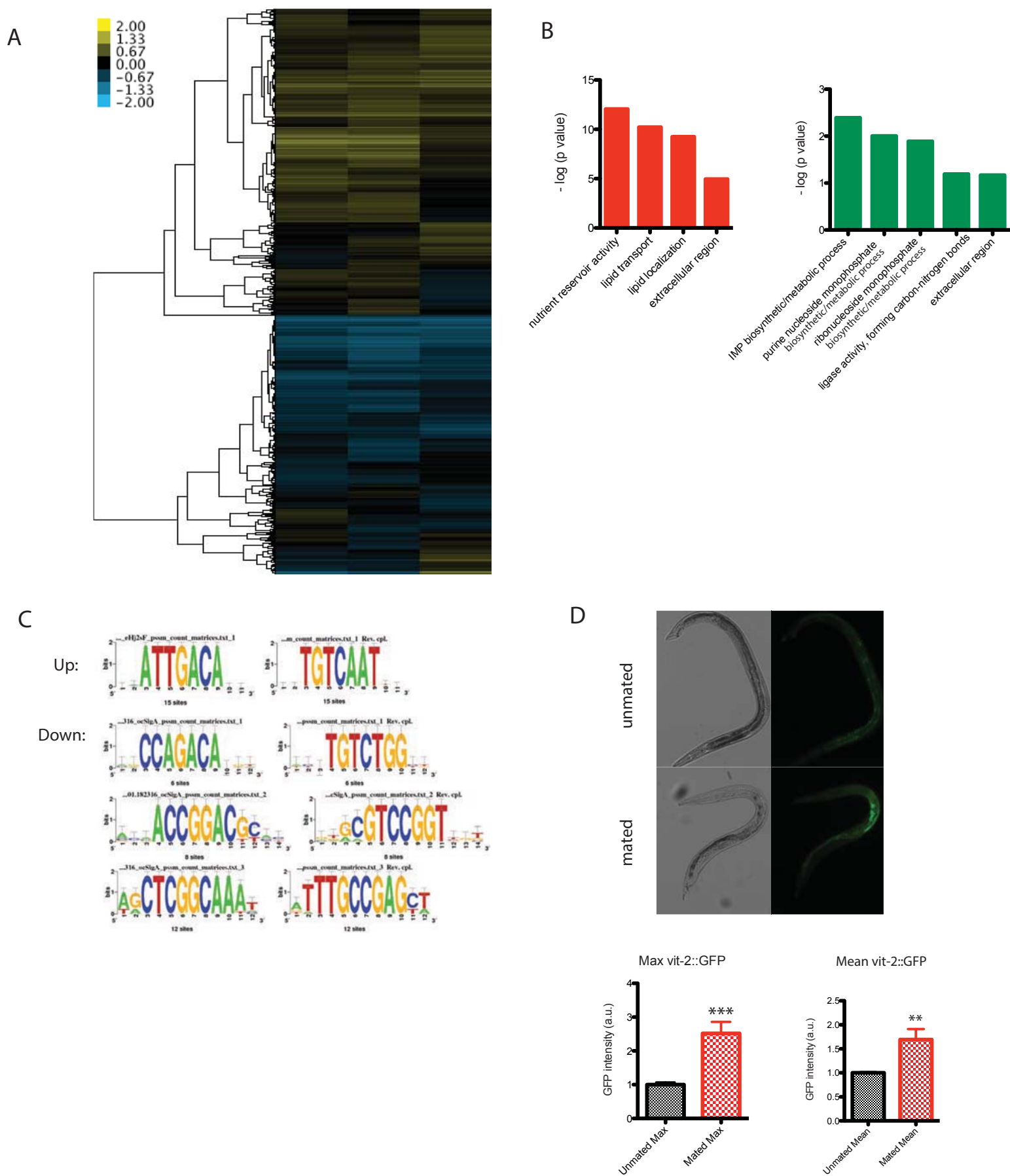


Figure 4 - figure supplement 2

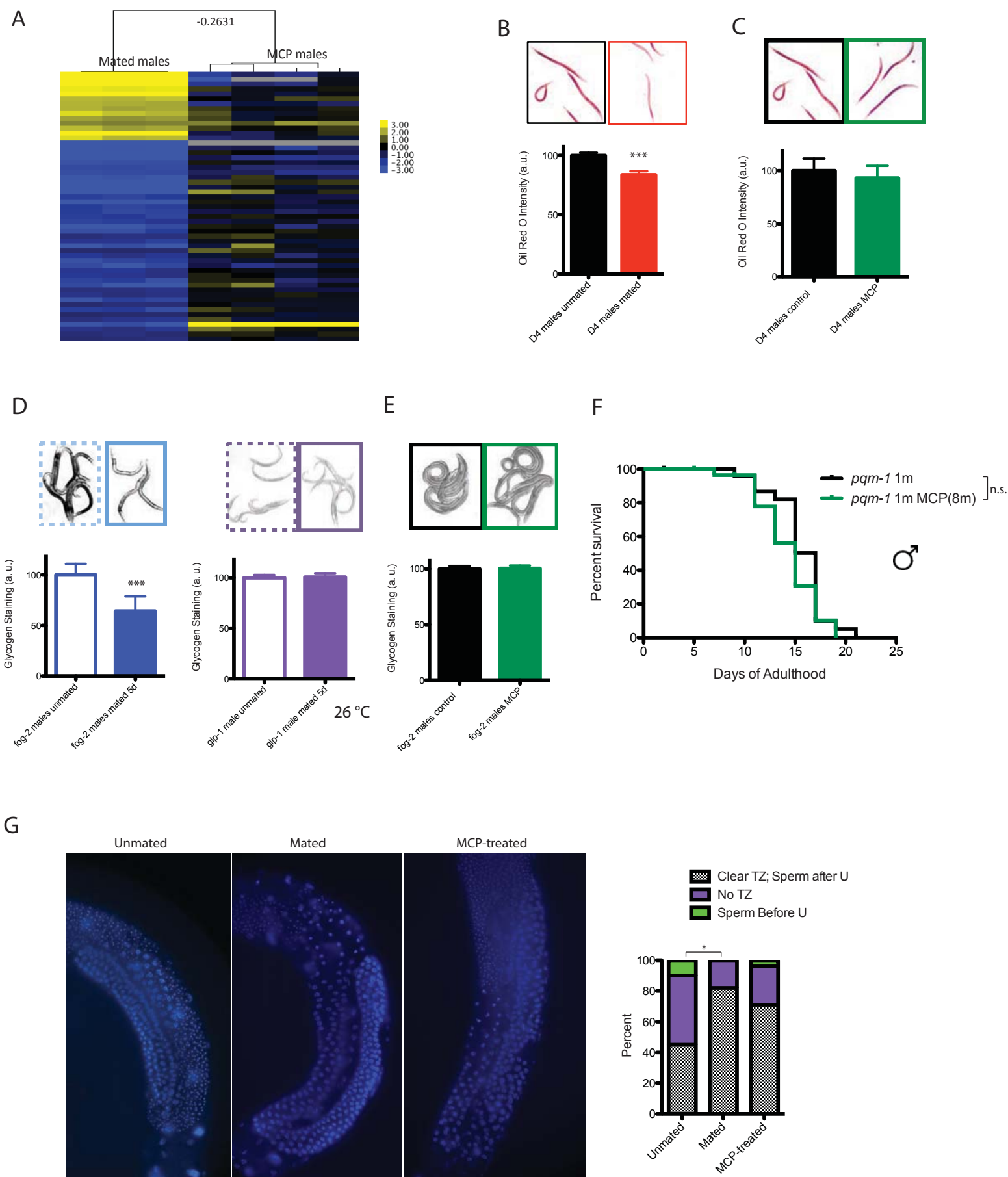


Figure 5

