in vitro egg production by the human parasite *Schistosoma mansoni*

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ABSTRACT

Schistosomes infect over 200 million people. The prodigious egg output of these parasites is the sole driver of pathology due to infection, yet our understanding of their sexual reproduction is limited because egg production is not sustained for more than a few days *in vitro*. Here, we describe culture conditions that support schistosome sexual development and sustained egg production *in vitro.* Female schistosomes rely on continuous pairing with male worms to fuel the maturation of their reproductive organs. Exploiting these new culture conditions we explore the process of male-stimulated female maturation and demonstrate that physical contact with a male worm, and not insemination, is sufficient to induce female development and the production of viable parthenogenetic haploid embryos. Furthermore, we show that RNAi can be used to robustly perturb the maintenance of the female reproductive system and blunt egg production *in vitro*. Taken together, these results provide a platform to study the fascinating sexual biology of these parasites on a molecular level, perhaps illuminating new strategies to control schistosome egg production.

body

INTRODUCTION

Schistosomes are blood-dwelling parasitic flatworms that cause serious disease in millions of people in the developing world (1). The pathology caused by these parasites is entirely due to the parasite's prodigious egg output (2). Although the goal of the parasite is to pass these eggs from the host to ensure the continuity of the parasite's complex life cycle, approximately half of these eggs become trapped in host tissues inducing inflammation that represents the primary driver of disease (3). Since parasites incapable of producing eggs produced little pathology in infected hosts, understanding the biology of schistosome egg production could suggest new therapeutic strategies against these devastating parasites.

Schistosomes are unique among flatworms as they do not sexually reproduce as hermaphrodites instead they have evolved separate male and female sexes (2, 4, 5). This transition from hermaphroditism to dioecism has led to some intriguing biological phenomena, in particular the observation that female schistosomes rely on continuous pairing with a male worm to become sexually mature and produce eggs (2, 6-8). Indeed, females grown in the absence of male worms are developmentally stunted and their reproductive organs are undeveloped. Upon pairing with a male worm the female's sexual organs become mature and egg production commences. Interestingly, this process is reversible since females deprived of male contact will regress to an immature state (9). Although the process of male-induced female maturation was described almost a century ago little is known about the nature of the molecular signals that induce female maturation upon pairing with a male worm.

A major bottleneck for understanding the biology of egg production and female sexual development is that normal egg production ceases within days of removal of the parasite from the host (10-13). While work by numerous investigators has established robust conditions for the maintenance (14-17) and growth of adult-staged parasites (12, 18, 19), no *in vitro* conditions that sustain continuous egg production have been described. Here, we report conditions that support long-term schistosome egg production *in vitro*. As a proof of principle, we use these culture conditions to explore the process by which male worms stimulate female maturation. We find that direct contact with a male worm along the female worm's entire body is essential for sexual maturation. Furthermore, we demonstrate that in the absence of sperm transfer that contact with a male worm is sufficient for female worms to produce viable parthenogenetic haploid embryos. These studies provide new insights in the biology of schistosome egg production and lay the groundwork for the application of a growing molecular tool kit to understanding the fascinating sexual biology of these important pathogens.

RESULTS AND DISCUSSION

Media containing ascorbic acid, red blood cells, and cholesterol supports egg production *in vitro*. The most successful systematic effort for culturing schistosomes *in vitro* are those of Basch (12, 18, 19). While Basch's "medium 169" (BM169) was able to support the *in vitro* growth of larval parasites to adulthood (18), it was insufficient for maintaining sexually mature egg-laying female parasites (12, 19). Nevertheless, given the success of BM169 to support parasite growth, we reasoned BM169 was the ideal place to begin optimizing conditions for egg production. As previously reported (12), adult schistosomes recovered from mice and cultured in

93 BM169 progressively lost the ability to lay eggs with the morphological characteristics of those
94 laid *in vivo* or immediately *ex vivo* (Fig. 1A).

95 A schistosome egg is constructed from cells derived from two organs: the ovary, that
96 contributes an oocyte, and the vitellaria, that provide 20-30 vitellocytes (2, 20). Although female
97 worms paired with male worms retained the ability to produce oocytes during *in vitro* culture
98 (Fig. S1A,B), we noted a rapid loss in the ability of cultured parasites to generate vitellocytes.
99 Vitellocytes contain two types of large cytoplasmic inclusions: lipid droplets and vitelline
100 droplets that coalesce to form the eggshell (21, 22). Using Fast Blue BB to label vitelline
101 droplets we found that vitellaria progressively ceased production of large numbers of vitellocytes
102 in BM169 (Fig. 1B,C). Similarly, BODIPY 493/503 labeling found that the vitellaria of females
103 in BM169 possessed few lipid droplets at D20 (Fig. 1C). Examination of a panel of genes
104 expressed in mature vitellocytes (23) by whole mount *in situ* hybridization (Fig. 1D) and
105 quantitative PCR (Fig. 1E) found a significant decrease in the expression of vitellaria-specific
106 transcripts during culture, similar to previous studies (13). Thus, the capacity for vitellogenesis is
107 rapidly lost *in vitro*.

108 To improve the rate of vitellogenesis and egg production we examined supplements that
109 could potentially satisfy either known metabolic requirements for egg production (e.g., lipids(24))
110 or documented auxotropies of the worm (e.g., polyamines, fatty acids, sterols(25-27)). From
111 these analyses we observed no qualitative effects on egg production from supplements, including:
112 albumin (e.g., Lactalbumin or Linoleic Acid-Oleic Acid-Albumin), spermidine, commercially
113 available lipid supplements, commercially available antioxidant supplements, red blood cells
114 (RBCs), low density lipoprotein, L-carnitine, N-acetyl-cysteine, or sera from various species
115 (chicken, bovine, or horse). During this process, however, we came across a report detailing the
116 formation of abnormal eggs in schistosome-infected guinea pigs fed an Vitamin C (L-ascorbic
117 acid) deficient diet (28). Strikingly, addition of ascorbic acid to BM169 led to a marked increase
118 in vitelline development (Fig. 1C, Fig. S1C) and the production of eggs morphologically similar
119 to those laid by parasites immediately *ex vivo* (Fig. 1C).

120 Although L-ascorbic acid had profound effects on the quality of eggs generated *in vitro*,
121 the rate of egg production and development of the vitellaria remained inferior to that of fresh *ex*
122 *vivo* parasites (Fig. 1C). Thus, we re-examined some of the previous assessed media
123 supplements. Given the critical role for lipid metabolism in egg production (24), we reasoned
124 that adding complex sources of lipids (and other nutrients) that the parasite encounters *in vivo*
125 might act synergistically with ascorbic acid. We found that supplementation with either red
126 blood cells or a commercial "cholesterol" concentrate containing purified Low Density
127 Lipoprotein (LDL) increased lipid stores along the intestine but had little effect on vitelline
128 development or the production of normal eggs (Fig. 1C). However, combination of red blood
129 cells, the cholesterol/LDL concentrate, and ascorbic acid produced a dramatic increase in the rate
130 of vitellogenesis (Fig. 1C), the rate of egg production (Fig. 1C), and a marked increase in the
131 expression of vitellaria-specific transcripts (Fig. 1D,E). From here on we refer to this formulation
132 as ABC169 (Ascorbic Acid, Blood Cells, Cholesterol and Basch Media 169).

133 In vertebrate cells, L-ascorbic acid acts not only as an antioxidant, but an essential co-
134 factor for a variety of enzymes (29). Mammals deficient for L-ascorbic acid cannot perform key
135 enzymatic reactions (e.g., collagen hydroxylation) leading to symptoms commonly known as
136 scurvy (29). Interestingly, the effects of vitamin C to prevent scurvy are stereoselective as D-
137 isoascorbic acid cannot replace L-ascorbic acid at equimolar concentrations (30). Similarly, we

138 observed that D-isoascorbic acid could not replace L-ascorbic acid in egg production (Fig. 1F),
139 suggesting that either L-ascorbic acid is selectively transported into cells or that it acts in a
140 stereoselective fashion to facilitate one or more enzymatic reactions within the cell. Future
141 studies aimed at determining the function of vitamin C in schistosome cells could suggest new
142 approaches to blunt egg production.

