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

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

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

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

In vertebrate cells, L-ascorbic acid acts not only as an antioxidant, but an essential cofactor for a variety of enzymes (29). Mammals deficient for L-ascorbic acid cannot perform key enzymatic reactions (e.g., collagen hydroxylation) leading to symptoms commonly known as scurvy (29). Interestingly, the effects of vitamin C to prevent scurvy are stereoselective as D-isoascorbic acid cannot replace L-ascorbic acid at equimolar concentrations (30). Similarly, we
observed that D-isoascorbic acid could not replace L-ascorbic acid in egg production (Fig. 1F),
suggesting that either L-ascorbic acid is selectively transported into cells or that it acts in a
stereoselective fashion to facilitate one or more enzymatic reactions within the cell. Future
studies aimed at determining the function of vitamin C in schistosome cells could suggest new
approaches to blunt egg production.

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

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

Physical contact with a male worm is sufficient for female worms to produce viable
parthenogenetic haploid embryos. Several theories have been put forward to explain the
mechanism by which male worms stimulate female development (2, 33-35). However, the
experiments supporting (or refuting) these hypotheses were conducted using sub-optimal culture
conditions (34) and in many cases have not been subject to extensive reproduction in the modern literature. Thus, we were compelled to revisit key observations using ABC169. The prevailing thought is that female development requires direct contact with a male worm (7, 34, 35). The most intriguing study supporting this hypothesis are those of Popiel and Basch (32). These authors observed that small segments of male worms could stimulate vitelline development. Interestingly, vitelline development was confined to regions in direct contact with the male segment (32). Consistent with these observations, we found a large fraction of small posterior fragments could pair with immature females (Fig. 4A,B, Movie S3). These posterior segments often paired with posterior regions of female worms, and consistent with observations of Popiel and Basch, vitelline maturation occurred only in regions in direct contact the male segment (Fig. 4B). Thus, pairing with a male induces a signal that induces localized female vitelline maturation.

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

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

**RNA interference can be used to study schistosome reproduction in vitro.** Given the rapid rate at which the vitellaria degenerate in vitro, the use of RNA interference to study sexual reproduction is restricted to the first few days of culture (24, 38). This approach is not ideal since the functional consequences of certain RNAi perturbations (39) may not manifest in the narrow window before control parasites cease normal egg production. Therefore, we examined the
efficacy of RNAi using ABC169. The production of both oocytes and vitellofocytes is dependent on dedicated populations of stem cells: oogonia in the ovaries (40) and S1 cells in the vitellaria (21). Despite the fact that oocyte and vitellofocyte production depends on these stem cells there are no direct studies of the function of either of these stem cell populations. Therefore, we examined the effect of RNAi against Histone H2B that is expressed in both S1 cells (23) and oogonia (41) and was previously reported to be essential for proliferative somatic stem cell maintenance due presumably to its role in cell cycle progression (42). For these studies we treated parasites with dsRNA and monitored egg production and the status of the reproductive organs after 14 days of culture in ABC169. Consistent with studies in male worms, we observed a complete ablation of somatic stem cells (54/54 female parasites). Similarly, we observed a massive depletion of the EdU incorporation in the stem cell compartments of the vitellaria (50/54 parasites) and ovaries (48/54 parasites) (Fig. 5A). As anticipated, stem cell depletion led to a loss of mature vitellofocytes (Fig. 5B) and a substantial regression in the size of the ovary (Fig. 5A). Consistent with the effects of Histone H2B RNAi treatment on the reproductive organs we noted a significant decline in the rate of egg production in Histone H2B (RNAi) worm pairs (Fig. 5C). Given these observations, we suggest the combination of ABC169 with approaches such as RNAi will provide a platform to dissect schistosome reproductive biology on a molecular level.

Conclusions

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

Parasites and in vitro maintenance. Adult NMRI Schistosoma mansoni (6-7 weeks post infection) were recovered from infected mice by perfusion through hepatic portal vein with 37°C DMEM (Mediatech, Manassas, VA) plus 5% serum and heparin (200–350 U/ml). Single sex infections were obtained by infecting mice with male or female cercariae recovered from NMRI Biomphalaria glabrata snails infected with single miracidia. Following recovery from the host, worms were rinsed several times in DMEM + 5% serum before placing into culture media. The base medium for these studies was BM169 (18) with the addition of 1x Antibiotic-Antimycotic (Gibco/Life Technologies, Carlsbad, CA). Although Basch included red blood cells in BM169, it has been noted that erythrocytes, which schistosomes consume in vivo, are not sufficient to sustain egg production (13, 19, 24) and were initially omitted from the base medium. We explored sera from a variety of sources (horse, fetal bovine, chicken) and determined that newborn calf serum (Sigma-Aldrich, St. Louis, MO) both performed well and was cost efficient. For ABC169, BM169 was supplemented with 200 μM ascorbic acid (Sigma-Aldrich, St. Louis, MO), 0.2% V/V bovine washed red blood cells (10% suspension, Lampire Biological Laboratories, Pipersville, PA) and 0.2% V/V Porcine cholesterol concentrate (Rocky Mountain Biologicals, Missoula, MT). Ascorbic acid was added from a 500 mM stock dissolved in BM169 that was stored at 4°C in the dark for less than 2 weeks. For regular maintenance, 4-6 worm pairs were cultured at 37°C in 5% CO2 in 3 ml media in a 12-well plate; media was changed every other day. To count egg-laying rates, individual worm pairs were maintained in 24 well plates in 1 ml of media and eggs were removed for counting every 24 h during a media change. Worms that separated or became ill during cultivation were excluded from analysis.

