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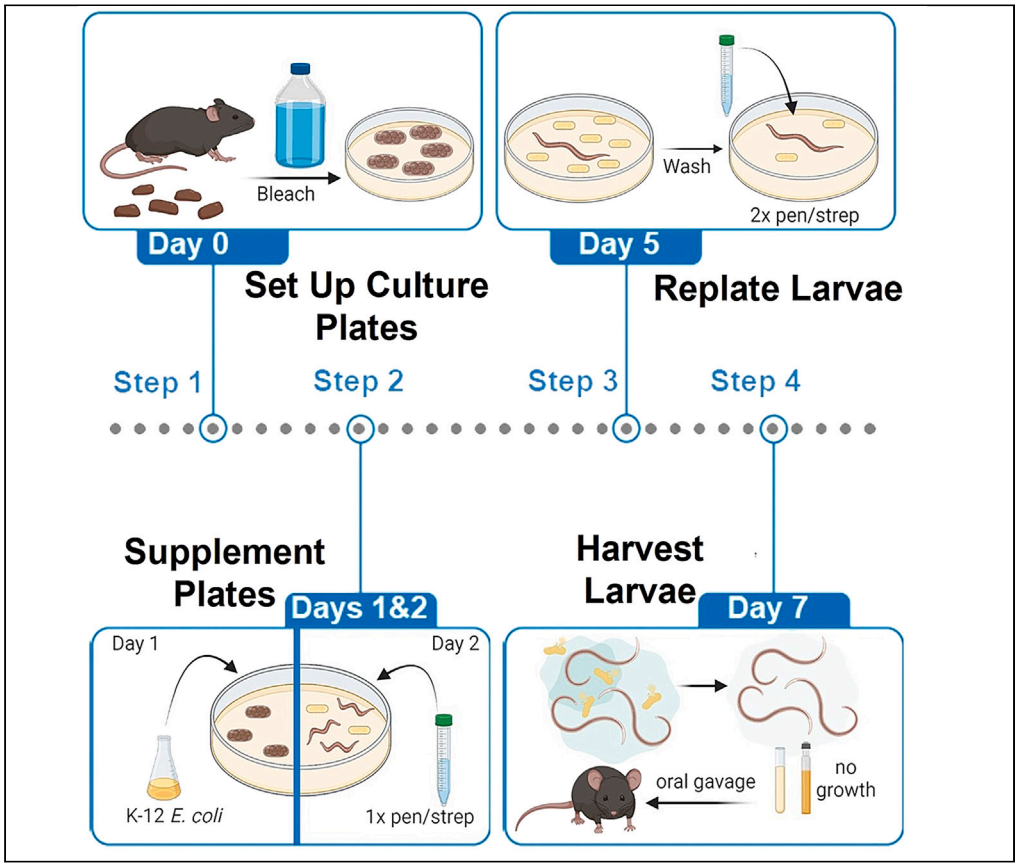
A simplified protocol for deriving sterile, infectious murine *Heligmosomoides polygyrus bakeri* larvae



Gastrointestinal helminth infection occurs within a diverse microbiome, complicating the interpretation of whether effects are caused by the parasite versus the microbial community. Here, we present a protocol for deriving sterile larvae of the murine helminth, *Heligmosomoides polygyrus bakeri* (*H. polygyrus*), providing experimental control of the microbiome. We describe steps for sterilizing with a bleach solution and developing into infectious larvae using *E. coli*. We then detail procedures for removing bacterial contaminants before harvesting to ensure the generation of germ-free larvae.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Steps for the isolation of eggs from the helminth, *Heligmosomoides polygyrus bakeri*

Steps for sterilizing eggs and performing axenic maturation of larvae with *E. coli*

Steps for sterilizing larvae and validating sterility and infectivity in murine hosts

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A simplified protocol for deriving sterile, infectious murine *Heligmosomoides polygyrus bakeri* larvae

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SUMMARY

Gastrointestinal helminth infection occurs within a diverse microbiome, complicating the interpretation of whether effects are caused by the parasite versus the microbial community. Here, we present a protocol for deriving sterile larvae of the murine helminth, *Heligmosomoides polygyrus bakeri* (*H. polygyrus*), providing experimental control of the microbiome. We describe steps for sterilizing with a bleach solution and developing into infectious larvae using *E. coli*. We then detail procedures for removing bacterial contaminants before harvesting to ensure the generation of germ-free larvae.

BEFORE YOU BEGIN

H. polygyrus is a murine-specific helminth that is associated with bacteria through all stages of its life cycle.[1] It establishes a long, immune-limiting infection in mice and is well-known for its use as a model of the hygiene hypothesis, which states that the eradication of "old friends" may have contributed to a rise in autoimmune and inflammatory diseases.[2] In this context, *H. polygyrus* has been shown to attenuate a variety of autoimmune diseases.[3–5] Additionally, *H. polygyrus* provides a useful model with which to study host-pathogen interactions[6] and has been used to identify interesting immune-evasion strategies employed by multicellular pathogens such as release of a TGF-β mimic[7] and secreted exosomes containing miRNA thought to influence host gene regulation.[8]

H. polygyrus infection is associated with alteration of the gut microbiome.[9–11] Interestingly, studies have also demonstrated that the *H. polygyrus*-altered gut microbiome recapitulates a variety of effects originally attributed to the parasite when it is transferred to a naïve host in the absence of *H. polygyrus*.[5,12,13] This raises the exciting possibility that alterations in the gut microbiome help attenuate autoimmune diseases in addition to the direct effects of *H. polygyrus* infection. These findings underscore the need for a germ-free *H. polygyrus* infection model that can help differentiate between immunomodulatory helminth vs. microbiome factors.

In late 2021, a protocol for generating germ-free *H. polygyrus* was described[14] that employed antibiotic sterilization of adult *H. polygyrus* worms (via bactericidal neomycin, penicillin/streptomycin, gentamycin, metronidazole, ampicillin, vancomycin as well as the fungicide amphotericin B). In this model, sterile adult helminths successfully produced viable eggs *in vitro*. This protocol also relies on larval maturation on nematode growth agar plates colonized with the auxotrophic *E. coli* strain, HA107 which is not removed prior to murine infection. Thus, mice are infected with non-sterile

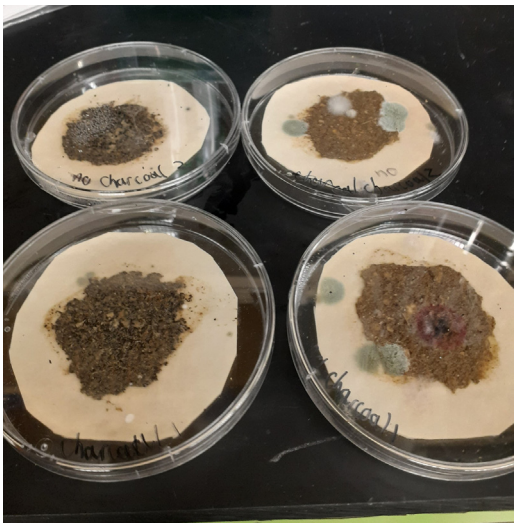

Figure 1. Fecal slurry plates used to grow conventional infectious *H. polygyrus* larvae (related to before you begin)

larvae, exposing the host to bacterial antigen. Though HA107 cannot survive in the murine gut this model leaves the possibility of confounding (non-helminth) immune stimulation. Here, we describe a protocol for generating *bona fide* germ-free, infective *H. polygyrus* larvae. Using common lab reagents, we demonstrate germ-free larvae have similar infectivity compared to their conventionally reared counterparts.