143 **Parasites cultured in ABC169 produce eggs capable of developing to miracidia.** After an
144 initial peak, egg production in BM169 dropped precipitously and by D7 of culture parasites laid
145 ~13 egg-like masses per day (Fig. 2A,B). We noted a similar peak in egg production using
146 ABC169, however, after D7 these parasites sustained production of ~44 morphologically normal
147 eggs per day (Fig. 2A). Indeed, eggs laid in ABC169 possessed a lateral spine typical of *S.*
148 *mansoni* eggs, had smooth shells, and contained a "germinal disc" corresponding to the early
149 embryo (Fig. 2B). Eggs freshly laid in ABC169 media were larger on average than those laid in
150 BM169 (Fig. 2C) and contained similar numbers of nuclei as eggs laid by parasites freshly
151 recovered from mice (Fig. 2D). Due to a high concentration of phenolic proteins that originate in
152 the vitelline droplets that form the eggshell, schistosome eggshells are highly autofluorescent
153 (20). In contrast to the egg-like masses from BM169, where parasites produce few vitelline
154 droplets (Fig. 1C), eggs from parasites in ABC169 possessed autofluorescence comparable to
155 those from parasites freshly *ex vivo* (Fig. 2E). Furthermore, nearly half of the eggs laid by
156 parasites at D18-20 of culture in ABC169 contained clusters of proliferative embryonic cells
157 visualized by labeling with thymidine analog EdU (Fig. 2F, G). *S. mansoni* eggs are passed from
158 the host to release larvae called miracidia (2). Approximately 10-20% of eggs laid on the first
159 day cultured either in BM169 or ABC169 produced miracidia (Fig. S2). However, this rate
160 dropped during time in culture (Fig. S2). While eggs laid in BM169 after D7 were incapable of
161 liberating miracidia, about 1-2% of eggs laid in ABC169 produced viable and morphologically
162 normal miracidia (Fig. 2H, Movie S1-2). Given the disparity between the rate of entering
163 embryonic development (Fig. 2G) and the capacity for these embryos to mature to miracidia (Fig.
164 S2), it is possible that cues from the host are necessary for efficient development of *S. mansoni*
165 embryos to miracidia.

166 **Immature virgin females sexually mature and produce viable eggs upon pairing with male**
167 **worms in ABC169 medium.** Studying the process by which male schistosomes stimulate female
168 development has traditionally been challenging since females experience incomplete
169 development following pairing with a male *in vitro* (31, 32). To examine sexual maturation in
170 ABC169, we recovered immature virgin females from mice and paired these females with
171 sexually mature virgin male worms. The vitellaria of paired immature females grown in BM169
172 were poorly developed (Fig. 3A) and these parasites laid small numbers of morphologically
173 abnormal eggs (Fig. 3B). However, the vitellaria of newly paired immature virgin females
174 cultured in ABC169 developed normal vitellaria (Fig. 3A) and these parasites were capable of
175 laying morphologically normal eggs beginning between D6-D7 of culture (Fig. 3B-C). These
176 eggs could initiate embryogenesis (Fig. 3D, 42.7% n=1010 from D16 to D22) and about 2%
177 could develop to miracidia (eggs from D11-14, n=2807 eggs). Thus, ABC169 supports female
178 development following pairing with a male worm.

179 **Physical contact with a male worm is sufficient for female worms to produce viable**
180 **parthenogenetic haploid embryos.** Several theories have been put forward to explain the
181 mechanism by which male worms stimulate female development (2, 33-35). However, the
182 experiments supporting (or refuting) these hypotheses were conducted using sub-optimal culture

183 conditions (34) and in many cases have not been subject to extensive reproduction in the modern
184 literature. Thus, we were compelled to revisit key observations using ABC169. The prevailing
185 thought is that female development requires direct contact with a male worm (7, 34, 35). The
186 most intriguing study supporting this hypothesis are those of Popiel and Basch (32). These
187 authors observed that small segments of male worms could stimulate vitelline development.
188 Interestingly, vitelline development was confined to regions in direct contact with the male
189 segment (32). Consistent with these observations, we found a large fraction of small posterior
190 fragments could pair with immature females (Fig. 4A,B, Movie S3). These posterior segments
191 often paired with posterior regions of female worms, and consistent with observations of Popiel
192 and Basch, vitelline maturation occurred only in regions in direct contact the male segment (Fig.
193 4B). Thus, pairing with a male induces a signal that induces localized female vitelline maturation.

194 While culturing male posterior segments with female worms we observed that these
195 female worms laid morphologically normal eggs (Fig. 4C). However, examination of these eggs
196 found that they contained no embryos capable of incorporating EdU (Fig. 4C). To explore this
197 observation in more detail we examined the ovaries of the female worms paired with male
198 segments and found no evidence of mature oocyte production (Fig. 4D). Since the schistosome
199 ovary is located anterior to the vitellaria, and this region was not in contact with male posterior
200 segments, we reasoned that ovaries, like the vitellaria, might also require local contact with a
201 male worm to mature and begin oocyte production. To test this model, we amputated males
202 behind the testes (Fig. 4E) and paired the decapitated and castrated fragments with immature
203 female worms. Since these large posterior fragments typically ensheathed the entire female
204 worm (Fig. 4E), we reasoned that this pairing might be sufficient to stimulate oogenesis.
205 Consistent with this model, we found that ovaries of females paired with decapitated males
206 produced oocytes (Fig. 4F). Furthermore, these parasites had fully developed vitellaria along
207 their entire length (Fig. 4G) and laid morphologically normal eggs (Fig. 4H).

208 Despite the fact that females paired with castrated male segments had no chance of being
209 inseminated, we observed that eggs laid by these parasites possessed the ability to initiate
210 embryogenesis (Fig. 4H) and could even give rise to miracidia (0.23%, n=4704 eggs). Previous
211 studies have suggested that female schistosomes can produce parthenogenetic offspring
212 containing a haploid set of maternal chromosomes when mated with males of distantly related
213 schistosome species (36, 37). From these experiments it is not clear if simply coming into
214 contact with a male schistosome of another species is sufficient to induce the production of
215 parthenogenetic offspring or if parthenogenesis occurs only following the transfer of sperm (36,
216 37). Therefore, we examined the karyotypes of eggs laid by females paired with castrated males.
217 Unlike the diploid karyotypes of embryos from eggs laid by freshly *ex vivo* parasites (2n=16),
218 mitotic cells from eggs laid by females paired with castrated males were haploid containing only
219 8 chromosomes (Fig. 4I, Fig. S3). These results suggest that only contact with a male worm
220 along the entire length of the female body, and not insemination, is sufficient for the production
221 of viable haploid embryos. These data highlight the value of ABC169 media to enhance our
222 understanding of schistosome reproductive biology.

223 **RNA interference can be used to study schistosome reproduction *in vitro.*** Given the rapid
224 rate at which the vitellaria degenerate *in vitro*, the use of RNA interference to study sexual
225 reproduction is restricted to the first few days of culture (24, 38). This approach is not ideal since
226 the functional consequences of certain RNAi perturbations (39) may not manifest in the narrow
227 window before control parasites cease normal egg production. Therefore, we examined the

228 efficacy of RNAi using ABC169. The production of both oocytes and vitellocytes is dependent
229 on dedicated populations of stem cells: oogonia in the ovaries (40) and S1 cells in the vitellaria
230 (21). Despite the fact that oocyte and vitellocyte production depends on these stem cells there
231 are no direct studies of the function of either of these stem cell populations. Therefore, we
232 examined the effect of RNAi against *Histone H2B* that is expressed in both S1 cells (23) and
233 oogonia(41) and was previously reported to be essential for proliferative somatic stem cell
234 maintenance due presumably to its role in cell cycle progression (42). For these studies we
235 treated parasites with dsRNA and monitored egg production and the status of the reproductive
236 organs after 14 days of culture in ABC169. Consistent with studies in male worms, we observed
237 a complete ablation of somatic stem cells (54/54 female parasites). Similarly, we observed a
238 massive depletion of the EdU incorporation in the stem cell compartments of the vitellaria (50/54
239 parasites) and ovaries (48/54 parasites) (Fig. 5A). As anticipated, stem cell depletion led to a
240 loss of mature vitellocytes (Fig. 5B) and a substantial regression in the size of the ovary (Fig.
241 5A). Consistent with the effects of *Histone H2B* RNAi treatment on the reproductive organs we
242 noted a significant decline in the rate of egg production in *Histone H2B (RNAi)* worm pairs (Fig.
243 5C). Given these observations, we suggest the combination of ABC169 with approaches such as
244 RNAi will provide a platform to dissect schistosome reproductive biology on a molecular level.

