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Embryo collection, cultivation, and gene silencing via zygotic RNAi in harvestman embryos

INTRODUCTION

The spider (*Cupiennius* and *Parasteatoda*) and acariform mite (*Archegozetes* and *Tetranychus*) models previously advanced for developmental study are useful for contrasting developmental processes between chelicerates—the ramus of the arthropod tree that includes horseshoe crabs, spiders, mites, and scorpions—and mandibulates—the remaining arthropods. But representation of two arachnid orders alone does not suffice to enable polarization of developmental character states within Chelicerata; this requires representation of at least three lineages spanning the most recent common ancestor of Arachnida. The harvestman *Phalangium opilio*, also called the brown daddy-long-legs, offers promising prospects for conducting comparative developmental studies within chelicerates, due to (a) phylogenetic placement with respect to spiders and acariform mites (the most recent common ancestor of the three corresponds to the arachnid ancestor), and (b) retention of various plesiomorphies not present in spiders or acariform mites. Gene expression studies in the harvestman have facilitated inference of conserved and divergent processes in arachnid body and appendage patterning (Sharma et al., 2012a, b), and in turn engendered hypotheses of gene function that require testing, ideally by gene silencing. This protocol describes techniques for embryo collection and gene silencing in *P. opilio* via zygotic RNA interference (RNAi), i.e., embryonic injection of double-stranded RNA (dsRNA).

RELATED INFORMATION

The format and methods presented here are based on the protocol for gene silencing in the spider *Cupiennius salei*, by Prpic et al. (2008).

MATERIALS

Reagents

Bleach solution (Clorox, diluted to 50% in distilled water)
Double-sided Scotch tape
dsRNA against target gene (synthesis using Ambion T7 kit recommended)
Halocarbon-700 oil (Sigma-Aldrich)
Heptane (100%; Sigma-Aldrich)
Phenol red or food-grade dye
Water, RNase-free

Equipment

Agar plates, 2%
Capillaries, 1/0.58mm, 1B100F-4 (World Precision Instruments)
Capillary puller (e.g., P-97 micropipette puller; Sutter Instruments Co.)
Capillary-filling pipette tips (Eppendorf)
Cotton
Coverslips (22 × 22 mm)
Eppendorf tubes, 1.5 mL
Forceps, coarse and fine
Ice
Incubator, set to 28 °C
Microinjector (e.g., IM 300, Narashige)
Micromanipulator (e.g., MMO-202ND, Narashige)
Paper tissue
Petri dishes (various sizes)
Pipettes (Pasteur, plastic)
Plastic containers (e.g., Ziploc, large rectangle)
Platform shaker
Soil, sterile (potting)
Stereomicroscope
Whatman filter paper
Worm pick (or eyelash/fine brush tool)

METHODS

Embryo collection

1. Place thin layer of potting soil on the bottom of plastic container. Add cotton balls soaked with distilled water to corners of container.

2. Hand collect adults of *P. opilio* and place in plastic container.

    a. Collecting is best conducted at night with a flashlight, between 2100 and 0300 hours. *P. opilio* are often found by the sides of houses, parking lots, or other disturbed localities.

    b. *Identification: P. opilio* is a large brown species (body of female up to 10 mm) with long legs, identified by the sinuous dark markings on the dorsum.

    c. *Sexing:* Males of *P. opilio* have cheliceral horns and longer legs; females have no cheliceral horns and larger bodies.

Male

Female

    d. Females of *P. opilio* are communal. Keep one male per 3-5 females in each container. Keeping multiple males in a small space will result in male-male combat and the death of the weaker male.

3. Keep container closed and away from the light to maintain humidity (add damp cotton as needed, or place in humidified room).

4. Feed *ad libitum*; adults will feed and drink water every day. Freshly killed soft-bodied arthropods work best.

5. Visually inspect underside of container to spot clutches. Clutches will appear as large clusters of >1 mm, yellow-white eggs. A healthy female will lay clutches of 60-200 eggs every few days.