Institutional permissions

Studies involving vertebrate animals require appropriate institutional approvals, which need to be in place prior to initiating experiments. All mouse experiments described here were approved by the Montana State University Institutional Animal Care and Use Committee. All mice were *Mus musculus*, C57BL/6 line (Jackson Labs, Inc). Germ-free mice were reared from birth inside HEPA-filtered isolators (Park Bio Inc.) and monitored routinely for microbial contamination as described.[15]

Establish conventional *H. polygyrus* infection

Timing: 3–4 weeks

Stool from conventional, specific pathogen free (SPF) mice infected with *H. polygyrus* serves as the starting material for sterile larvae. C57BL/6 mice are traditionally used as hosts given their susceptibility to infection, but other genotypes should theoretically produce similar results.

1. Collect stool from mice infected with *H. polygyrus* for 2–16 weeks.
   a. Transfer mice into paper-towel lined cage for at least 5–7 h.
   b. Collect stool with forceps into 15 mL falcon tube or another sterile sample container.
2. Culture conventional *H. polygyrus*.
   a. Rehydrate infected stool by adding approximately 2 mL water per gram of stool.
   b. Allow rehydration for at least 15 min.
   c. Add activated charcoal approximately equal to the amount of stool. Mix stool and charcoal until homogenous with a metal spatula.
      i. The addition of charcoal to fecal slurry plates is optional, but advised as it suppresses bacterial outgrowth originating from stool in our hands.
      ii. If including, rinse activated charcoal for 30 min prior to use (Figure 1).
   d. Prepare circles of Whatman filter paper cut from a sheet or manufactured circular.
   e. Place circle of Whatman filter paper in 100 mm × 15 mm petri dish.

f. Leave approximately one-half inch of space between the edge of the filter paper and the inside edge of the petri dish.
g. Using metal spatulas, spread a thin layer of fecal slurry in the center of the filter paper.
    i. Leave another one-half inch between the stool layer and the edge of the filter paper.
    ii. Repeat until all slurry is spread onto Whatman paper in dishes.
h. Add water to the center of the filter paper until the paper is fully moistened.
    i. When the plate is tilted, you should observe a bead of water formed at the edge of the filter paper.
i. Place the fecal slurry plates in a dark, humid, room-temperature environment, such as a Styrofoam box containing a beaker with a paper towel in water.
j. After seven days, harvest infectious larvae by rinsing the edges of the Whatman filter paper with water.
k. Use a transfer pipette to rinse each edge five to 10 times avoiding the stool-charcoal mix as you go.
    i. Rotate the plate 120° and repeat.
l. Harvest rinsed liquid containing larvae into a sterile 15 mL falcon tube.
m. Larvae may be harvested for three days (days 7–9 post-plating) and stored for up to 6 months at 4°C. Viable, motile larvae should be observable under an inverted light microscope at 10× magnification (exact make and model: Nikon eclipse TS100, used throughout the protocol for visualizing eggs and larvae).

3. Infect mice.
    a. Prepare harvested larvae for infection by washing three times with 15 mL of water. Pellet larvae in between washes by centrifuging at 200 RCF for 5 min.
    b. Resuspend larvae in 3–5 mL of water.
    c. Count the number of infectious larvae in four 50 μL aliquots in a flat-bottomed 96-well plate or other suitable sample container under 10× magnification.
    d. Determine the number of larvae in suspension.
    e. Resuspend the larvae at a concentration of 300 larvae per 100 μL of water. Pellet by centrifuging at 200 × *g* for 5 min.
    f. Infect C57BL/6 mice by oral gavage. Administer 100 μL of the prepared larval suspension to each mouse.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Agar A	Fisher Scientific	9002-18-0
Sodium chloride	Fisher Scientific	7647-14-5
Sucrose	Fisher Scientific	S2-212
Dulbecco's phosphate-buffered saline 1× (PBS)	Sigma-Aldrich	D5773
Magnesium sulfate heptahydrate	Sigma-Aldrich	10034-99-8
Calcium chloride dihydrate	Sigma-Aldrich	10035-04-8
Cholesterol	Sigma-Aldrich	C3045-5G
Potassium phosphate monobasic	Sigma-Aldrich	7778-77-0
Potassium phosphate dibasic	Sigma-Aldrich	7758-11-4
Penicillin-Streptomycin solution	Sigma-Aldrich	P4458-100ML
Essendant 121oz. Clorox germicidal bleach	Fisher Scientific	50371500
YCFA media	Anaerobe Systems	AS-680
Granular activated charcoal (coconut) 20 × 50 mesh	Charcoal House	SKU# C-142
Bacto Proteose Peptone No. 2	Fisher Scientific	212120

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Ethanol, absolute (200 proof), molecular biology grade, Fisher BioReagents	Fisher Scientific	BP28184
Fisher BioReagents Microbiology media: LB broth, Miller	Fisher Scientific	BP1426-500
Axygen agarose LE	Fisher Scientific	14-223-081
Thermo Scientific DreamTaq Green PCR master mix	Fisher Scientific	FERK1081
Experimental models: Organisms/strains		
Heligmosomoides polygyrus bakeri	Weinstock Laboratory[9]	N/A
Escherichia coli K-12	Carolina Biologicals	155067
Mouse strain C57BL/6, male and female, 7–8 weeks of age at infection	Jackson Laboratory	000664
Germ-free mouse strain C57BL/6, female, 12 weeks of age at infection	Bred in-house	N/A
Oligonucleotides		
16S forward	5′-agagtttgatcctggctcag-3′	N/A
16S reverse	5′-accttgttacgactt-3′	N/A
Software and algorithms		
ImageJ	Scale and Freehand Trace	Freely Available at: https://imagej.net/ij/
Other		
Petri dish (100 × 15 mm)	Fisher Scientific	FB0875712
Cell dissociation screen cup	Sigma-Aldrich	S1145
Mesh screen (380 μm)	Sigma-Aldrich	S0770
Transfer pipettes, sterile	Sigma-Aldrich	HS206373B
Microscope slide (26 × 75 × 1 mm)	Sigma-Aldrich	BR474701
Rectangular coverslip (13 × 54 mm)	Fisher Scientific	50-194-4687
96-well flat bottomed polystyrene microplates	Fisher Scientific	07-001-000
Screw cap centrifuge tube, 15 mL	Sigma-Aldrich	AXYSCT15ML500
Disposable polypropylene pestles	Fisher Scientific	13-717-270
Whatman filter paper circles (90 mm diameter)	Sigma-Aldrich	WHA5201090
Sterile inoculating loops (1 μL)	Fisher Scientific	50-225-3018
Polystyrene spectrophotometer cuvettes	Sigma-Aldrich	C5291-100EA
Aluminum foil	Sigma-Aldrich	Z185159
MSC Ziploc freezer bag	Fisher Scientific	50-111-3769
PowerSoil Pro Kit	QIAGEN	47017
GENESYS 30 Vis spectrophotometer	Fisher Scientific	14-380-442
Labconco Biosafety cabinet (class II A2)	Fisher Scientific	30-231-0001
Sorvall Legend XF centrifuge	Fisher Scientific	75-393-839
Fisherbrand Isotemp Direct heat CO_2 incubator, 184 L, stainless steel	Fisher Scientific	11-676-603
Laxco LMI-3000 Series Routine inverted microscope	Fisher Scientific	LMI3PH2
Corning LSE shaking incubator	Fisher Scientific	07-202-151
Analytik Jena Biometra TOne thermal cyclers	Fisher Scientific	84-640-70301
iScript cDNA Synthesis Kit	Bio-Rad	1708890
iTaq Universal SYBR Green Supermix	Bio-Rad	1725120
CFX384 Touch real-time PCR detection system with starter package	Bio-Rad	1840138
Direct-zol RNA Miniprep	Zymo Research	R2051