245 **Conclusions**

246 During his extensive studies to develop *in vitro* culture conditions for *S. mansoni*, Paul
247 Basch lamented that the production of viable eggs *in vitro* "remains a formidable challenge" (19).
248 We find three additions to Basch's base media formulation (ascorbic acid, blood cells, and LDL)
249 can sustain the production of viable eggs for several weeks *in vitro*. Although ABC169 does not
250 fully replicate the *in vivo* reproductive potential of the parasite, it allows us to recapitulate
251 important aspects of schistosome sexual biology *in vitro* and thus represents a significant
252 improvement over existing culture methods. Taken together, the ability to sustain sexual maturity
253 *in vitro,* coupled with a growing understanding of the molecular programs associated with
254 distinct reproductive states (43, 44), and tools such as RNAi, will expedite our understanding of
255 the molecular factors governing parasite sexual development and egg production, perhaps
256 suggesting new therapeutic opportunities.
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262 **Materials and Methods**

263 **Parasites and *in vitro* maintenance.** Adult NMRI *Schistosoma mansoni* (6-7 weeks post
264 infection) were recovered from infected mice by perfusion through hepatic portal vein with 37°C
265 DMEM (Mediatech, Manassas, VA) plus 5% serum and heparin (200–350 U/ml). Single sex
266 infections were obtained by infecting mice with male or female cercariae recovered from NMRI
267 *Biomphalaria glabrata* snails infected with single miracidia. Following recovery from the host,
268 worms were rinsed several times in DMEM + 5% serum before placing into culture media. The
269 base medium for these studies was BM169 (18) with the addition of 1x Antibiotic-Antimycotic
270 (Gibco/Life Technologies, Carlsbad, CA). Although Basch included red blood cells in BM169, it
271 has been noted that erythrocytes, which schistosomes consume *in vivo*, are not sufficient to
272 sustain egg production (13, 19, 24) and were initially omitted from the base medium. We
273 explored sera from a variety of sources (horse, fetal bovine, chicken) and determined that
274 newborn calf serum (Sigma-Aldrich, St. Louis, MO) both performed well and was cost efficient.
275 For ABC169, BM169 was supplemented with 200 µM ascorbic acid (Sigma-Aldrich, St. Louis,
276 MO), 0.2% V/V bovine washed red blood cells (10% suspension, Lampire Biological
277 Laboratories, Pipersville, PA) and 0.2% V/V Porcine cholesterol concentrate (Rocky Mountain
278 Biologicals, Missoula, MT). Ascorbic acid was added from a 500 mM stock dissolved in BM169
279 that was stored at 4°C in the dark for less than 2 weeks. For regular maintenance, 4-6 worm pairs
280 were cultured at 37°C in 5% CO_2 in 3 ml media in a 12-well plate; media was changed every
281 other day. To count egg-laying rates, individual worm pairs were maintained in 24 well plates in
282 1 ml of media and eggs were removed for counting every 24 h during a media change. Worms
283 that separated or became ill during cultivation were excluded from analysis.

284 **Molecular biology.** For quantification of gene expression, RNA was reverse transcribed (iScript,
285 Biorad) and Quantitative PCR was performed using an Applied Biosystems Quantistudio3
286 instrument and PowerUp SYBR Green Master mix (ThermoFisher, Carlsbad, CA). Gene
287 expression was normalized to the expression of a proteasome subunit (Smp_056500) and relative
288 gene expression values and statistical analyses were performed using the Quantistudio Design
289 and Analysis software (ThermoFisher, Carlsbad, CA). The heatmap depicting relative gene
290 expression was generated using R. Oligonucleotide sequences were listed in Dataset S1.

291 **Parasite labeling and imaging.** Whole-mount *in situ* hybridization was performed as previously
292 described (23, 41). ImageJ was used to quantify egg size, autofluorescence, and nuclei number
293 from images of eggs acquired on a Nikon A1+ laser scanning confocal microscope. Like
294 previously described diazo salts (45, 46), Fast Blue BB strongly labeled the polyphenol rich
295 vitelline droplets of mature vitellocytes and could be visualized as both by bright field and
296 fluorescence microscopy. For Fast Blue BB staining, female worms were separated from males
297 using 0.25% tricaine in BM169 and fixed for 4 hrs in 4% Formaldehyde in PBS+0.3% Triton X-
298 100 (PBSTx). Parasites were washed in PBSTx for 10 mins, stained in freshly-made filtered 1%
299 Fast Blue BB in PBSTx for 5 mins and rinsed in PBSTx 3 times. Worms were incubated with 1
300 µg/ml DAPI in PBSTx for 2hrs, cleared in 80% glycerol in PBS and mounted on slides. For Fast
301 Blue BB/BODIPY 493/503 staining, parasites were processed similarly expect detergents were
302 omitted from all buffers; parasites were labeled with 1 µg/ml BODIPY 493/503 (ThermoFisher,
303 Carlsbad, Ca) in PBS for 1hr following Fast Blue BB staining. For EdU labeling, eggs within 24-
304 48 hours of being laid were incubated in Medium 199 supplemented with 10 µM 5-Ethynyl-2′-
305 deoxyuridine (EdU) and incubated overnight. The following day the eggs were collected by
306 centrifugation at 10,000g for 1min, fixed in 4% formaldehyde in PBSTx for 4hrs, EdU was

307 detected as previously described (41), and washed in PBSTx. *in situ* hybridizations and
308 colormetic detection of Fast Blue BB were imaged using a Zeiss AxioZoom V16 equipped with
309 a transmitted light base and a Zeiss AxioCam 105 Color camera. All other images were acquired
310 using a Nikon A1+ laser scanning confocal microscope.

311 **Egg culture, miracidia hatching, and labeling.** Eggs were collected on 10 µm cell strainer
312 rinsed into Medium 199 (Corning) containing 10% Fetal Bovine Serum and 1x Antibiotic-
313 Antimycotic (Gibco/Life Technologies, Carlsbad, CA) for 7 days at 37°C in 5% CO_2. After
314 culture, eggs were collected on 10 µm cell strainer and rinsed with artificial pond water 1-2 times
315 and placed in artificial pond water under light. A small aliquot of eggs was counted to determine
316 the total number of eggs examined. Active miracidia were observed and counted under light
317 microscopy every 30 mins for up to 4hrs. Miracidia fixation and labeling was performed as
318 previously described (47).

319 **Karyotype analysis**
320 Karyotypes from schistosome embryos were determined using a modified version of a previously
321 published method (48). Briefly, eggs were incubated in M199 for two days after deposition and
322 then incubated in 5 µM Nocodazole for 1 hour at 37°C. Eggs were pelleted at 20,000 g for 1 min,
323 rinsed in DI water, pelleted, resuspended in 1ml of water, and incubated for 20 min at RT.
324 Following centrifugation, the pelleted eggs were resuspended in 1 ml of fixative (3:1
325 methanol:acetic acid) for 15 minutes. Eggs were again pelleted and resuspended in 100 µl of
326 fixative and the eggshells were disrupted using a Kontes pellet pestle (DWK Life Sciences,
327 Rockwood, TN). The disrupted eggshells were allowed to settle for 10 min, the supernatant was
328 collected, and centrifuged for 8 min at 240 g. All but ~20 µl of supernatant was removed and the
329 remaining liquid was pipetted dropwise on to a glass slide (Superfrost Plus, ThermoFisher,
330 Carlsbad, CA) freshly dipped in PBS. After air-drying chromosomes were labeled with
331 Vectashield containing DAPI (Vector Laboratories, Burlingame, CA) and imaged on a Nikon
332 A1+ laser scanning confocal microscope with a 60x/1.4 NA objective.
333
334 **RNA Interference**
335 Double stranded RNA was generated as previously described (41, 49). For dsRNA treatment
336 worm pairs were electroporated with a square-wave pulse (125 V for 20 ms (50)) on a BTX
337 Gemini in 4 mm cuvettes with 160 µg of dsRNA in 400 µl of DMEM. The worms were then
338 supplemented with 60 µg/ml of dsRNA on day 0, 1, 2, 6 and 10. At D14, parasites were pulsed
339 for 4 hours with EdU and processed as previously described (41). Eggs laid between D12 to D14
340 were collected to determine egg-laying rates.
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343 **ACKNOWLEDGMENTS.** We dedicate this manuscript to the memory of Paul Basch. We
344 thank Melanie Cobb, Michael Reese and members of Collins laboratory for comments on the
345 manuscript. Mice and *B. glabrata* snails were provided by the NIAID Schistosomiasis Resource
346 Center of the Biomedical Research Institute (Rockville, MD) through NIH-NIAID Contract
347 HHSN272201000005I for distribution through BEI Resources. The work was supported by the
348 National Institutes of Health (R01 R01AI121037), the Wellcome Trust (107475/Z/15/Z) and the
349 Welch Foundation (I-1948-20180324).
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468
469