Molecular biology. For quantification of gene expression, RNA was reverse transcribed (iScript, Biorad) and Quantitative PCR was performed using an Applied Biosystems Quantstudio3 instrument and PowerUp SYBR Green Master mix (ThermoFisher, Carlsbad, CA). Gene expression was normalized to the expression of a proteasome subunit (Smp_056500) and relative gene expression values and statistical analyses were performed using the Quantstudio Design and Analysis software (ThermoFisher, Carlsbad, CA). The heatmap depicting relative gene expression was generated using R. Oligonucleotide sequences were listed in Dataset S1.

Parasite labeling and imaging. Whole-mount in situ hybridization was performed as previously described (23, 41). ImageJ was used to quantify egg size, autofluorescence, and nuclei number from images of eggs acquired on a Nikon A1+ laser scanning confocal microscope. Like previously described diazo salts (45, 46), Fast Blue BB strongly labeled the polyphenol rich vitelline droplets of mature vitellocytes and could be visualized as both by bright field and fluorescence microscopy. For Fast Blue BB staining, female worms were separated from males using 0.25% tricaine in BM169 and fixed for 4 hrs in 4% Formaldehyde in PBS+0.3% Triton X-100 (PBSTx). Parasites were washed in PBSTx for 10 mins, stained in freshly-made filtered 1% Fast Blue BB in PBSTx for 5 mins and rinsed in PBSTx 3 times. Worms were incubated with 1 μg/ml DAPI in PBSTx for 2hrs, cleared in 80% glycerol in PBS and mounted on slides. For Fast Blue BB/BODIPY 493/503 staining, parasites were processed similarly expect detergents were omitted from all buffers; parasites were labeled with 1 μg/ml BODIPY 493/503 (ThermoFisher, Carlsbad, Ca) in PBS for 1hr following Fast Blue BB staining. For EdU labeling, eggs within 24-48 hours of being laid were incubated in Medium 199 supplemented with 10 μM 5-Ethynyl-2'-deoxyuridine (EdU) and incubated overnight. The following day the eggs were collected by centrifugation at 10,000g for 1min, fixed in 4% formaldehyde in PBSTx for 4hrs, EdU was
detected as previously described (41), and washed in PBSTx. *in situ* hybridizations and colorimetric detection of Fast Blue BB were imaged using a Zeiss AxioZoom V16 equipped with a transmitted light base and a Zeiss AxioCam 105 Color camera. All other images were acquired using a Nikon A1+ laser scanning confocal microscope.

**Egg culture, miracidia hatching, and labeling.** Eggs were collected on 10 μm cell strainer rinsed into Medium 199 (Corning) containing 10% Fetal Bovine Serum and 1x Antibiotic-Antimycotic (Gibco/Life Technologies, Carlsbad, CA) for 7 days at 37°C in 5% CO₂. After culture, eggs were collected on 10 μm cell strainer and rinsed with artificial pond water 1-2 times and placed in artificial pond water under light. A small aliquot of eggs was was counted to determine the total number of eggs examined. Active miracidia were observed and counted under light microscopy every 30 mins for up to 4hrs. Miracidia fixation and labeling was performed as previously described (47).

**Karyotype analysis**
Karyotypes from schistosome embryos were determined using a modified version of a previously published method (48). Briefly, eggs were incubated in M199 for two days after deposition and then incubated in 5 μM Nocodazole for 1 hour at 37°C. Eggs were pelleted at 20,000 g for 1 min, rinsed in DI water, pelleted, resuspended in 1 ml of water, and incubated for 20 min at RT. Following centrifugation, the pelleted eggs were resuspended in 1 ml of fixative (3:1 methanol:acetic acid) for 15 minutes. Eggs were again pelleted and resuspended in 100 μl of fixative and the eggshells were disrupted using a Kontes pellet pestle (DWK Life Sciences, Rockwood, TN). The disrupted eggshells were allowed to settle for 10 min, the supernatant was collected, and centrifuged for 8 min at 240 g. All but ~20 μl of supernatant was removed and the remaining liquid was pipetted dropwise on to a glass slide (Superfrost Plus, ThermoFisher, Carlsbad, CA) freshly dipped in PBS. After air-drying chromosomes were labeled with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA) and imaged on a Nikon A1+ laser scanning confocal microscope with a 60x/1.4 NA objective.