MATERIALS AND EQUIPMENT

NGM Agar

Reagent	Final concentration	Amount
NaCl	3 mg/mL	3.0 g
Bacto Peptone	2.5 mg/mL	2.5 g
Agar A	17 mg/mL	17 g
$MgSO_4$*(1 M)	1 μM	1 mL
$CaCl_2$*(1 M)	1 μM	1 mL

(*Continued on next page*)

Continued

Reagent	Final concentration	Amount
Cholesterol (1 mg/mL)**	0.05 μg/mL	5 mL
Potassium phosphate buffer (PPB)***	25 μM	25 mL
ddH_2O	N/A	968 mL
Total	N/A	1000 mL

Autoclave media containing ingredients without an asterisk. After making, store plates at 4°C and use within 2 months. Recipe makes enough for 60 plates (15 mL per plate).
*Filter-sterilize. Add 1 mL per L agar after autoclaving and cooling.
**Dissolve cholesterol to 1 mg/mL in 100% ethanol. Filter-sterilize. Add 5 mL per L agar after autoclaving and cooling.
*** Mix 132 mL of 1 M K_2HPO_4 with 868 mL of 1 M KH_2PO_4 to make PPB. Filter-sterilize and add after autoclaving and cooling.

Sheather's sugar solution

- Saturated sugar solution: add 98.5 g sucrose in 75 mL ddH_2O.

Stir over low heat to fully dissolve sucrose, scale as necessary, store at 4°C.

Filtering is not necessary.

Alternatives: Available for purchase at Jorgenson Laboratories (category number J1028).

STEP-BY-STEP METHOD DETAILS

Preparation of axenic larval culture

Timing: 10 h

Timing: 2–3 h (for steps 2–5)

Eggs will be isolated from the stool of conventional mice infected with *H. polygyrus* and sterilized with a 25% bleach solution. Egg sterilization has been optimized to kill bacteria without affecting viability. Sterilized eggs will be plated on nematode growth medium (NGM) agar plates. Prepared culture plates will be incubated at ambient temperature (15–30°C).

1. Collect stool from conventional mice infected with *H. polygyrus.*
   a. Transfer infected mice to a paper towel-lined cage for 5–7 h.
   b. Collect stool in 15 mL falcon tube or another appropriate sample container.
   c. Rehydrate stool for a minimum 15 min by adding reverse osmosis (RO) water by adding approximately 2 mL water per gram of stool.
2. Isolate eggs from stool.
   a. Place cell dissociation cup with 380 μm mesh screen insert inside one half of a 100 mm × 15 mm petri dish. Troubleshooting.
   b. Use a metal spatula to break up rehydrated stool into smooth slurry inside of 15 mL falcon tube.
   c. Fill the majority of stool container with Sheather's Sugar Solution and invert to mix with rehydrated stool slurry.
   d. Pour or use a transfer pipette to strain the sugar-stool slurry over the metal mesh while keeping the mesh held to the cup with the spatula or small pestle.
      i. The slurry should remain in the cup.
   e. Vigorously mix the stool by holding down the mesh screen with the spatula and break up clumps with a small pestle.

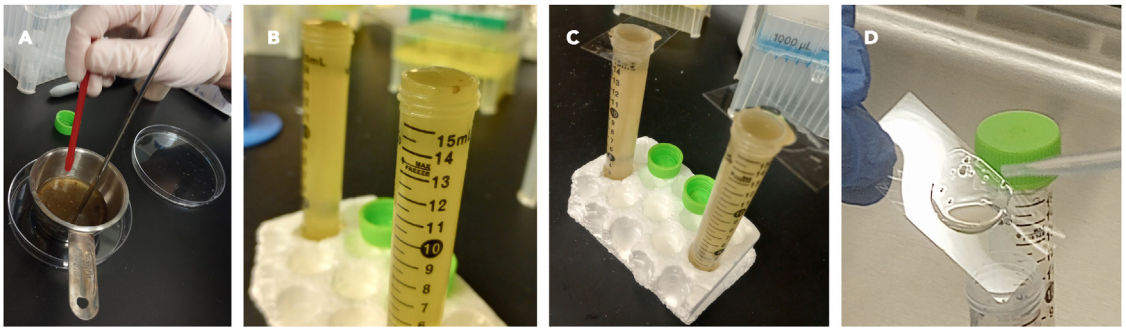

Figure 2. Visualization of the major steps of egg isolation

Cover glass can be washed and reused across protocol repetitions.

(A) Egg straining setup with cell cup and metal mesh.

(B) Sheather's sugar solution meniscus for egg collection.

(C) Cover glass placed on meniscus.

(D) Rinsing of cover glass containing eggs with sterile PBS into 15 mL falcon tube. (related to step 2).

f. Pick up the cup and filter the sugar-stool mixture through the mesh screen into the petri dish below (Figure 2).

g. Transfer the filtrate to 15 mL falcon collection tubes with a transfer pipette.

h. Fill all collection tubes to 15 mL with filtered slurry.

i. Continue straining stool on mesh screen until approximately 1 tube per infected mouse has been collected.

j. Centrifuge collection tubes at 200 × *g* for 5 min at ambient temperature (15–30°C). Do not remove the supernatant.

k. Add Sheather's Sugar Solution dropwise to the centrifuged collection tube(s) until a convex meniscus forms above the top of the falcon tube.

l. Place a microscope slide coverslip over the meniscus for 2–4 min then remove, invert, and transfer the coverslip to a biosafety cabinet.

   i. Take care to keep the egg-sugar mixture on the coverslip until you rinse it off (Figure 2).

   ii. Eggs will float to the top and stick to the coverslip. Validate the presence of eggs by placing coverslip onto a microscope slide and viewing under an inverted light microscope at 4–10× magnification.

m. Rinse the coverslip holding the egg-sugar mixture into a new 15 mL falcon tube inside the biosafety cabinet using sterile 1× phosphate buffered saline (PBS) and a sterile transfer pipette. Troubleshooting.

n. Repeat previous step until no more eggs are seen under an inverted light microscope or the PBS rinse tubes are full. You should generate approximately one full PBS-rinse tube per slurry collection tube.

o. Centrifuge egg suspension at 200 × *g* for 5 min at ambient temperature (15–30°C) and discard supernatant.

   i. If multiple PBS-rinse tubes were generated, combine resulting egg pellets into one 15 mL falcon tube in a volume of 1–3 mL of PBS-egg suspension.

3. Determine the total number of eggs in suspension. This step can be performed in parallel with step 4.

   a. Dilute an aliquot of stock suspension by a factor of 4–100. Dilution must be sufficient to easily count eggs.

      i. Egg production varies throughout infection period with peak production at 7 weeks post infection.[16]

   b. Count eggs in diluted aliquots using an inverted light microscope at 4–10× magnification in a McMaster counting chamber, flat-bottomed 96-well plate, or another appropriate container/stage.

   c. Multiply the average number of counted eggs per microliter by the dilution factor and total stock suspension volume to determine the total egg number.