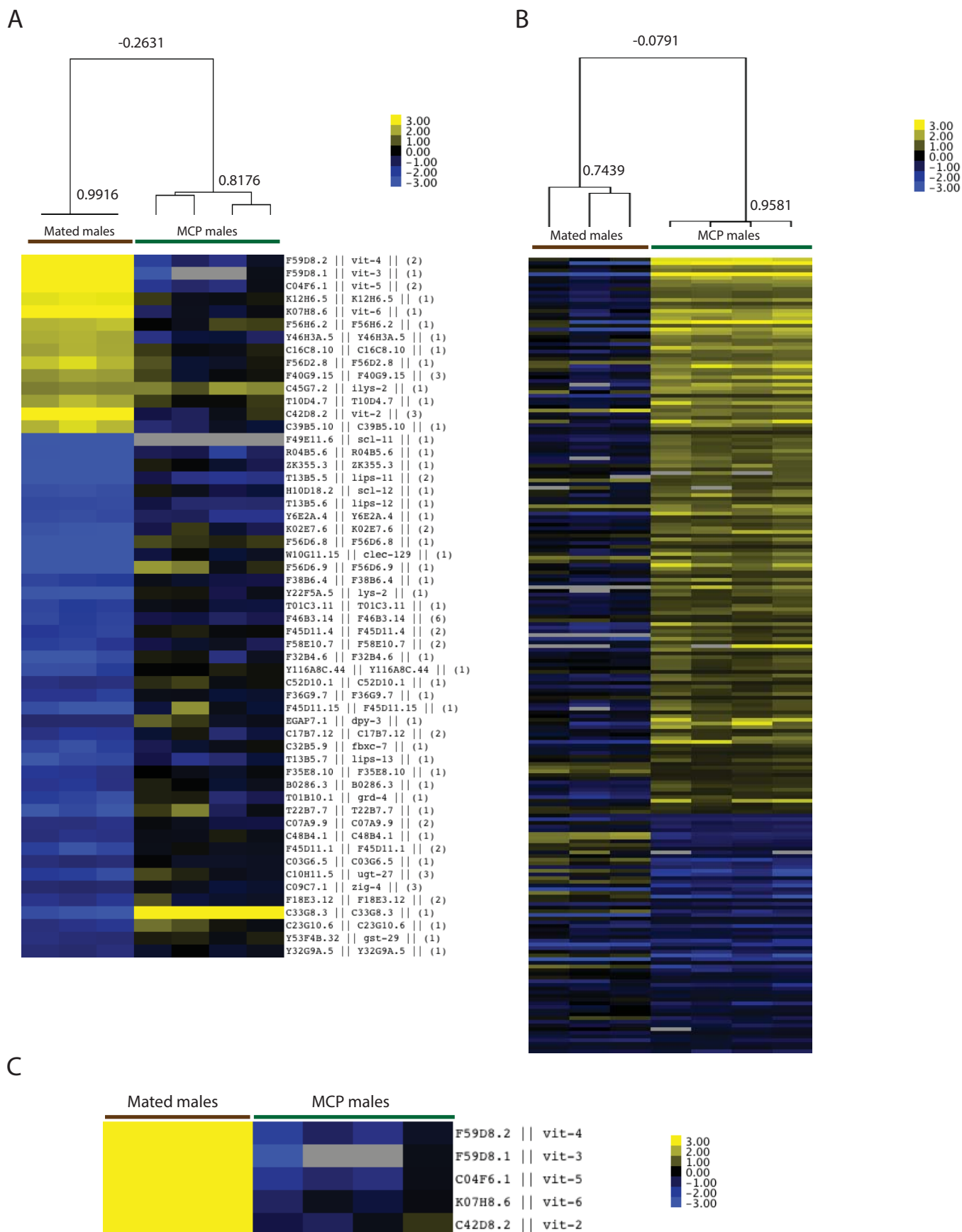


Figure 5 - figure supplement 1

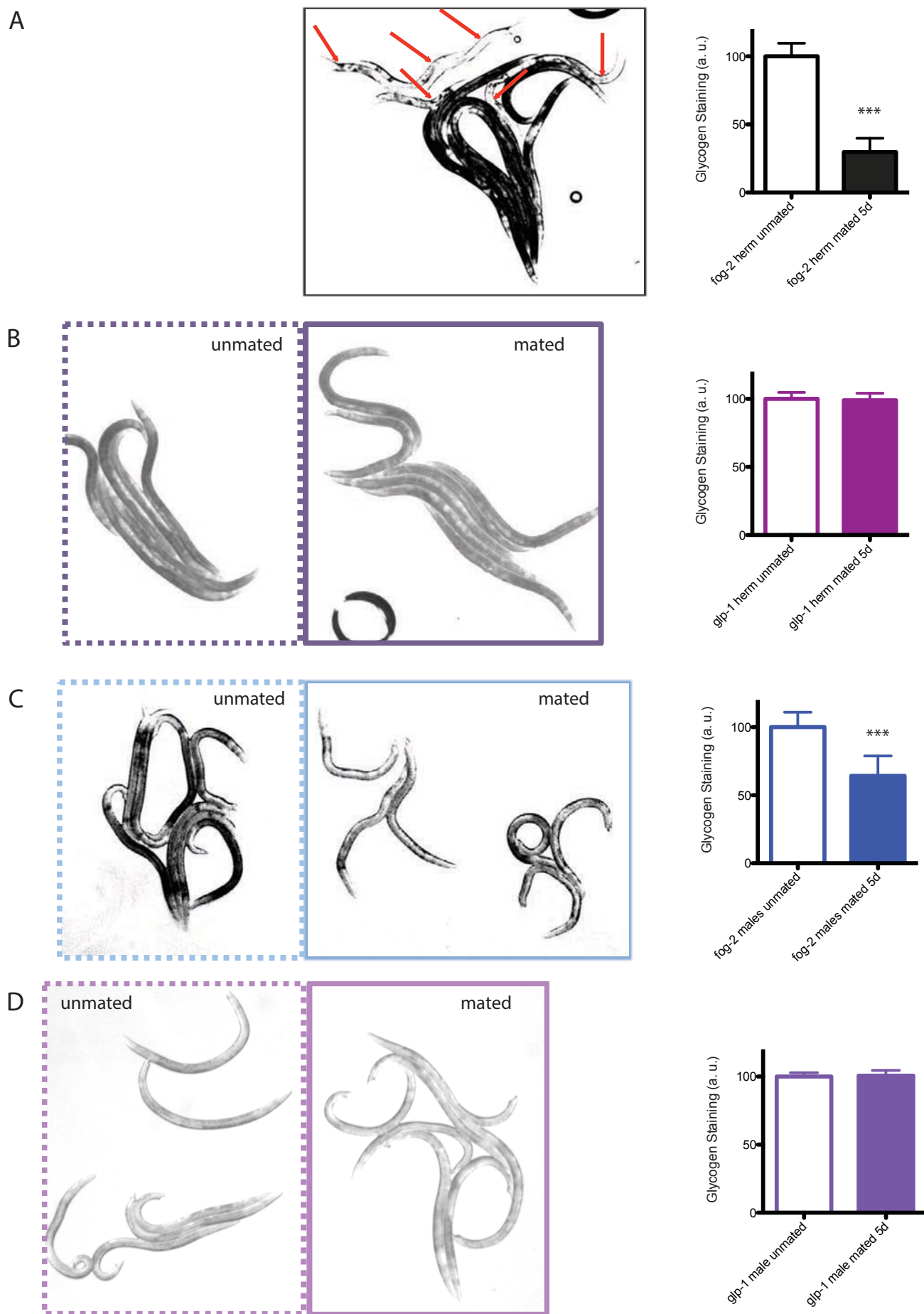