**RNA Interference**
Double stranded RNA was generated as previously described (41, 49). For dsRNA treatment worm pairs were electroporated with a square-wave pulse (125 V for 20 ms (50)) on a BTX Gemini in 4 mm cuvettes with 160 μg of dsRNA in 400 μl of DMEM. The worms were then supplemented with 60 μg/ml of dsRNA on day 0, 1, 2, 6 and 10. At D14, parasites were pulsed for 4 hours with EdU and processed as previously described (41). Eggs laid between D12 to D14 were collected to determine egg-laying rates.

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FIGURE LEGENDS

Fig. 1. ABC169 supports the maintenance of schistosome vitellaria.
(A) Morphological changes of eggs laid by worm pairs maintained in BM169. After D6 parasites began laying abnormally formed eggs (arrow). These eggs were small, usually did not contain a lateral spine, and did not possess a smooth surface.
(B) Fast Blue BB staining (brownish-red labeling) showing loss of mature vitelloocytes during culture in BM169. Representative images from three experiments with n > 10 parasites.
(C) Confocal slice showing Fast Blue BB and BODIPY labeling in the vitellaria of freshly recovered parasites and in schistosomes at D20 of cultivation in BM169 supplemented with RBCs (Red Blood Cells), LDL, and/or ascorbic acid. Representative images from three experiments with n > 10 parasites.
(D) Whole-mount in situ hybridization showing expression of vitellaria-enriched genes in freshly perfused female worms and parasites cultured in ABC169 or BM169 for 20 days. Representative images from three experiments with n ≥ 9 parasites.
(E) Heat-map showing relative expression of vitellaria-enriched transcripts in freshly perfused female worms (“Fresh”) and parasites cultured in ABC169 or BM169 for 20 days. Each column represents an independent biological replicate; samples are normalized to the expression of an arbitrarily chosen biological replicate from the BM169 group. Changes in expression between BM169 and ABC169 were statistically significant (p<0.05, T-test).
(F) Morphology of eggs laid by paired adult females in medium ABC169 supplemented with L-ascorbic acid or D-isoascorbic acid on D20. Representative of 3 experiments. Scale bars: A, B, D, F 100 µm; C 25 µm.

Fig. 2. Eggs laid in ABC169 develop and hatch.
(A) Rate of egg production per worm pair in BM169 or medium ABC169 during in vitro culture. Dotted line indicates the point at which parasites beginning laying morphologically abnormal eggs. n ≥ 19 worm pairs examined in 4 experiments. Error bars represent 95% confidence intervals.
(B) Morphology of eggs (Left, bright field; Right, DIC) laid by paired adult females in BM169 or medium ABC169 on D20. Red arrows show early embryos in eggs laid in ABC169.
(C-E) Quantification of the (C) size, (D) number of DAPI-labeled nuclei, and (E) autofluorescence intensity from eggs laid by freshly perfused female worms (Fresh, n=41), parasites cultured in BM169 (n=61), or ABC169 (n=59) on D20. **** p<0.0001, T-Test. Error bars represent 95% confidence intervals.
(F) EdU-labeled embryonic cells of an egg laid by a paired adult female in ABC169 on D20.
(G) Percentage of eggs with clusters of cycling EdU+ embryonic cells from eggs laid between D16 and D22 by paired adult females in BM169 or ABC169. >2000 eggs from 6 experiments. Error bars represent 95% confidence interval.
(H) Miracidia from eggs laid in ABC169. Left, miracidium (pseudocolored red) inside an egg laid on D20 of culture in ABC169. Right, hatched miracidium from D20 egg labeled with DAPI and phalloidin. These miracidia appear grossly normal in morphology possessing 2 pairs of flame cells (arrows).
Scale bars: B, F, H 20 µm; C-E 50 µm.
**Fig. 3.** ABC169 supports the maturation of immature female schistosomes following pairing with a male worm.

(A) Vitellaria development visualized by Fast Blue BB labeling in immature female schistosomes in the presence or absence of male worms in BM169 or ABC169 at D20 of culture. Black pigment in ABC169 parasites represents digested RBCs.

(B) Eggs laid by immature female in BM169 or ABC169 on day 20 after pairing with a male.

(C) Proportion of females laying eggs (left axis, magenta) and number of eggs laid per worm pair (right axis, green) from D1 to D20 following pairing of immature females with male worms in ABC169. n=31 worm pairs examined in 4 separate experiments. Error bars represent 95% confidence intervals.