Figure 3. Picture of bleached egg culture plates

Sterilized eggs are distributed across ambient temperature (15–30°C) NGM agar plates. The culture plates are wrapped in aluminum foil and placed inside a sealable bag. Aseptic technique and 70% ethanol ensure the sterility of the bag and aluminum foil which are left to incubate inside the biosafety cabinet (related to step 4g).

4. Sterilize eggs with 25% bleach. Example given with a 5 mL solution.
   a. Bring the eggs to a volume of 3.75 mL with sterile, autoclaved RO water and add 1.25 mL of bleach. Move immediately to the next step.
      i. The volume can be adjusted as needed but be sure the concentration of bleach in solution remains 25%.
   b. **Immediately** place the bleached egg solution in centrifuge at 200 × *g* for 5 min at ambient temperature (15–30°C). See Critical note below. Troubleshooting.
   c. Once finished, **immediately** remove bleach solution from pelleted eggs and quench the bleach reaction by resuspending eggs in sterile, autoclaved RO water.
   d. Wash the eggs a total of two times with sterile water centrifuging at 200 × *g* for 5 min at ambient temperature (15–30°C) between washes to pellet eggs. Troubleshooting.
   e. Resuspend eggs in sterile, autoclaved RO water at a volume of 2 mL per NGM culture plate.
      i. Culture no more than 10,000 eggs per NGM plate to avoid consumption of *E. coli* in subsequent steps.
   f. Add 2 mL of egg suspension fluid to the middle of each NGM plate. Note the number of eggs per plate.
   g. Wrap each plate in aluminum foil and place in a sealed bag. Leave the culture bag flat in a sterile, ambient temperature (15–30°C) environment such as biosafety cabinet for the duration of the culture period (Figure 3).
5. Inoculate 5–10 mL of Luria Broth (LB) with K-12 *E. coli* from frozen glycerol stock.
   a. Secure in a shaking incubator at 200 rpm and 37°C and leave for at least 15 h.
      i. This step must be performed on the same day that the eggs are plated on NGM agar so that the *E. coli* culture reaches an appropriate density in time for the next step.

⚠ CRITICAL: Eggshells are thinned by bleach treatment and are negatively affected by concentrations higher than 25% and exposure times longer than 5 min (Figure 4). Care must be taken to ensure treatment falls within this range. Additionally, bleach must be removed from the solution before combining eggs with *E. coli* so that supplemented bacteria are not eliminated by egg sterilization methods.

Mature and sterilize *H. polygyrus* larvae

Timing: 1 week

Timing: 1–2 h (for steps 10 and 11)

In this step K-12 *E coli* is added to the plate, which is necessary for larval development into the infectious L3 stage. Treatment with a penicillin and streptomycin (pen/strep) cocktail is used to chemically sterilize the larvae prior to infection of mice.

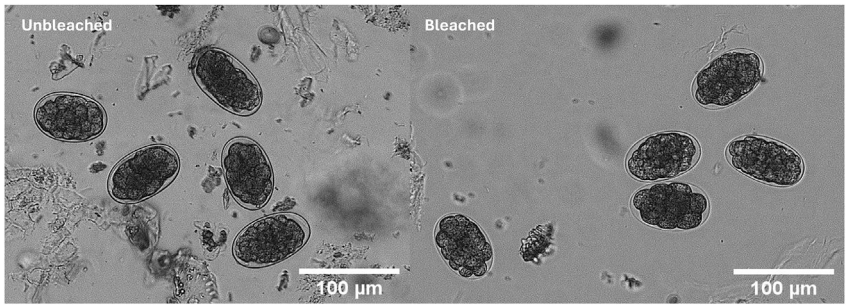

Figure 4. Visual comparison of untreated eggs to those exposed to 25% bleach for 5 min at 10× magnification and a field of view of 0.9 mm using an inverted light microscope with an attached camera (exact make and model: ZEISS Axioscope 5)

Eggshell thickness differs across treatment. Scale bars at 100 µM (related to step 4).

6. Adjust *E. coli* cultured in the previous step to an optical density of 0.3–0.4 (600 nm). Work in a biosafety cabinet to maintain sterility.
7. Add 1 µL diluted *E. coli* per 1,000 eggs up to a maximum of 5 µL per plate (i.e., for egg counts above 5,000, do not add more than 5 µL *E. coli* to the plate).
8. Rewrap plates and return to bag to incubate at ambient temperature (15–30°C) inside the biosafety cabinet. Troubleshooting.
9. One day after addition of *E. coli*, flood each NGM plate with 2 mL of a pen/strep cocktail consisting of 100 unit/mL penicillin and 100 µg/mL streptomycin (1× solution).
   a. Ensure entire plate is covered by pen/strep solution.
   b. If the plate contains 1 mL or more of liquid, add 1 mL of a 200 unit/mL penicillin and 200 µg/mL streptomycin solution instead so that the concentration is halved to a 1× solution and 2 mL of liquid is present on the plate.
   c. Rewrap plates in aluminum foil and place back into culture bag to incubate at ambient temperature (15–30°C) in the biosafety cabinet.
10. On day five of culture (five days after the eggs are first plated onto NGM), wash, and transfer plate contents to new NGM plate to remove remaining *E. coli*. Perform all steps in a biosafety cabinet to avoid contamination.
    a. Prop up the plate bottom with petri dish lid and add 2 mL of sterile, autoclaved RO water to each plate.
    b. Rinse plates of all worms by pipetting sterile, autoclaved RO water across the top of the angled plate with p1000 pipette or sterile transfer pipette. Use a fresh pipette for each NGM plate to reduce the risk of cross-contamination.
    c. With a sterile pipette, transfer the contents of each plate to a labeled 15 mL falcon tube, generating one tube per NGM culture plate.
    d. Fill the tube up to the 15 mL mark with sterile, autoclaved RO water and centrifuge at 200 × *g* for 5 min at ambient temperature (15–30°C).
    e. Remove wash liquid from larval pellet and repeat sub steps d and e for a total of five washes (Table 1).
    f. Do not cross-contaminate larvae plates during wash steps.
    g. Remove final wash volume so that pellet is left in 0.5–1 mL of sterile, autoclaved RO water. Add 3 mL of 200 unit/mL penicillin and 200 µg/mL streptomycin pen/strep cocktail to each 15 mL falcon tube.
    h. Transfer the washed and antibiotic-treated *H. polygyrus* larvae to new, labeled NGM plates that have reached ambient temperature using a sterile pipette.
    i. Tilt the plate to ensure the larval suspension covers the entire surface of the new NGM plate.