470 **FIGURE LEGENDS**
471
472 **Fig. 1. ABC169 supports the maintenance of schistosome vitellaria.**
473 (A) Morphological changes of eggs laid by worm pairs maintained in BM169. After D6 parasites
474 began laying abnormally formed eggs (arrow). These eggs were small, usually did not contain a
475 lateral spine, and did not possess a smooth surface.
476 (B) Fast Blue BB staining (brownish-red labeling) showing loss of mature vitellocytes during
477 culture in BM169. Representative images from three experiments with n > 10 parasites.
478 (C) Confocal slice showing Fast Blue BB and BODIPY labeling in the vitellaria of freshly
479 recovered parasites and in schistosomes at D20 of cultivation in BM169 supplemented with
480 RBCs (Red Blood Cells), LDL, and/or ascorbic acid. Representative images from three
481 experiments with n > 10 parasites.
482 (D) Whole-mount *in situ* hybridization showing expression of vitellaria-enriched genes in freshly
483 perfused female worms and parasites cultured in ABC169 or BM169 for 20 days. Representative
484 images from three experiments with n ≥ 9 parasites.
485 (E) Heat-map showing relative expression of vitellaria-enriched transcripts in freshly perfused
486 female worms ("Fresh") and parasites cultured in ABC169 or BM169 for 20 days. Each column
487 represents an independent biological replicate; samples are normalized to the expression of an
488 arbitrarily chosen biological replicate from the BM169 group. Changes in expression between
489 BM169 and ABC169 were statistically significant ($p<0.05$, T-test).
490 (F) Morphology of eggs laid by paired adult females in medium ABC169 supplemented with L-
491 ascorbic acid or D-isoascorbic acid on D20. Representative of 3 experiments.
492 Scale bars: A, B, D, F 100µm; C 25µm.
493
494 **Fig. 2. Eggs laid in ABC169 develop and hatch.**
495 (A) Rate of egg production per worm pair in BM169 or medium ABC169 during *in vitro* culture.
496 Dotted line indicates the point at which parasites beginning laying morphologically abnormal
497 eggs. n ≥ 19 worm pairs examined in 4 experiments. Error bars represent 95% confidence
498 intervals.
499 (B) Morphology of eggs (Left, bright field; Right, DIC) laid by paired adult females in BM169
500 or medium ABC169 on D20. Red arrows show early embryos in eggs laid in ABC169.
501 (C-E) Quantification of the (C) size, (D) number of DAPI-labeled nuclei, and (E)
502 autofluorescence intensity from eggs laid by freshly perfused female worms (Fresh, n=41),
503 parasites cultured in BM169 (n=61), or ABC169 (n=59) on D20. **** $p<0.0001$, T-Test. Error
504 bars represent 95% confidence intervals.
505 (F) EdU-labeled embryonic cells of an egg laid by a paired adult female in ABC169 on D20.
506 (G) Percentage of eggs with clusters of cycling EdU^+ embryonic cells from eggs laid between
507 D16 and D22 by paired adult females in BM169 or ABC169. >2000 eggs from 6 experiments.
508 Error bars represent 95% confidence interval.
509 (H) Miracidia from eggs laid in ABC169. Left, miracidium (pseudocolored red) inside an egg
510 laid on D20 of culture in ABC169. Right, hatched miracidium from D20 egg labeled with DAPI
511 and phalloidin. These miracidia appear grossly normal in morphology possessing 2 pairs of
512 flame cells (arrows).
513 Scale bars: B, F, H 20 µm; C-E 50 µm.
514

515 **Fig. 3. ABC169 supports the maturation of immature female schistosomes following**
516 **pairing with a male worm.**
517 (A)Vitellaria development visualized by Fast Blue BB labeling in immature female schistosomes
518 in the presence or absence of male worms in BM169 or ABC169 at D20 of culture. Black
519 pigment in ABC169 parasites represents digested RBCs.
520 (B) Eggs laid by immature female in BM169 or ABC169 on day 20 after pairing with a male.
521 (C) Proportion of females laying eggs (left axis, magenta) and number of eggs laid per worm pair
522 (right axis, green) from D1 to D20 following pairing of immature females with male worms in
523 ABC169. n=31 worm pairs examined in 4 separate experiments. Error bars represent 95%
524 confidence intervals.
525 (D) Eggs laid by previously immature females commence embryonic development. Left,
526 representative developing EdU-labeled embryo from egg laid in ABC169 on D20.
527 Scale bars: A,B 100 µm; D 20 µm.
528
529 **Fig. 4. Using ABC169 to study schistosome male-induced female sexual maturation.**
530 (A) Pairing of immature females with amputated male segments. Left, cartoon showing
531 approximate positions of male amputations. Most often we observed posterior segments pairing
532 with posterior region of immature females, shown to right.
533 (B) Fast blue BB labeling showing female vitellaria development after pairing with posterior
534 male segments. Left panels, female after separation from male segment showing vitellaria
535 development in posterior region. Right panel, female before separation from male segment
536 showing vitellaria development is restricted to paired region. Representative of > 40 female
537 parasites examined in 4 separate experiments.
538 (C) Eggs laid by immature female worms paired with male segments are morphologically normal
539 (left) but do not contain developing embryos as measured by EdU labeling (right). n > 500 eggs
540 examined in 4 separate experiments.
541 (D) DAPI staining to examine ovary development in immature females paired with intact male
542 worms or an amputated posterior male segment. Females paired with intact males possess
543 differentiated oocytes (DO) (n=23), whereas females paired with male segments produce no
544 differentiated oocytes (n=41).
545 (E) Pairing of immature females with decapitated and castrated male segments. Left, cartoon
546 showing approximate positions of amputation, removing both the head and testes. These
547 decapitated and castrated segments paired with female along most of the female body, shown to
548 right.
549 (F) DAPI staining to examine ovary development in immature females paired with decapitated
550 and castrated male segments. These ovaries contain differentiated oocytes (DO). Representative
551 images from n = 22 parasites examined in 3 separate experiments.
552 (G) Fast Blue BB showing vitellaria development in females after pairing with decapitated and
553 castrated male segments. Representative images n=22 parasites from 3 separate experiments.
554 (H) EdU labeling showing embryonic development in eggs from females paired with decapitated
555 and castrated male segments. 273/602 eggs laid between D16-D22 contained clusters of EdU^+
556 cells.
557 (I) DAPI labeling of metaphase spreads from eggs laid by (left) fresh *ex vivo* female parasites
558 paired with intact males and (right) immature female parasites paired with decapitated and
559 castrated male segments. Embryonic cells from fresh *ex vivo* parasites were diploid (2n=16),
560 whereas those from unfertilized females are haploid (n=8).