(D) Eggs laid by previously immature females commence embryonic development. Left, representative developing EdU-labeled embryo from egg laid in ABC169 on D20.

Scale bars: A,B 100 µm; D 20 µm.

**Fig. 4. Using ABC169 to study schistosome male-induced female sexual maturation.**

(A) Pairing of immature females with amputated male segments. Left, cartoon showing approximate positions of male amputations. Most often we observed posterior segments pairing with posterior region of immature females, shown to right.

(B) Fast blue BB labeling showing female vitellaria development after pairing with posterior male segments. Left panels, female after separation from male segment showing vitellaria development in posterior region. Right panel, female before separation from male segment showing vitellaria development is restricted to paired region. Representative of > 40 female parasites examined in 4 separate experiments.

(C) Eggs laid by immature female worms paired with male segments are morphologically normal (left) but do not contain developing embryos as measured by EdU labeling (right). n > 500 eggs examined in 4 separate experiments.

(D) DAPI staining to examine ovary development in immature females paired with intact male worms or an amputated posterior male segment. Females paired with intact males possess differentiated oocytes (DO) (n=23), whereas females paired with male segments produce no differentiated oocytes (n=41).

(E) Pairing of immature females with decapitated and castrated male segments. Left, cartoon showing approximate positions of amputation, removing both the head and testes. These decapitated and castrated segments paired with female along most of the female body, shown to right.

(F) DAPI staining to examine ovary development in immature females paired with decapitated and castrated male segments. These ovaries contain differentiated oocytes (DO). Representative images from n = 22 parasites examined in 3 separate experiments.

(G) Fast Blue BB showing vitellaria development in females after pairing with decapitated and castrated male segments. Representative images n=22 parasites from 3 separate experiments.

(H) EdU labeling showing embryonic development in eggs from females paired with decapitated and castrated male segments. 273/602 eggs laid between D16-D22 contained clusters of EdU⁺ cells.

(I) DAPI labeling of metaphase spreads from eggs laid by (left) fresh ex vivo female parasites paired with intact males and (right) immature female parasites paired with decapitated and castrated male segments. Embryonic cells from fresh ex vivo parasites were diploid (2n=16), whereas those from unfertilized females are haploid (n=8).
Fig. 5. RNAi mediated stem cell depletion leads to regression of the female reproductive organs and cessation of egg production.

(A) EdU labeling of proliferative cells following control or Histone H2B RNAi treatment. Histone H2B(RNAi) animals possess few EdU-labeled nuclei. Insets show enlarged views of ovaries. Vitellaria are positioned to the right and white arrow indicates the proliferative stem cells in the vitellaria. Representative images from n > 35 parasites for each treatment examined in 6 separate experiments. White arrow indicates the vitellaria.

(B) Fast blue labeling following control or Histone H2B RNAi treatment. Histone H2B(RNAi) parasites produce few vitellocytes. Representative images from n > 50 parasites for each treatment examined in 6 separate experiments.

(C) Plot showing egg production per worm pair between D12-D14 of RNAi treatment. Error bars represent 95% confidence intervals. Representative images from n >100 parasites for each treatment examined in 6 separate experiments. **** p<0.0001, T-Test. Error bars represent 95% confidence intervals.

Scale bars: 50 µm.
Fig. 2

(A) Graph showing the number of eggs produced per day per pair over 20 days in vitro for BM169 and ABC169. The x-axis represents days in vitro, and the y-axis represents eggs produced per day per pair.

(B) Images of eggs stained with DAPI showing the distribution of nuclei.

(C) Scatter plots showing the area of eggs for BM169, ABC169, and Fresh.

(D) Scatter plots showing the number of nuclei per egg for BM169, ABC169, and Fresh.

(E) Scatter plots showing the intensity of autofluorescence for BM169, ABC169, and Fresh.

(F) Images showing the localization of Edu and DAPI in eggs.

(G) Bar chart showing the percentage of Edu-positive eggs for BM169 and ABC169.

(H) Images showing the staining of DAPI and DAPI/Phalloidin in eggs.
Fig. 3

A. 

B. 

C. 

D. EdU EdU EdU EdU DAPI

Fraction of females laying eggs 

Eggs laid per pair 

EdU DAPI
Fig. 4

A. Male segments

B. Pair with immature female

C. EdU DAPI

D. + Male segment + Intact male

E. Remove testes
Pair with immature female

F. DO

G. DO

H. EdU DAPI

I. Intact male Castrated male
**Fig. 5**

A. Control (RNAi) and Histone H2B (RNAi) labels for EdU and DAPI staining.

B. Fast blue BB staining for Control (RNAi) and Histone H2B (RNAi).

C. Graph showing the number of eggs produced per pair per day with control (RNAi) and Histone H2B (RNAi) treatments. The graph includes a statistical significance test with **** indicating a very significant difference.
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.