Table 1. Effect of washing on the larval culture *E. coli* load

CFU/μL *E. coli* pre-wash	CFU/μL *E. coli* post-wash	Percent change in CFU/μL	Number of eggs
4.80E+03	6.67E-01	−99.986	4000.00
3.00E+03	5.02E+01	−98.327	4000.00
1.50E+04	2.00E+01	−99.867	4000.00
1.46E+04	6.00E+00	−99.959	4000.00
2.70E+03	5.23E+01	−98.063	4000.00
3.35E+02	5.00E-01	−99.851	4000.00
3.75E+03	3.20E+01	−99.147	4000.00
tmtc	2.43E+02	NA	4000.00
tmtc	6.00E+01	NA	4000.00
tmtc	2.78E+01	NA	4000.00
1.80E+03	1.35E+01	−99.25	4000.00
3.60E+03	8.50E+00	−99.764	4000.00
tmtc	1.70E+02	NA	4000.00
1.55E+03	6.25E+00	−99.597	4000.00
tmtc	3.65E+02	NA	5000.00
2.25E+03	1.25E+01	−99.444	5000.00
9.00E+03	7.50E+01	−99.167	5000.00
4.03E+03	3.00E+01	−99.255	1000.00
tmtc	2.48E+02	NA	1000.00
2.35E+04	5.33E+01	−99.773	1000.00
1.55E+04	6.48E+01	−99.582	1000.00

Aliquots were taken from each larval wash suspension on the first and last (fifth) wash. There was no cross-contamination between plates. The aliquots were serially diluted and plated so that the amount of the *E. coli* before and after washing was determined for each culture plate. Those plates where bacterial overgrowth made counting colonies impossible labeled with too many to count (tmtc) (related to step 11).

j. Rewrap the plates in aluminum foil and seal in culture bag left at ambient temperature (15–30°C) in sterile biosafety cabinet.

11. On the seventh day of culture, harvest larvae into labeled 15 mL falcon tubes and check sterility. Troubleshooting.
    a. Prop plate up on edge of top lid and rinse with approximately 2 mL of sterile, autoclaved RO water as done before to rinse all larvae off the plate.
    b. Use p1000 pipettor or sterile transfer pipette to transfer each plate's contents into a labeled 15 mL falcon tube.
    c. Wash the pen/strep out of culture by filling collection tubes to the 15 mL mark, centrifuging at 200 × *g* for 5 min at ambient temperature (15–30°C) and removing the supernatant approximately down to the 2 mL mark.
    d. Repeat step c for a total of two washes.
    e. Resuspend the larvae in a final volume of 3–5 mL of sterile, autoclaved RO water.

Optional: The egg suspension may be streaked on an LB plate before and after *E. coli* spike to ensure the eggs were sterilized by bleach treatment. Incubate the plate for at least 15 h at 37°C and ensure there is no growth in the section corresponding to the bleached eggs before *E. coli* was added.

Infect germ-free mice with sterile larvae

Timing: 5–6 days

Harvested larvae are considered third stage (filariform) after a week of development. Earlier stages (rhabditiform) lack a sheath and do not exhibit characteristic twitching motion. Sterile L3 (third larvae

stage) are resuspended at the appropriate concentration for mouse infection. Germ-free mice are administered larvae via oral gavage and germ-free status is evaluated post-infection.

12. Evaluate sterility of L3 stocks using cultivation.
    a. Place an aliquot of larvae suspension onto an LB plate or in broth to check final sterility status. Ensure that the aliquot contains multiple larvae by verifying under an inverted light microscope.
        i. PCR for 16S rRNA encoding gene will still be positive in sterile larval stocks due to the presence of dead *E. coli*.
    b. Obtain second aliquot and test in anaerobic conditions.
        i. LB plates or other nutritional media can be pre-reduced (oxygen removed) inside of an anaerobic chamber to allow vegetation of spores. See Alternatives note.
    c. Allow at least 2 days of incubation of test aliquot to assure cultures are sterile.
    d. Discard any contaminated stocks and proceed with infection.
13. Count infectious larvae.
    a. Count four aliquots of pooled sterile larval suspension and determine the average count. (Methods video S1 and S2). Troubleshooting.
        i. Infectious larvae will exhibit a twitching motion, but harvested worms may only display this a short time after disturbance in suspension.
    b. Using the average count number, resuspend the larvae to a concentration of 100–300 per 100 μL of sterile, autoclaved RO water.
    c. Infect germ-free mice by oral gavage using sterile equipment. Gavage 100 μL larval suspension to infect mice with 100–300 infectious larvae.
    d. After 3–5 days, ensure germ-free status of mice by DNA extraction of stool with Powersoil Pro Kit and subsequent endpoint PCR for bacterial 16S rRNA encoding gene (using primers 8f, 1492r whose sequences are detailed in resource tables above).[17]
        i. Repeat this sterility check throughout infection to ensure mice have not become contaminated.
        ii. In our hands, weekly checks are sufficient.

Alternatives: Sealed, robber topped YCFA tubes provide anaerobic testing conditions without the need for an anaerobic chamber.

Alternatives: Culture of infected mouse stool in aerobic and anaerobic conditions, Gram staining, or a combination of these techniques along with endpoint PCR for the 16S rRNA encoding gene is appropriate to assure sterility of infected germ-free mice.

EXPECTED OUTCOMES

Sterilization with a bleach solution is sufficient to remove contamination

Exposing isolated eggs to a 25% bleach solution for 5 min removes microbes originating from the stool of infected mice. The concentration of the bleach solution is optimized to kill microbes while leaving *H. polygyrus* eggs unharmed (Figure 5). To test the robustness of this sterilization step, *E. coli* and spores produced by *Clostridium sporogenes* were spiked into isolated eggs before sterilization by bleach. Compared to untreated eggs, bleached eggs appeared sterile by culturing in LB and YCFA broth aerobically and anaerobically, respectively (Table 2). These findings are in line with bacterial bleach assays which found *E. coli* and *C. sporogenes* spores were unable to withstand exposure to 25% bleach for 5 min when tested separately from *H. polygyrus* eggs (Table S1; Figure S1). The *C. sporogenes* spores used in these experiments were generated using 70:30 sporulation plates[18] and confirmed by Sanger sequencing of the 16S rRNA encoding gene (using primers 8F,1492R whose sequences are detailed in resource tables above).[17]

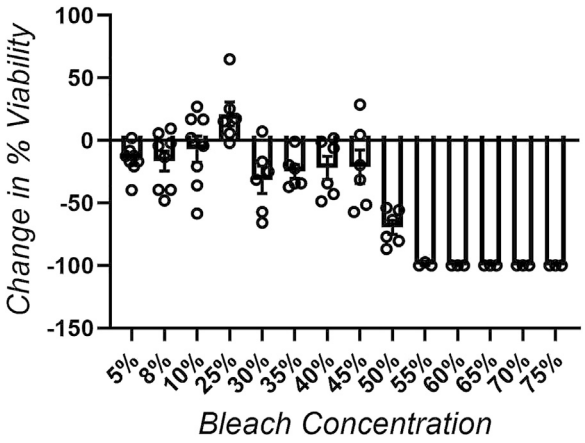

Figure 5. The effect of bleach on *H. polygyrus* larvae viability

The viability of larval cultures was determined by comparing the number of live worms collected after seven days of culture to the number of eggs plated per culture. Each treatment group was accompanied by a group treated with 0% bleach whose average viability was normalized to 100% allowing for a determination of the change in viability associated with bleaching the eggs. Mean with SEM.

Bleached eggs supplemented with *E. coli* develop similarly to conventionally grown larvae

Germ-free larvae grown by this procedure exhibit similar changes in size compared to conventionally cultured larvae (Figure 6). Heat-killed and sterile-filtered *E. coli* cultures did not spur larval growth, suggesting that live cells are required by *H. polygyrus* (Figure 6). Larval growth (size) was evaluated from images, where individual larvae were traced in ImageJ. These results were independently validated by another technician (Figure S2).