561 Scale bars: A, B 500 µm; C, D, F, H 50 µm, G 100 µm, I 10 µm.
562
563 **Fig. 5. RNAi mediated stem cell depletion leads to regression of the female reproductive**
564 **organs and cessation of egg production.**
565 (A) EdU labeling of proliferative cells following control or *Histone H2B* RNAi treatment.
566 *Histone H2B(RNAi)* animals possess few EdU-labeled nuclei. Insets show enlarged views of
567 ovaries. Vitellaria are positioned to the right and white arrow indicates the proliferative stem
568 cells in the vitellaria. Representative images from n > 35 parasites for each treatment examined
569 in 6 separate experiments. White arrow indicates the vitellaria.
570 (B) Fast blue labeling following control or *Histone H2B* RNAi treatment. *Histone H2B(RNAi)*
571 parasites produce few vitellocytes. Representative images from n > 50 parasites for each
572 treatment examined in 6 separate experiments.
573 (C)Plot showing egg production per worm pair between D12-D14 of RNAi treatment. Error bars
574 represent 95% confidence intervals. Representative images from n >100 parasites for each
575 treatment examined in 6 separate experiments. **** $p<0.0001$, T-Test. Error bars represent 95%
576 confidence intervals.
577 Scale bars: 50 µm.
578

Fig. 1

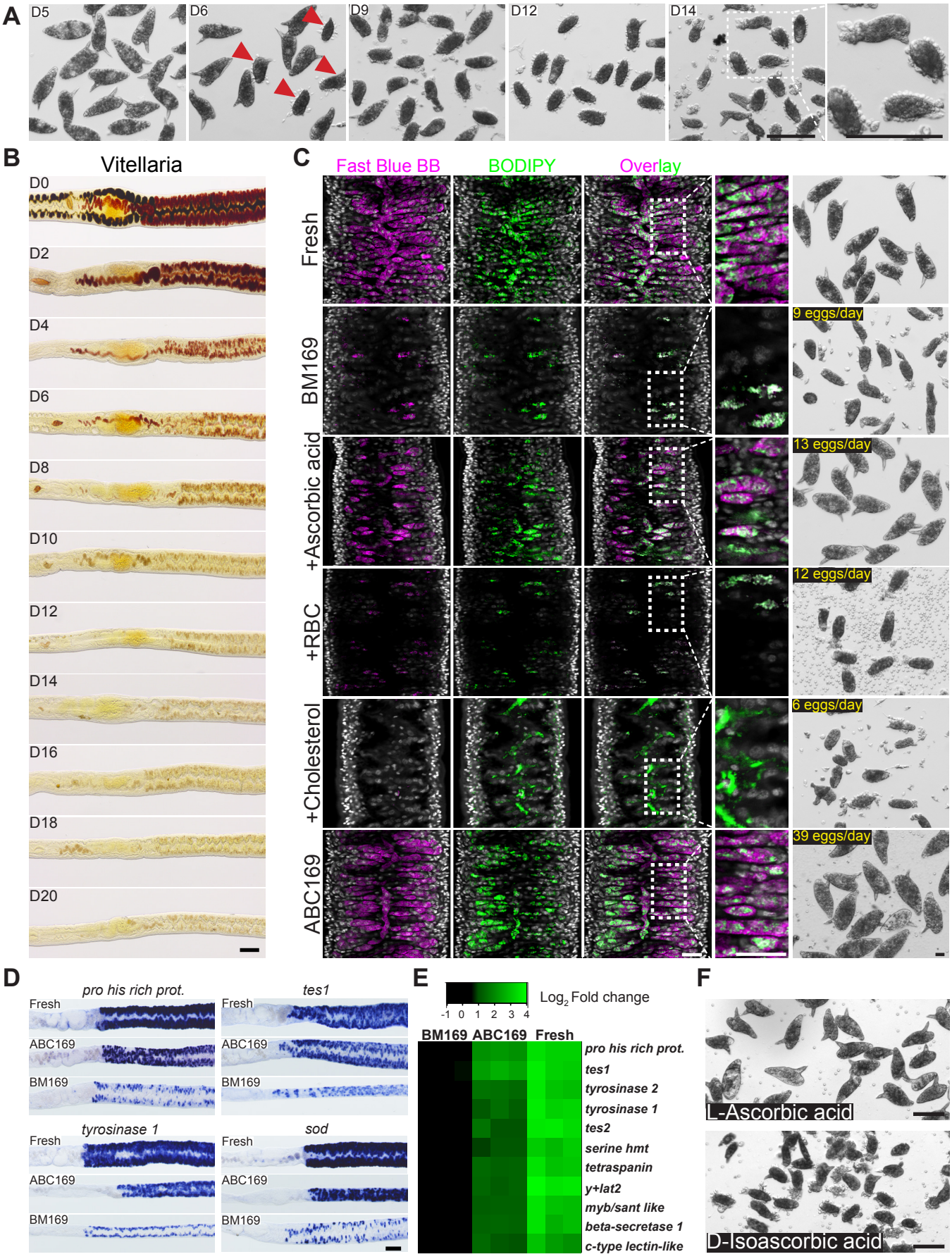

A
D5
D6
D9
D12
D14
B
Vitellaria
D0
D2
D4
D6
D8
D10
D12
D14
D16
D18
D20
C
Fast Blue BB
BODIPY
Overlay
Fresh
BM169
+Ascorbic acid
+RBC
+Cholesterol
ABC169
9 eggs/day
13 eggs/day
12 eggs/day
6 eggs/day
39 eggs/day
D
pro his rich prot.
tes1
tyrosinase 1
sod
Fresh
ABC169
BM169
E
Log2 Fold change
-1 0 1 2 3 4
BM169 ABC169 Fresh
pro his rich prot.
tes1
tyrosinase 2
tyrosinase 1
tes2
serine hmt
tetraspanin
y+lat2
myb/sant like
beta-secretase 1
c-type lectin-like
F
L-Ascorbic acid
D-Isoascorbic acid