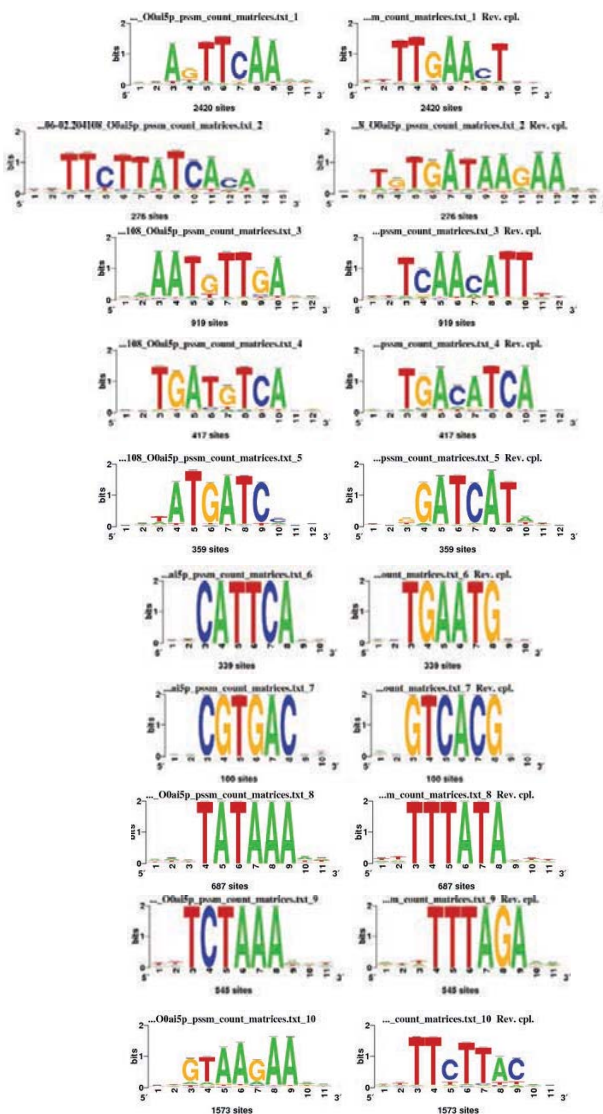


Figure 5 - figure supplement 2

Masculinized herm Grouped vs Single

daf-22 males MCP vs Ctrl

Up:



Down:

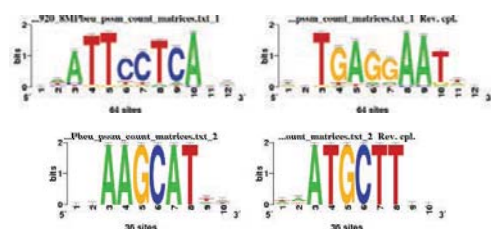
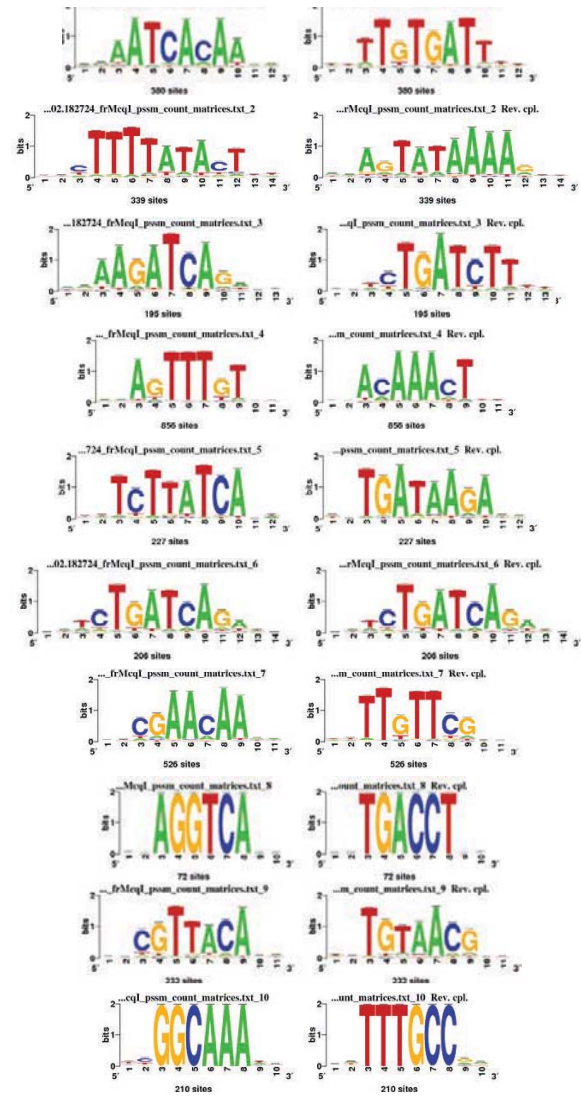
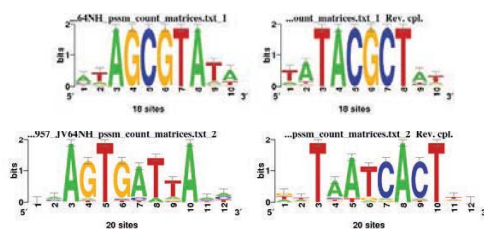