Germ-free larvae exhibit similar infectivity compared to conventional larvae

The ability of the germ-free larvae to infect SPF mice was compared to infection by conventionally cultured larvae (conv. larvae in SPF vs. GF larvae in SPF). Each group of mice was infected with approximately 300 infectious larvae. The intestinal worm burden of the germ-free larvae was indistinguishable from the conventionally cultured larvae at 14- and 28-days post-infection (Figures 7A and 7B). Similarly, McMaster counts at all days except the earliest time point surveyed (10 days post-infection) showed no marked difference between groups when considering the high degree of natural variability in this metric (Figure 7C). Egg production was also compared using the area under the curve defined by eggs per gram stool over time. No statistically significant differences were observed. Altogether, these results support the claim that germ-free larvae display similar infectivity to conventionally cultured larvae.

To demonstrate the sterility of larvae produced by the protocol, a group of germ-free mice was infected with approximately 200 infectious, germ-free larvae. The germ-free larvae used in this infection came from the same batch used to infect the SPF mice in the prior described results. The sterility of the mice across 28 days of infection was monitored weekly by extraction of stool and subsequent endpoint PCR of the full length 16s gene. If bacterial 16s is amplified, culture in YCFA and Gram stain can confirm whether the mice are contaminated. A productive infection was established in these germ-free mice post infection as shown by egg production throughout infection (Figure S3) and worm burden at day 28 post infection (Figure 7B). Mice retained their sterile status across the infection period.

Germ-free larvae elicit similar immune responses in mouse hosts

A successful parasitic immune response is often referred to as the "weep and sweep" response whereby goblet cell hyperplasia and recruitment of alternatively activated macrophages (AAMs) work together to expel the adult worms from the small intestine.[19] This response requires multiple

Table 2. Robustness of bleach sterilization step

E. coli dose	*C. sporogenes* dose	Growth in Luria broth	Growth in YCFA broth
6.95×10^8	8.79×10^4	No	No
Contamination error	8.79×10^4	No	No
2.23×10^8	8.79×10^4	No	No
2.17×10^{10}	8.79×10^4	No	No

The ability of the 25% bleach solution to kill contaminating microbes in the time frame given by the procedure was tested by a spike experiment. K-12 *E coli* grown at 37°C and 200 rpm for at least 15 h and spores isolated from *C. sporogenes* were both spiked into eggs isolated by the procedure prior to sterilization. A 250 μL aliquot of both bacterial suspensions was used as the spike volume. A 100 μL aliquot was removed from the eggs before and after the protocol bleach step and subsequent washes and spiked into LB broth aerobically and YCFA broth anaerobically. Unbleached egg sample spikes resulted in growth in both culturing conditions (related to step 4).

effector molecules. Expression of the STAT6-induced effector molecules Arginase-1 (*Arg1*), chitinase-like protein (Ym1, aka *Chil3)* , and Resistin-like molecule (RELM-alpha, aka *Retnla*) by AAMs has been found in cells isolated from the intestinal interface.[20] Additionally, increases in *Arg1* during *H. polygyrus* infection have been shown by qPCR in the spleen, peritoneal fluid, and duodenum[21,22] as well as staining of worm-induced intestinal granulomas.[23] Cells expressing RELMα were found to increase in *H. polygyrus*-infected mice by flow cytometry performed on duodenal digests[24] and staining of infected intestinal tissue.[23,25] Ym1 expression was also observed in granulomatous intestinal tissue at day 7 post *H. polygyrus* infection.[22] RELMβ, an effector molecule secreted by goblet cells, is similarly upregulated during *H. polygyrus* infection as shown by qPCR of infected duodenal tissue.[22] This molecule is important for worm expulsion as shown by increased worm burden in *H. polygyrus* infected genetic knockout mice.[26] Lastly, *H. polygyrus* induces goblet cell hyperplasia upon infection.[27] This can include secretion of *Muc5ac* which is not a component of the intestinal matrix at baseline.[28]

To validate that larvae generated by this protocol elicit a hallmark parasite immune response, the expression of genes encoding *Arg1* (for 5′-atgggcaacctgtgtccttt-3′, rev 5′-tctacgtctcgcaagccaat-3′), RELMα (Qiagen QuantiTect Primer Assay), RELMβ (for 5′-ctgatagtcccagggaacgc-3′, rev 5′-gtctgccagaagacgtgaca-3′), Ym1 (for 5′-aggaagccctcctaaggaca-3′, rev 5′-ctccacagattcttcctcaaaagc-3′), and *Muc5ac* (for 5′-tgtgcctgcctgtacaatgg-3′, rev 5′-ccagaacatgtgttggtgcag-3′) during conventional and germ-free larval *H. polygyrus* infection was determined by qPCR of infected and uninfected small intestinal tissue at days 14 and 28 post infection (Figures 8 and 9). These genes were normalized to the mouse housekeeping gene β-actin (for 5′-aaccctaaggccaaccgtgaa-3′, rev 5′-tcacgcacgatttccctctca-3′). There was no difference found between the expression of these molecules in SPF mice given either germ-free or conventional *H. polygyrus* larvae at either day 14 or 28 post infection as determined by a Dunn's rank mean test performed as a follow-up to non-parametric ANOVA (Kruskal-Wallis) performed in GraphPad PRISM 8 (Figures 8 and 9). Additionally, despite a smaller infectious dose, germ-free mice exhibit similar expression of these molecules to SPF mice at day 28 post infection (Figure 9).

LIMITATIONS

A major advantage of this protocol is its ability to be repeated several times across a conventional *H. polygyrus* infection cycle. However, the protocol is highly sensitive to contamination. Environmental microbes and antibiotic-resistant *E. coli* may lead to failed efforts on some plates. Contaminated cultures should be disposed of immediately. Users are advised to remain vigilant in microbial screening practices, sterile technique, and avoiding cross-contamination among plates. Fortunately, infected mice can generate source eggs for several weeks, providing a near-constant source of starting material. Increasing the number of plates can also promote isolation of contamination and reduce risk overall.

TROUBLESHOOTING

Problem 1

No eggs seen after isolation.