Fig. 2

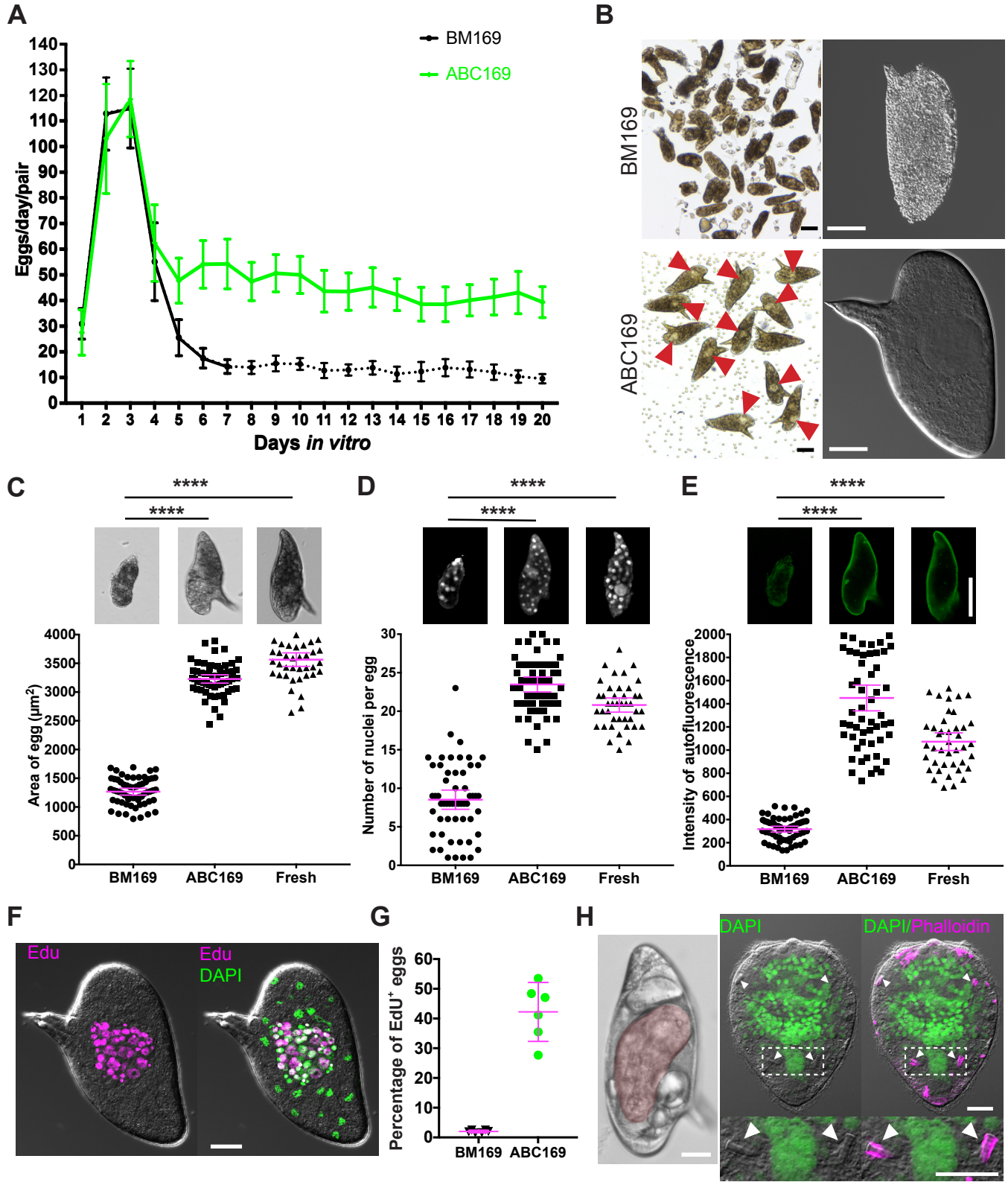

A
BM169
ABC169
Eggs/day/pair
Days in vitro
B
BM169
ABC169
C
****
****
Area of egg (μm²)
BM169
ABC169
Fresh
D
****
****
Number of nuclei per egg
BM169
ABC169
Fresh
E
****
****
Intensity of autofluorescence
BM169
ABC169
Fresh
F
Edu
Edu
DAPI
G
Percentage of EdU⁺ eggs
BM169
ABC169
H
DAPI
DAPI/Phalloidin

Fig. 3

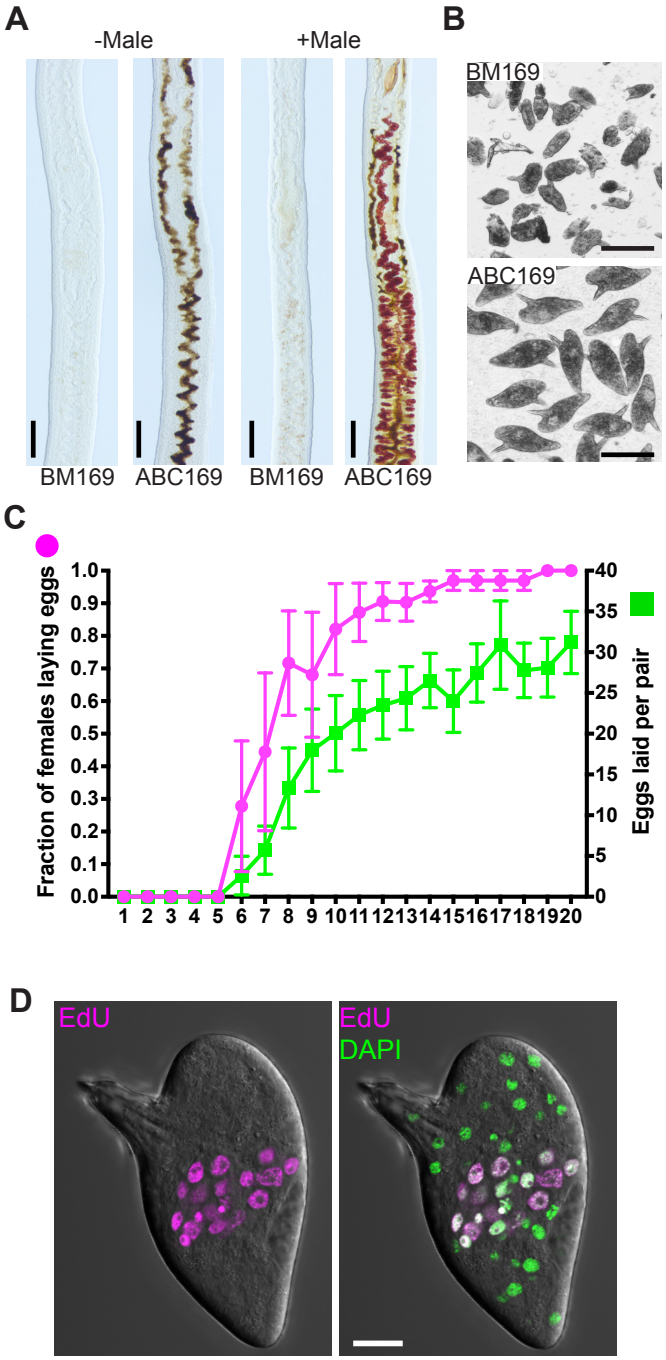

A
-Male
+Male
BM169
ABC169
BM169
ABC169
B
BM169
ABC169
C
Fraction of females laying eggs
Eggs laid per pair
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
40
35
30
25
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
D
EdU
EdU
DAPI