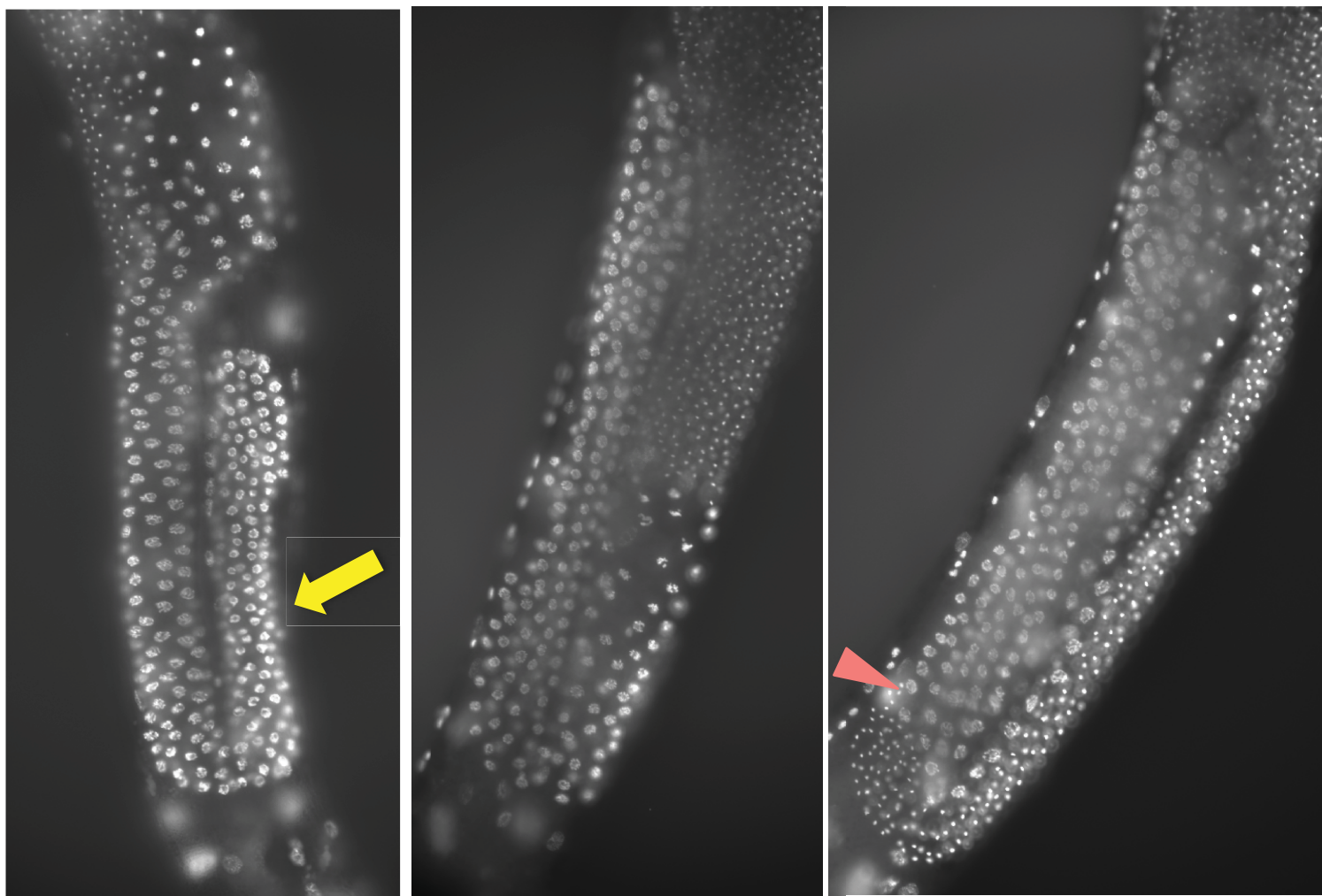
Figure 5 - figure supplement 3

A

Clear transition zone;
Sperm after U-turn

No transition zone

Sperm before U-turn



B