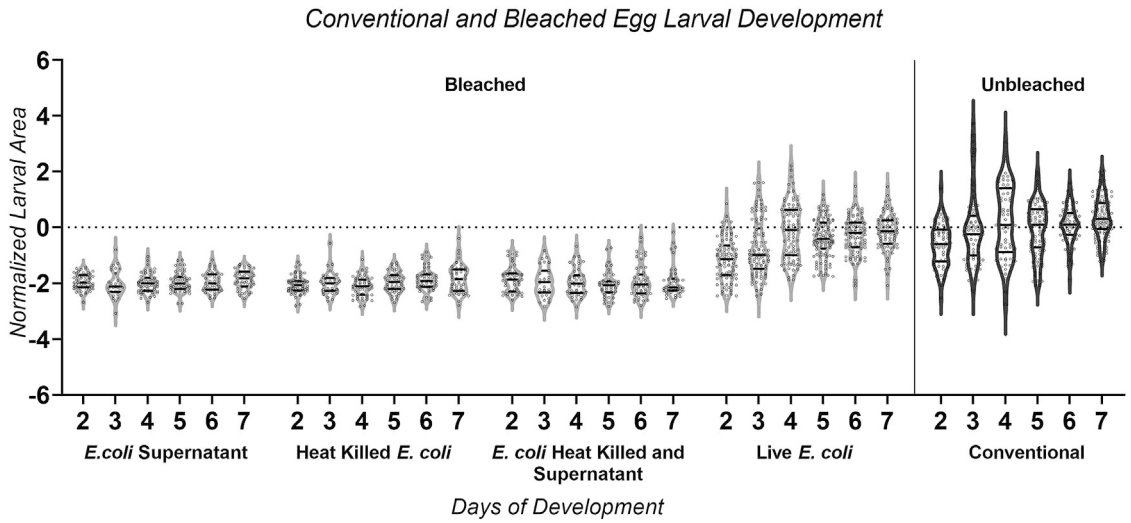

Figure 6. Comparison of larvae development from bleached or conventional eggs

Daily growth of conventionally cultured larvae was compared to larvae from sterilized eggs cultured on nematode growth agar plates (NGM) with live *E. coli* or sterile components of *E. coli* culture (heat-killed and/or sterile filtered culture broth). Samples of developing larvae were taken daily beginning on day 2 directly after larvae hatch. Larval samples were stored at 4°C and removed for viewing and photographing under an inverted light microscope with an attached camera (exact make and model: ZEISS Axioscope 5). The ImageJ freehand and analyze tools were used to trace and quantify individual worm areas on each picture. Larvae area was normalized to area measurements of a conventionally cultured larval developmental cycle.

Potential solution

- Ensure the presence of eggs before starting the procedure by examining a fecal slurry under an inverted light microscope. C57BL/6 mice used for farming *H. polygyrus* should be at least two weeks and no later than 16 weeks post infection with *H. polygyrus* (varies across genotypes).
- Ensure the sugar solution is approximately the correct density so that eggs float.
- Increase opening size of the wire mesh (if using 80-100) to ensure eggs are not caught on wire mesh (egg size ~75 μm by 50 μm[29]). Ensure eggs are passing through by viewing under an inverted light microscope (related to step 2).

Problem 2

Eggs do not hatch.

Potential solution

- Ensure bleach solution utilized is only 25% concentrated and eggs are exposed for only 5 min (related to step 4a).
- Immediately place egg suspension tube in the centrifuge after adding in bleach and remove bleach as soon as centrifugation ends (related to step 4c-d).
- Ensure there are enough eggs in culture to see hatched larvae. A proportion of eggs do not hatch naturally.

Problem 3

Few to no viable infectious larvae recovered.

Potential solution

- Check that NGM agar plate formulation is in accordance with the provided recipe and ensure plates are used within a 2-month time frame.

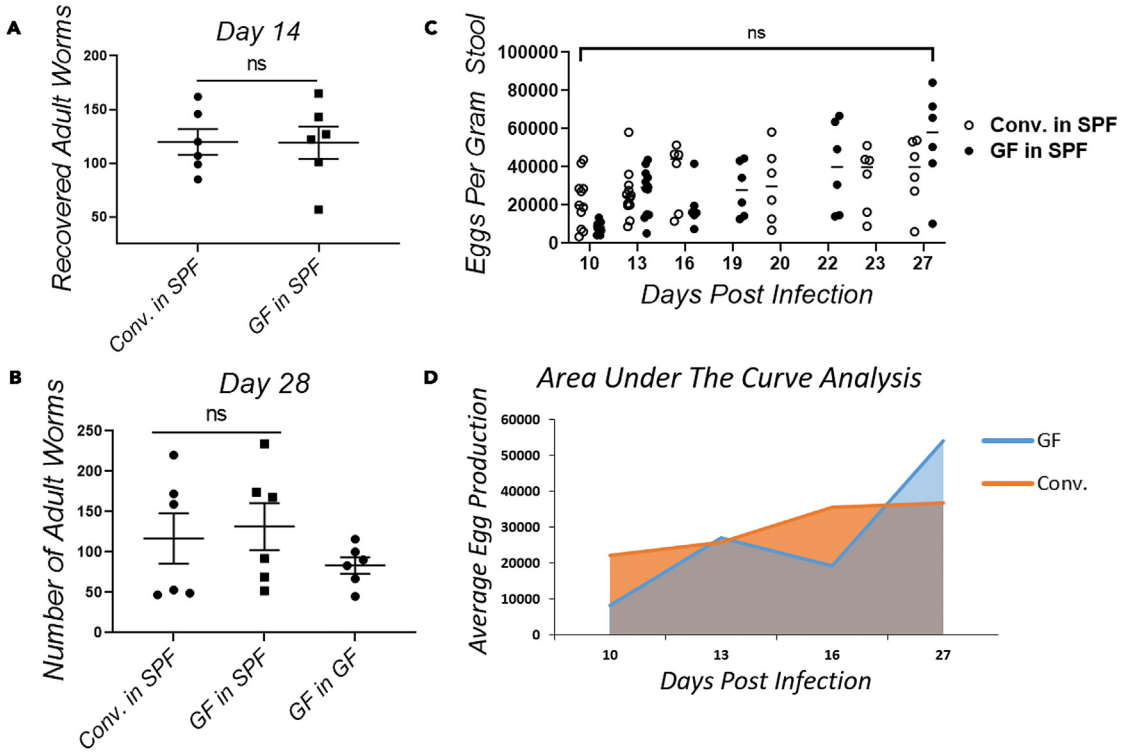

Figure 7. Comparison of helminth infection parameters in SPF C57BL/6 mice infected with conventional (conv.) or germ-free (GF) larvae developed using this protocol

(A and B) Worm burden at a given day post infection ($n = 6$). Small intestinal tissue from infected mice was flayed and draped over a parafilm hammock in a flat-bottomed 50 mL falcon tube filled with PBS. Worms fall out of the intestine over time. After 1–3 h of incubation at 37°C, the number of collected adult worms was quantified. Mean with SEM. B) A ratio of the number of adult worms collected to the initial larval dose for all three groups of infected mice. Differences between SPF groups assessed with a student's t-test. Mean with SEM performed in GraphPad PRISM 8.

(C) The number of eggs per gram stool produced by the larvae was determined by McMaster count across infection ($n = 12$ or 6 depending on day). Differences between groups tested with area under the curve analysis performed in GraphPad PRISM 8 ($p = 0.8700$) visualized in (D). ns ($p > 0.05$), * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$), **** ($p \leq 0.0001$).

- Check the old NGM plates after rinsing larvae off for washing. If there are many left on the old plate, more rigorous rinsing will be required (related to step 10b).
- Ensure that the pen/strep solution is no more concentrated than 100 units penicillin per mL and 100 mg streptomycin per mL or 200 units penicillin per mL and 200 mg streptomycin per mL on day 5 after washing the NGM plates.
- Do not centrifuge larvae at higher speeds than the 200 × *g* specified in the protocol.
- Larvae must be harvested after 7 days on the NGM culture plates and efforts ought to be made to minimize the storage of harvested larvae at 4°C (related to step 11).

Problem 4

Sterilized larvae failed to establish infection in mice.

Potential solution

- If not washed away, residual bleach in solution can kill the *E. coli* added for larval development. Larvae plated without the live *E. coli* will not fully develop and subsequently fail to establish infection (related to step 4d).
- If plating far more than 10,000 eggs per plate, ensure *E. coli* is not consumed by streaking culture liquid on an LB plate periodically throughout cultivation (related to step 7).