Fig. 4

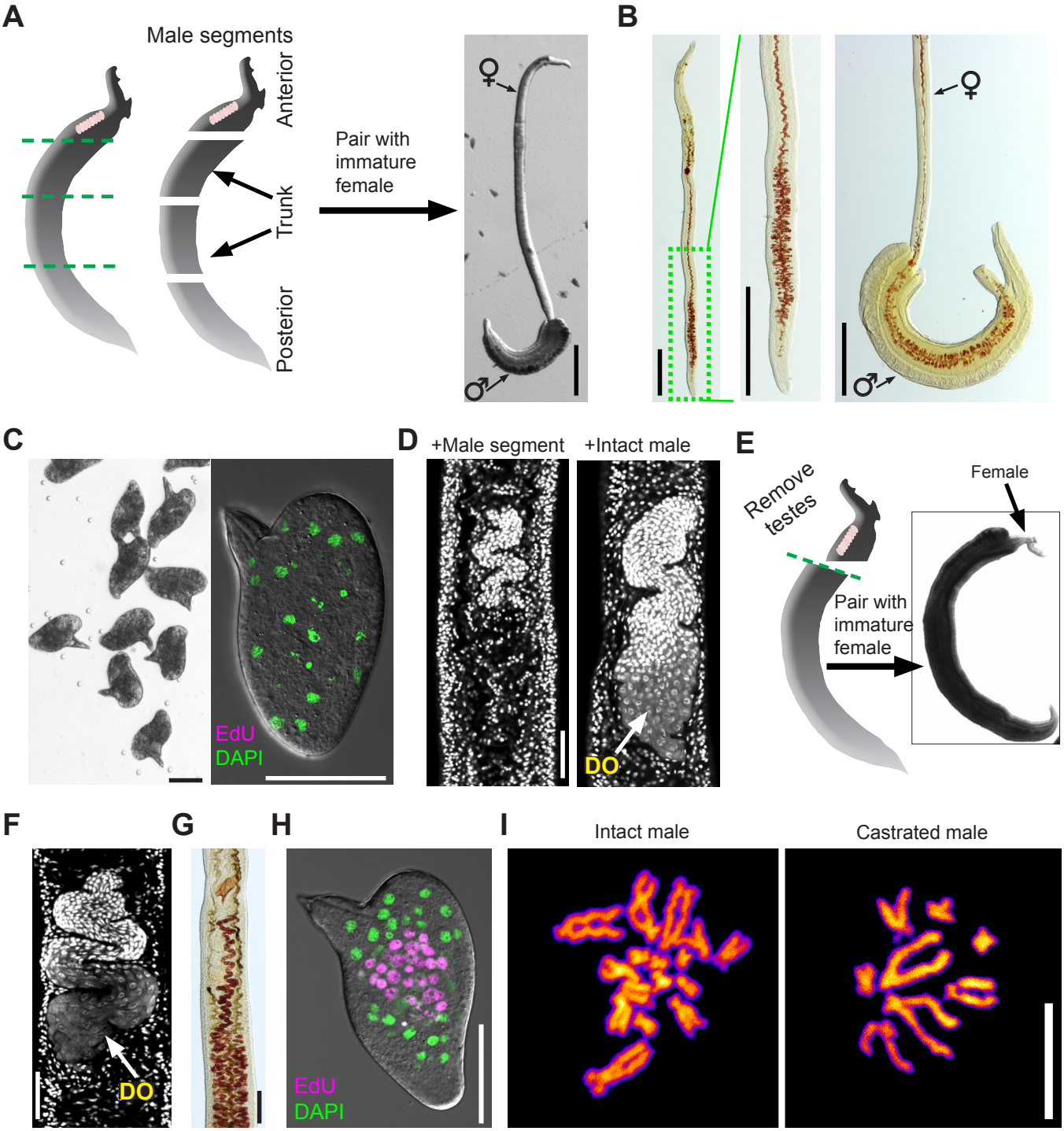
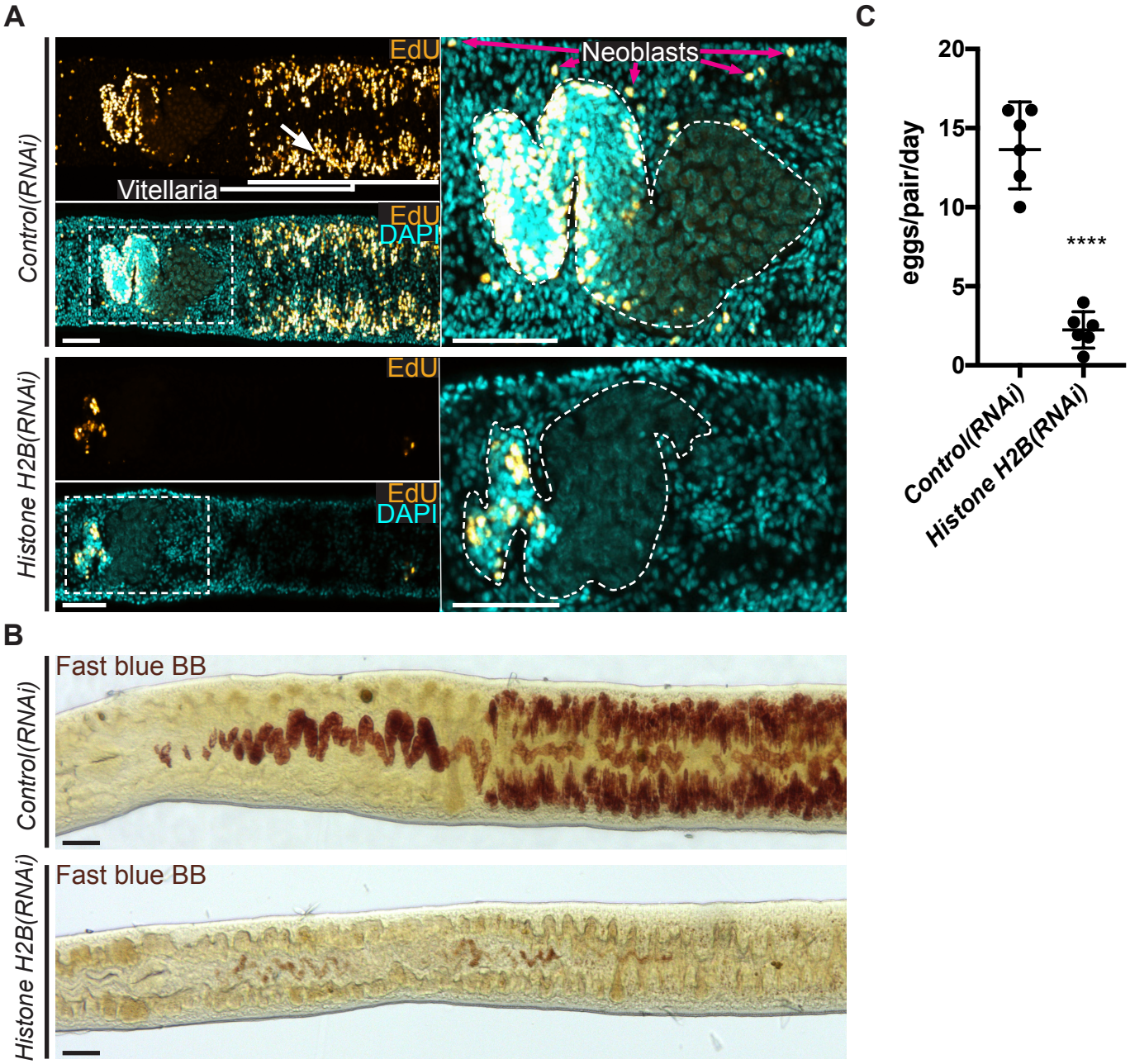

A
Male segments
Anterior
Trunk
Posterior
Pair with
immature
female
B
C
EdU
DAPI
D
+Male segment
+Intact male
DO
E
Remove
testes
Female
Pair with
immature
female
F
DO
G
H
EdU
DAPI
I
Intact male
Castrated male

Fig. 5


A
Control(RNAi)
Histone H2B(RNAi)
EdU
Vitellaria
EdU
DAPI
Neoblasts
B
Fast blue BB
C
eggs/pair/day
20
15
10
5
0
****
Control(RNAi)
Histone H2B(RNAi)