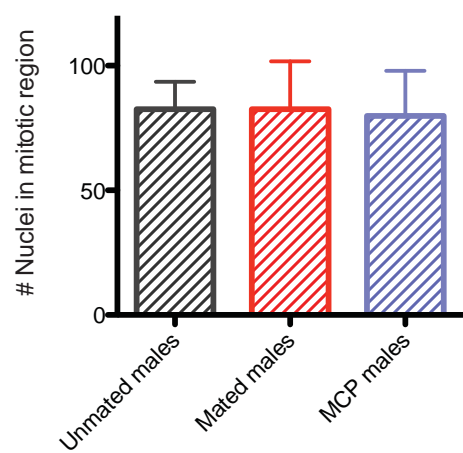


Figure 5 - figure supplement 4

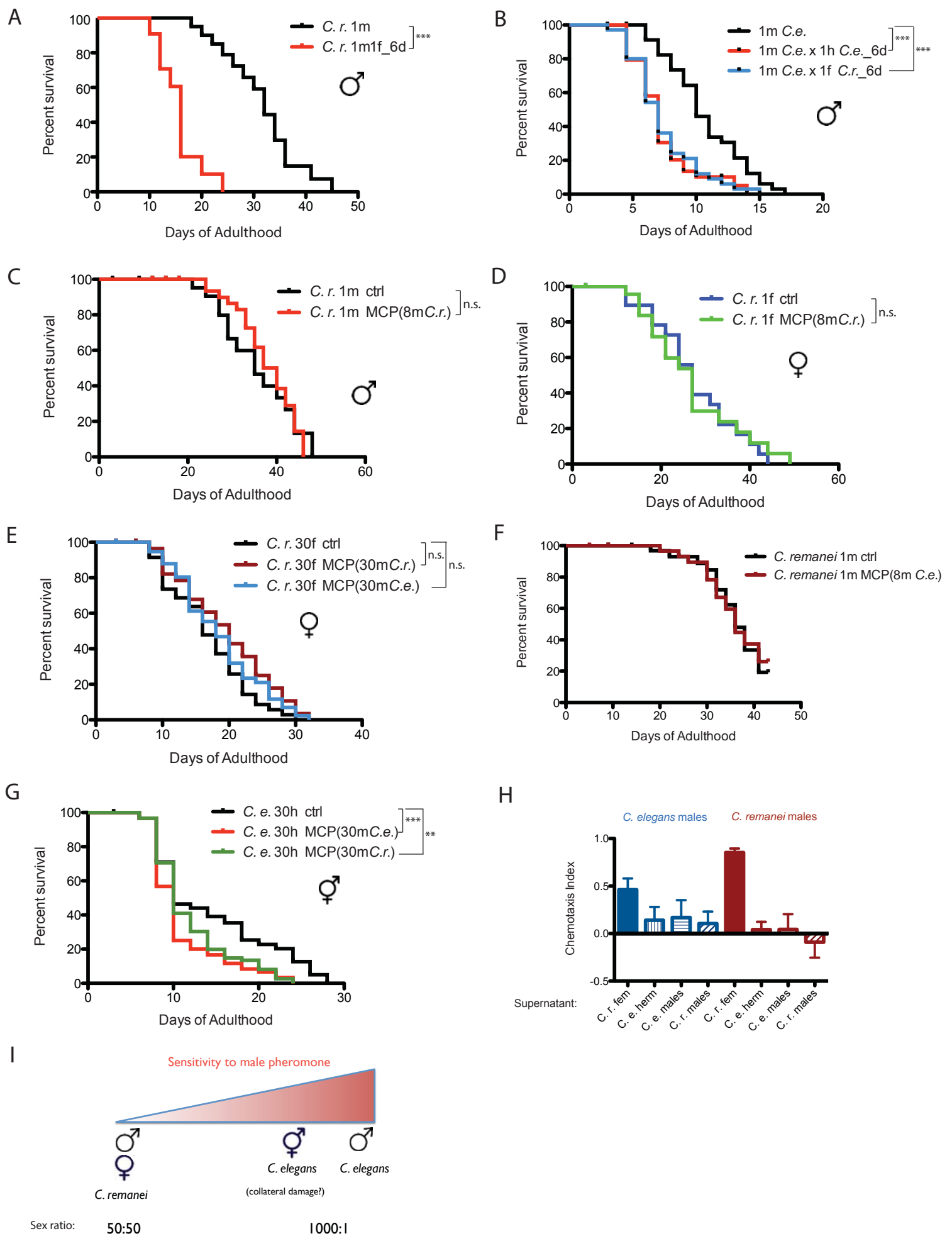


Figure 6

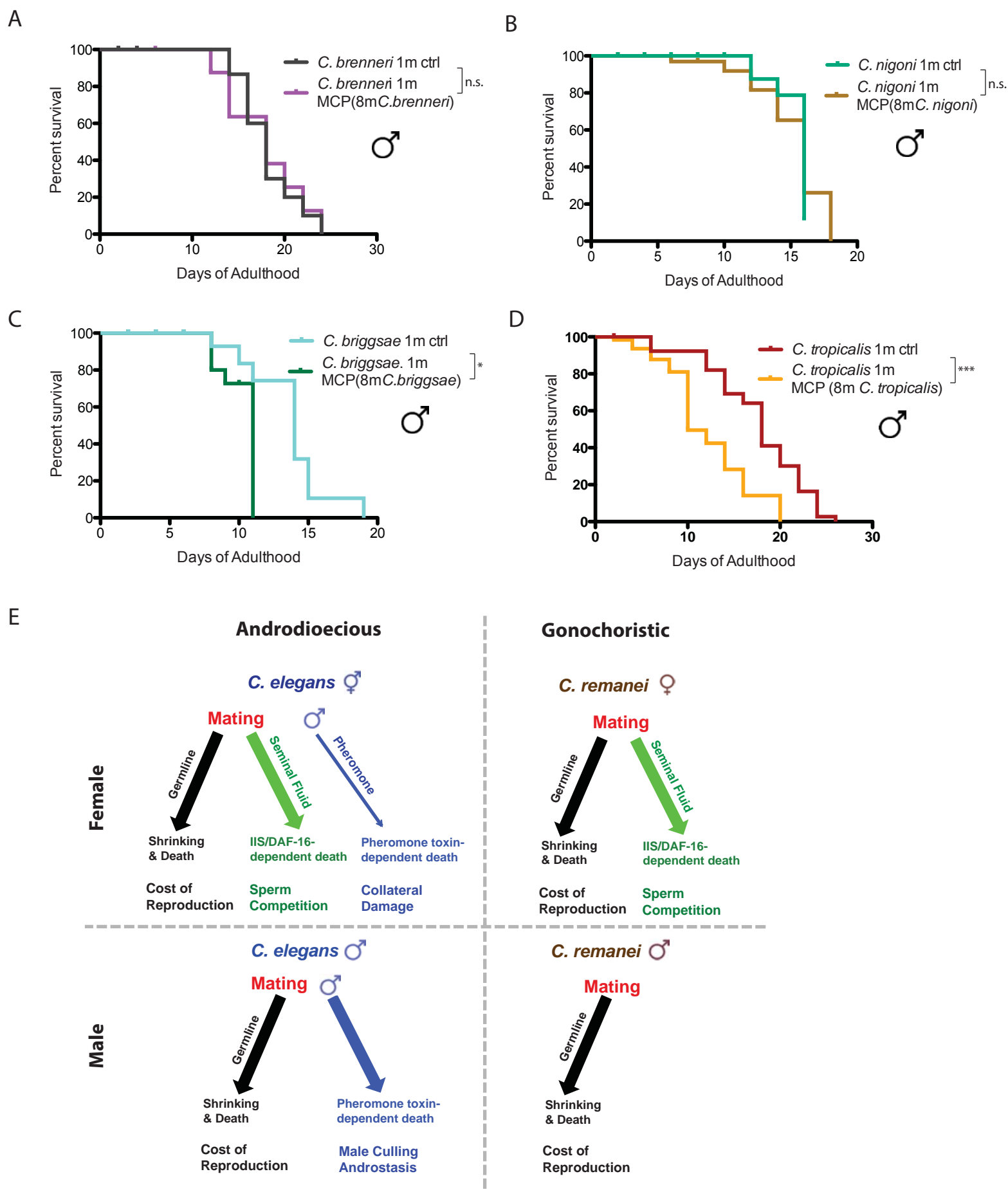


Figure 7

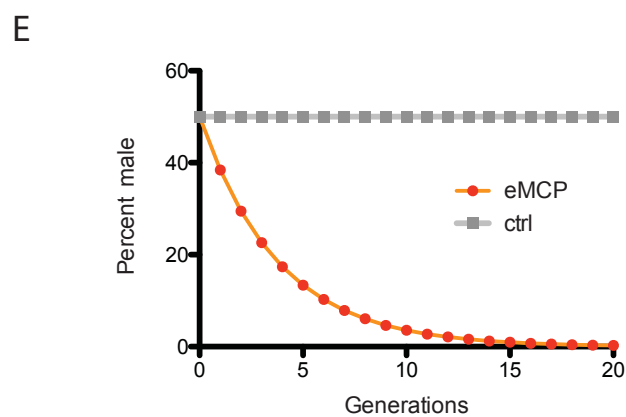