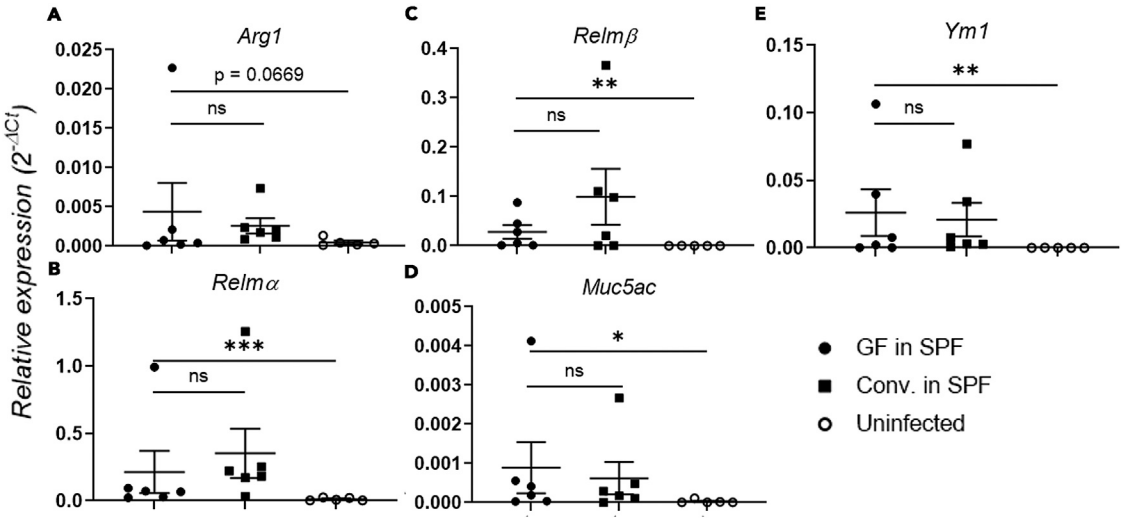

Figure 8. Type 2 immune response in C57BL/6 SPF mice 14 days post-infection with conventional (conv.) or germ-free (GF) larvae

(A–E) RNA isolated from the jejunum of infected (n = 6 per group) and uninfected (n = 5) mice was converted to cDNA by reverse-transcriptase and examined by qPCR for markers of anti-parasite immune response. Gene expression normalized to mouse β-actin. Mean with SEM. Differences between groups assessed with Kruskal-Wallis non-parametric ANOVA with Dunn's mean rank follow up test on the SPF mice. Mean with SEM. ns ($p > 0.05$), * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$), **** ($p \leq 0.0001$).

- Exposure to too much *E. coli* or concentrations of antibiotics outside the normal working range will harm larval infectivity. Ensure that steps for *E. coli* dilution are followed, *E. coli* is added the day after plating eggs, and pen/strep at the appropriate concentration is added on the second day of culture (related to step 6).
- Fully developed larvae display filariform characteristics and a twitching motion, though germ-free larvae tend to twitch less on average than those grown on fecal slurry. Mix aliquot with pipette or similar immediately before counting to ensure larval movement. Those larvae within the same batch that do not twitch as much ought to still be included in dose counting.
- Check larval viability before administration by oral gavage by viewing worms under an inverted light microscope and ensure that the dose falls in the range 100–300 infectious larvae per mouse (related to step 13a-c).

Problem 5

Larvae are not sterilized by the procedure.

Potential solution

- Ensure all steps past egg isolation are performed in a biosafety cabinet with proper aseptic technique. Check the sterility of reagents and materials used (related to step 2m).
- Test the potency of the pen/strep solution by spiking bacterial culture into tubes containing the working pen/strep concentration and ensuring there is no growth after at least 15 h of incubation at 37°C.
- Streak an LB plate with the sterilized egg solution before and after *E. coli* are spiked in to ensure that bleach treatment sterilized the eggs. In our hands, this has always been sufficient to kill off contaminating bacteria (related to step 7).
- Only 1 μL of *E. coli* culture diluted to an optical density at 600 nm between 0.3 – 0.4 should be added to every 1,000 eggs up to a maximum of 5 μL per plate (related to step 6).
- Ensure that cultured *E. coli* and prepared NGM plates are not exposed to unsterile environments which may introduce outside bacteria not sterilized by the procedure into larval cultures (related to step 6).

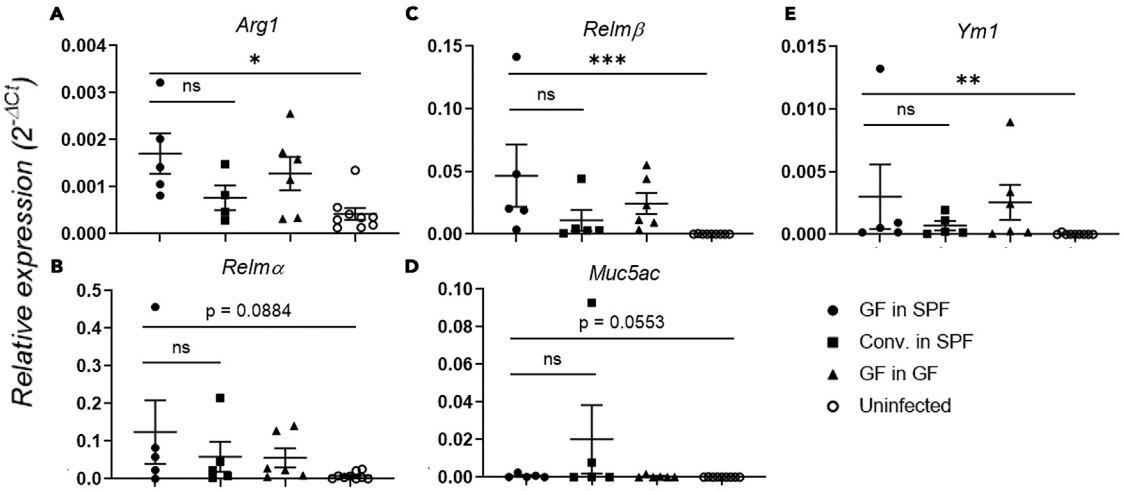

Figure 9. Immune response in C57BL/6 SPF and germ-free mice 28 days post-infection with conventional (conv.) or germ-free (GF) larvae

(A–E) RNA isolated from the jejunum of mice was converted to cDNA by reverse-transcriptase and examined by qPCR for markers of anti-parasite immune response. Gene expression normalized to mouse β-actin. Uninfected germ-free ($n = 6$) and SPF ($n = 5$) controls pooled. Mean with SEM. Differences between groups assessed with Kruskal-Wallis non-parametric ANOVA with Dunn's mean rank follow up test on the infected SPF mice. Mean with SEM. ns ($p > 0.05$), * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$), **** ($p \leq 0.0001$).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Seth Walk (seth.walk@montana.edu).

Technical contact

Further information and requests for clarification should be directed to and will be fulfilled by the technical contact, Karlin Blackwell (karlin.havlak@montana.edu).

Materials availability

This study did not generate any new reagents.

Data and code availability

Growth of larvae over culture time was generated using free software platform ImageJ version Java 1.8.0_172 [64-bit].

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103144.

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AUTHOR CONTRIBUTIONS

K.H.B. developed and optimized the methodology, performed validatory experiments including helminth infection, and wrote the protocol. L.R.P. helped with preliminary work establishing the protocol culturing method. H.M.G.W. and J.C.B. assisted in mouse experiments and harvests. E.M.G. helped troubleshoot the protocol and provided additional growth curve analyses. S.T.W. provided the germ-free mice. D.J.K. and S.T.W. conceived the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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