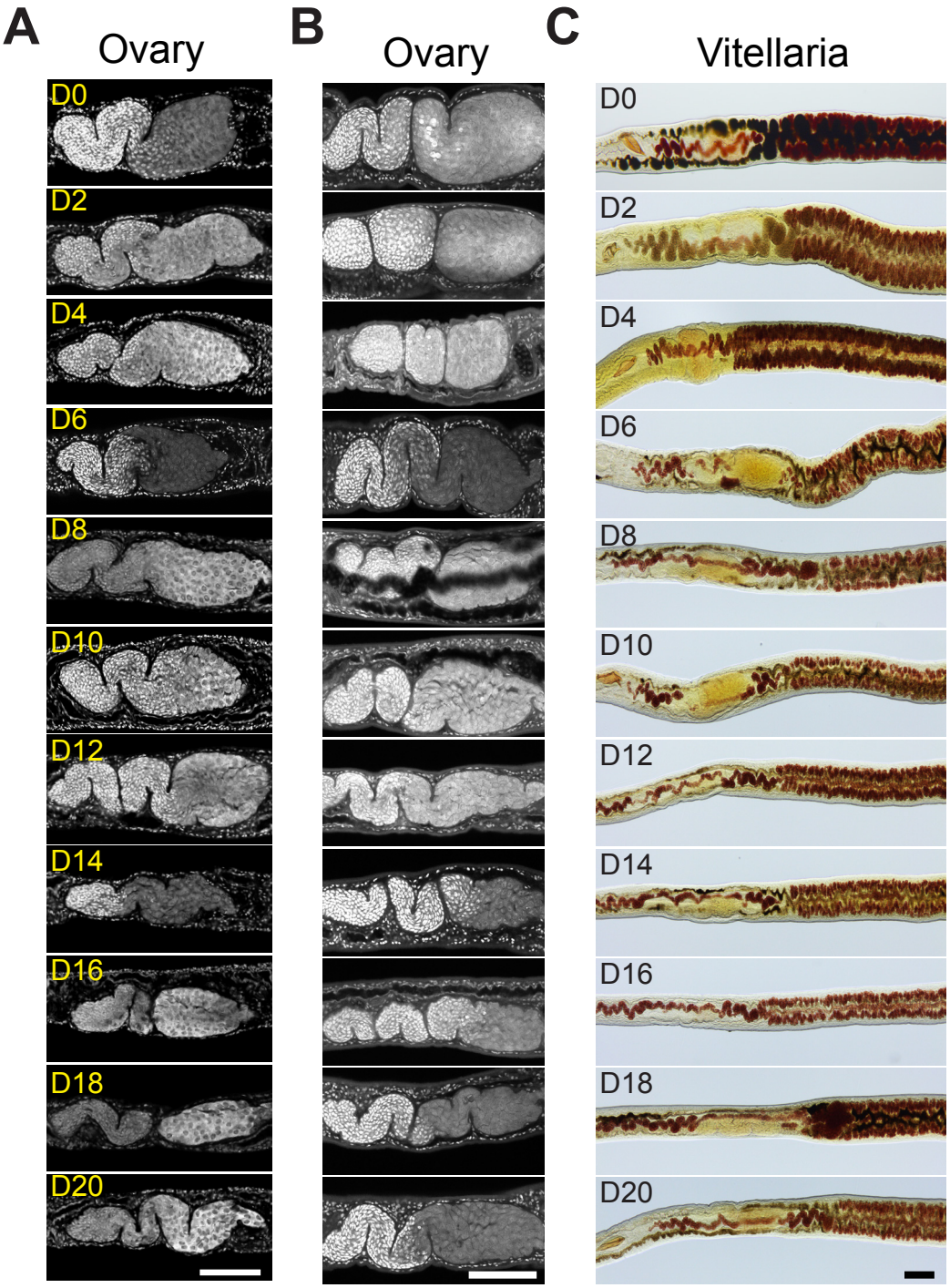


Fig. S1. Reproductive changes of paired adult female parasites during *in vitro* culture. Ovaries of females in (**A**) BM169 or (**B**) ABC169 between D0 to D20 of *in vitro* culture labeled with DAPI. Differentiated oocytes are present in the posterior regions (right) of ovaries during in vitro culture regardless of culture condition. (**C**) Fast Blue BB labeling showing the maintenance of mature vitellocytes during culture in BM169. Representative images from three biological replicates with n > 10 parasites. Scale bars: 100 µm.

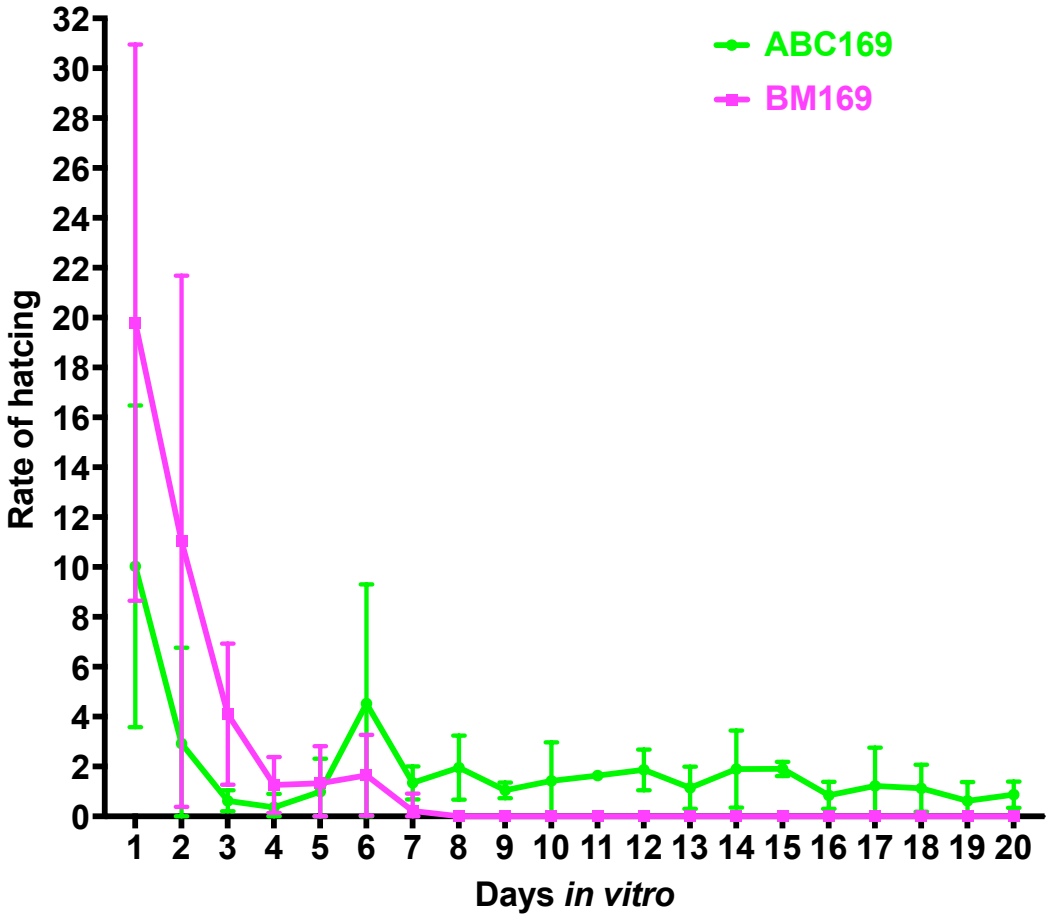


Fig. S2. **Hatching ratio of eggs laid on different days by worm pairs cultured in BM169 or ABC169.** Data points represent mean values from 4 independent experiments. Error bars represent 95% confidence intervals.

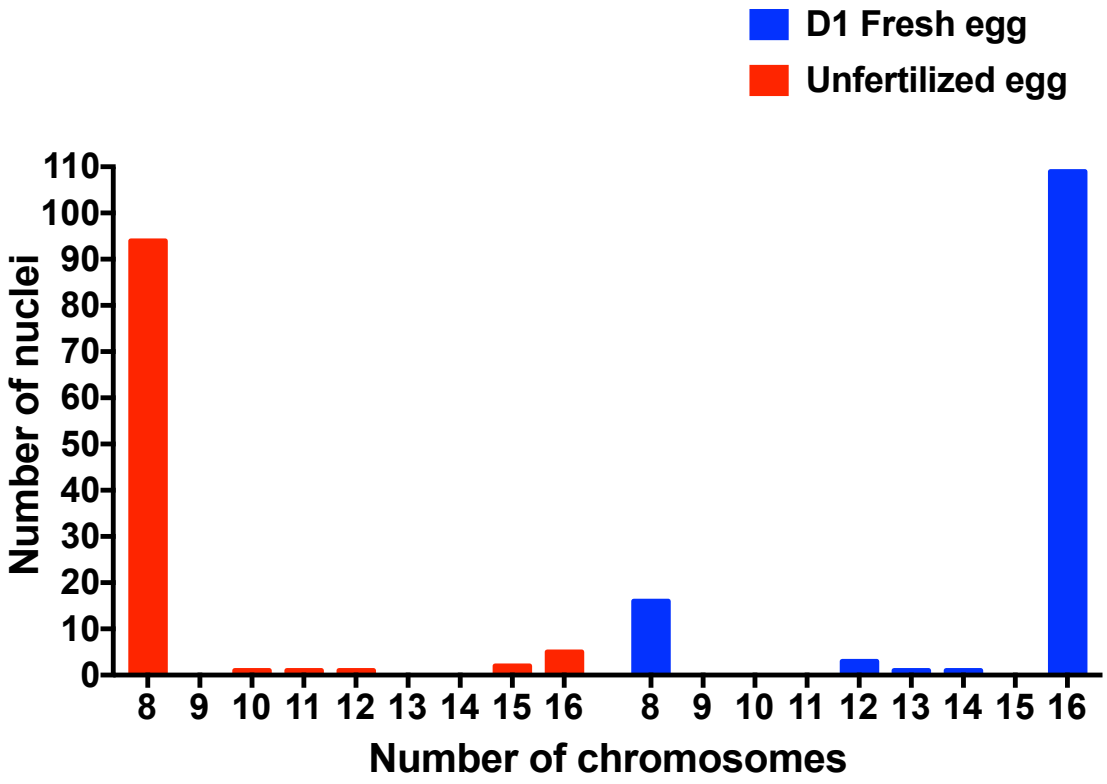


Fig. S3. Plot showing number of chromosomes from karyotypes obtained from eggs laid by freshly perfused worm pairs on D1 of culture ("D1 Fresh eggs", blue) or females paired with decapitated and castrated male segments ("unfertilized eggs", red)

Movie S1. Miracidium inside egg laid on D20 of culture in ABC169.

Movie S2. Miracidia after hatching from eggs laid on D20 of culture in ABC169.

Movie S3. Previously immature female paired with a male segment in ABC169.

Additional Dataset S1 (separate file). Oligonucleotide sequences for cDNA cloning and qRT-PCR analyses.