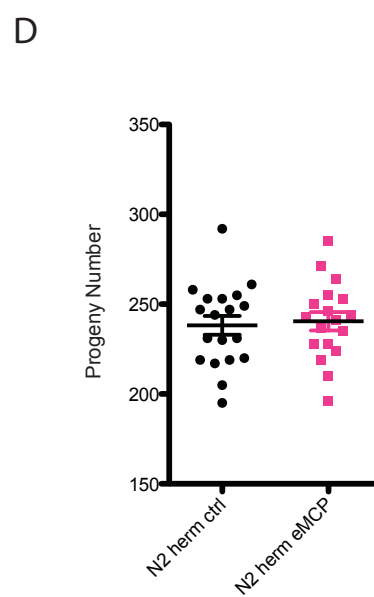
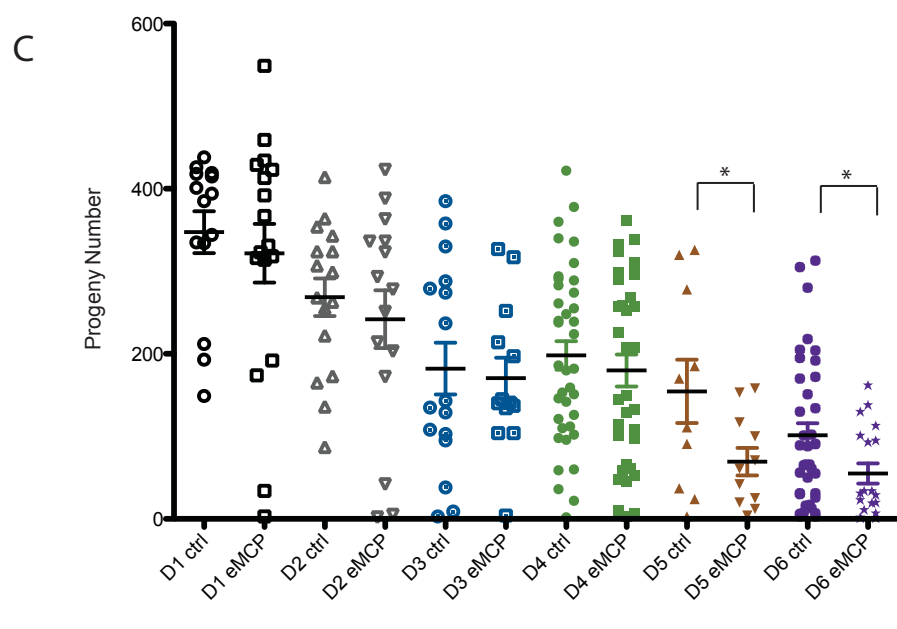
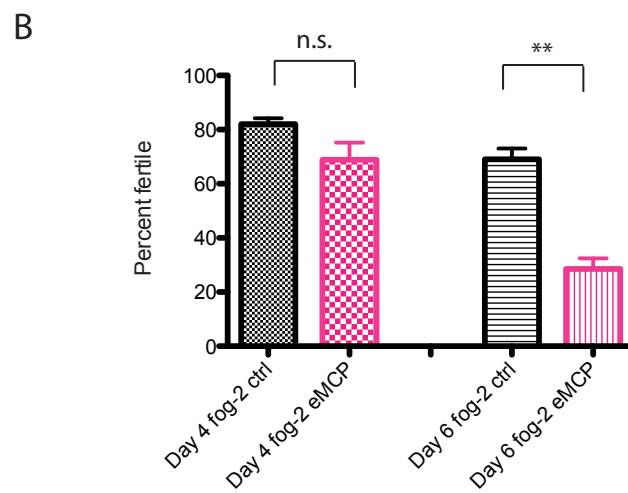
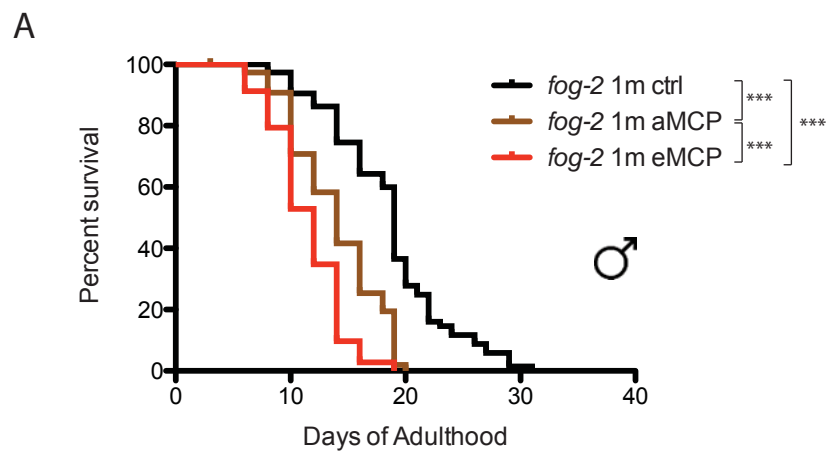


Figure 8

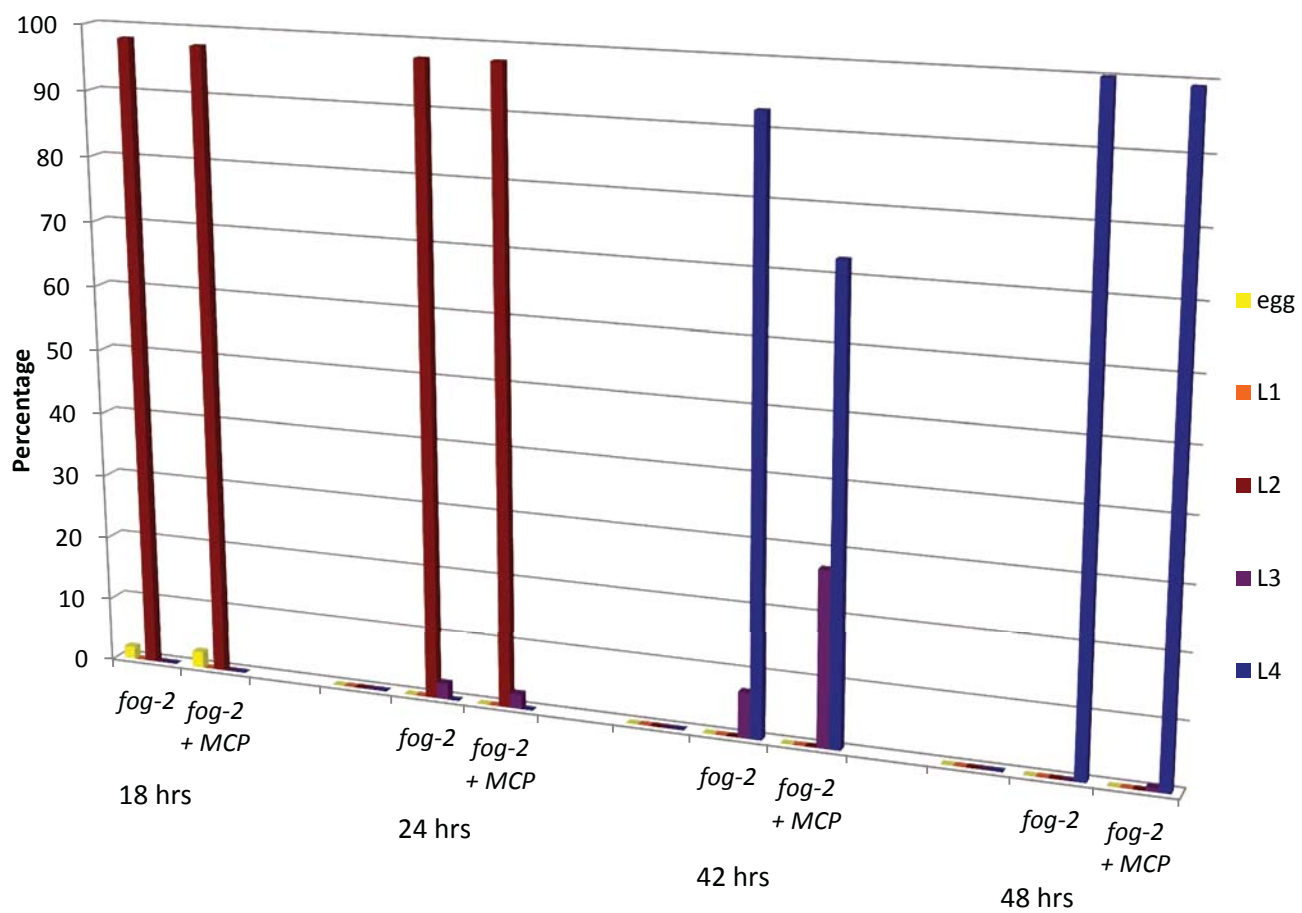


Figure 8 - figure supplement 1

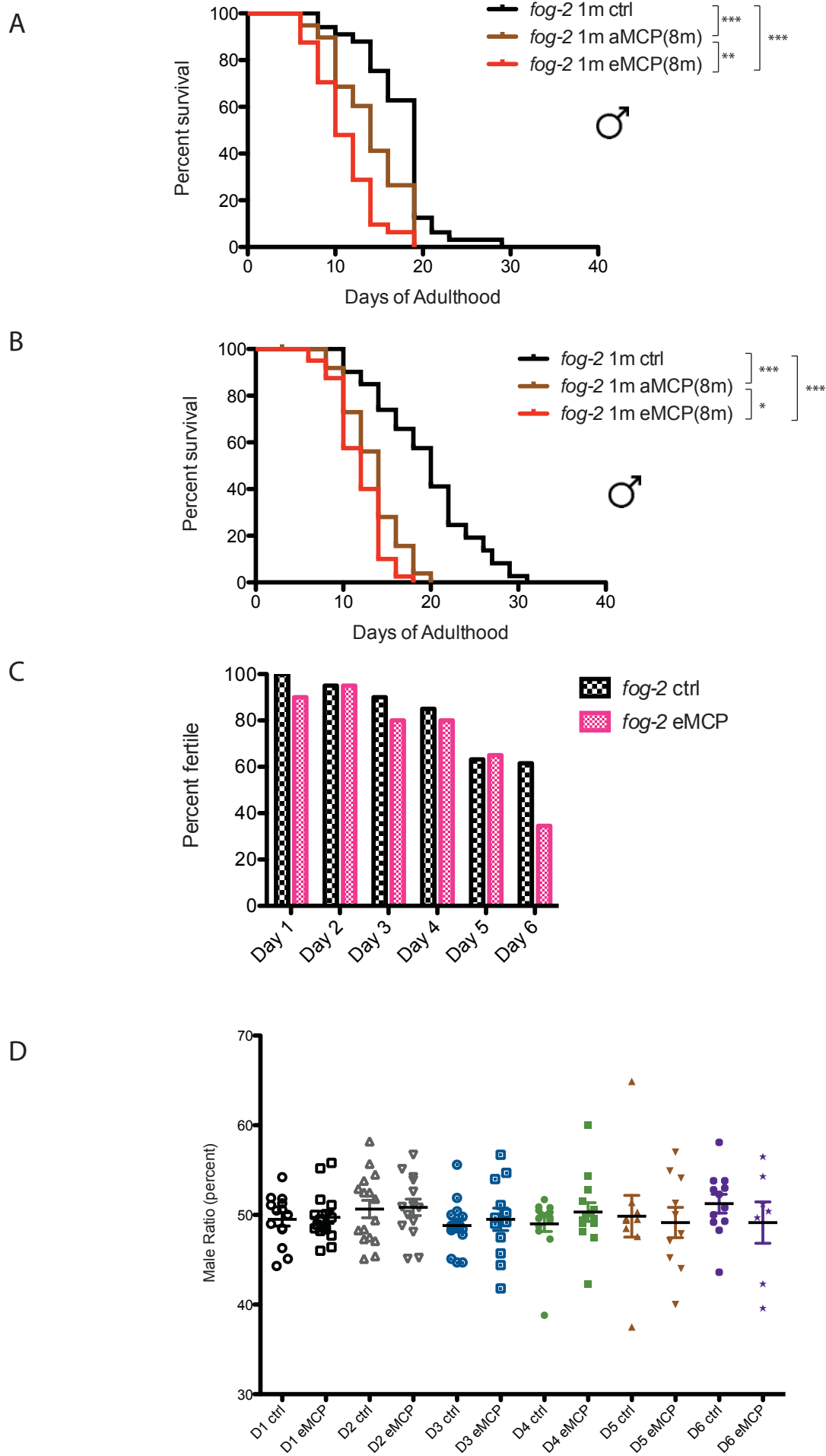


Figure 8 - figure supplement 2

A

Percent fertile	ctrl	eMCP
Day 1	100%	90%
Day 2	95%	95%
Day 3	90%	80%
Day 4 (3 replicates)	82 ± 2%	69 ± 6%
Day 5	63.2%	65%
Day 6 (3 replicates)	69 ± 4%	29 ± 4%

B

Progeny produced	ctrl	eMCP
Day 1	347 ± 25	322 ± 36
Day 2	269 ± 23	242 ± 35
Day 3	182 ± 31	171 ± 25
Day 4	198 ± 17	180 ± 19
Day 5	155 ± 38	69 ± 17
Day 6	101 ± 15	55 ± 12

C

	ctrl	eMCP	eMCP/ctrl
Day 1	3470 = 10*100%*347	2898 = 10*90%*322	83.5%
Day 2	2556 = 10*95%*269	2299 = 10*95%*242	90%
Day 3	1638 = 10*90%*182	1368 = 10*80%*171	83.5%
Day 4	1624 = 10*82%*198	1242 = 10*69%*180	76.5%
Day 5	980 = 10*63.2%*155	449 = 10*65%*69	45.8%
Day 6	697 = 10*69%*101	160 = 10*29%*55	23.0%
Total	10965	8416	76.8%

Figure 8 - figure supplement 3