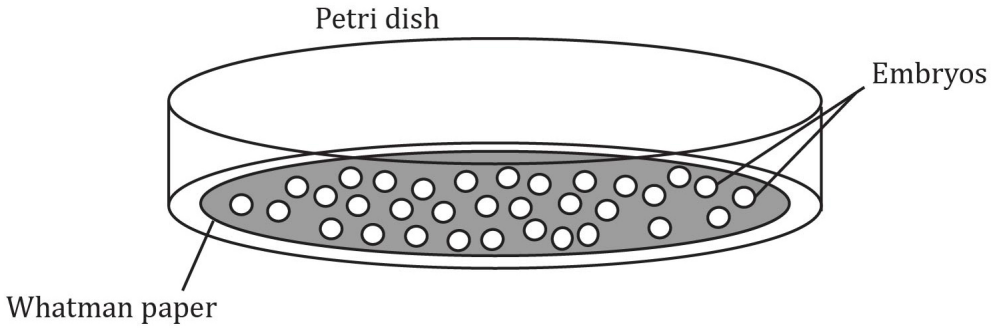
Embryo cultivation

1. Cut a circle of Whatman paper *ca*. 2 cm in diameter and place it within a 2 cm Petri dish. Fully moisten Whatman paper with distilled water.

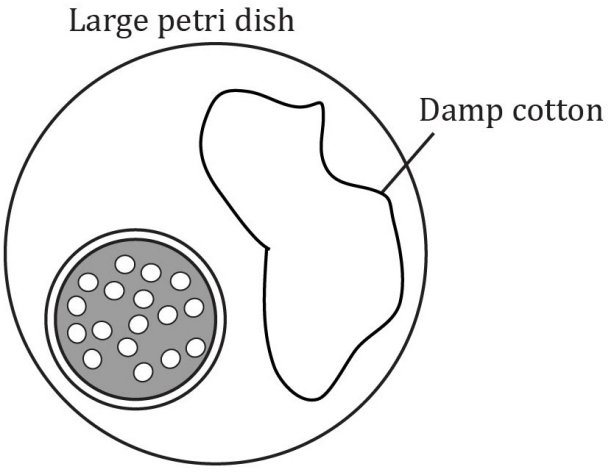
2. Remove adults from container temporarily. With forceps, dig away soil over the concealed clutch, being careful not to displace embryos.

3. Transfer embryos to Whatman paper with forceps, touching the tips of the forceps to wet cotton between each transfer. Embryos will adhere to sides of wet forceps; grasping embryos by forceps can break embryos.

4. Keep embryos slightly apart from each other to minimize losses in the event of fungal attack. The setup will resemble the schematic below.



5. Cover the petri dish and place it in a larger petri dish. Surround with soaked cotton wad to maintain humidity and place in 28 °C incubator.



6. Every 1-2 days: Monitor and remove dead eggs. Keep Whatman paper moist.

7. Every 18 hours: remove 3-5 eggs from clutch and dechorionate under a stereomicroscope using weak bleach solution to check clutch stage

Preparation for injection

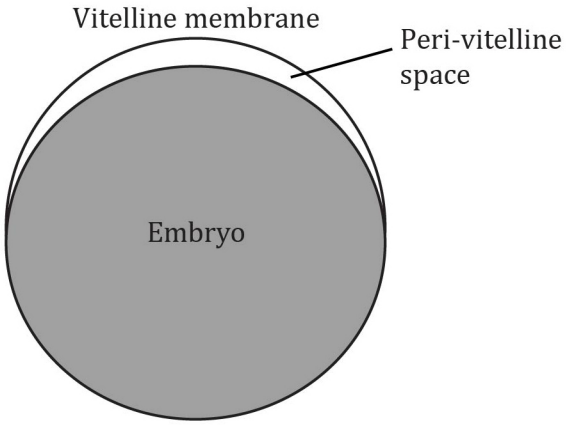
1. While working under a hood with protective gear: Place large amount of double-sided tape into a bottle of heptane (as much as can be accommodated in bottle) and rotate bottle on platform shaker for 3-4 hours.

2. While working under a hood with protective gear: Remove tape from bottle and discard (heptane solid waste). "Heptane glue" should now act as a volatile liquid adhesive.

3. While working under a hood with protective gear: Pipette a few drops of heptane glue onto 22 × 22 mm coverslips and wait until dry (2-4 minutes).

4. Repeat Step 3 to make two coats of adhesive. When dry, coverslip should stick instantly to inert dry surfaces (e.g., latex gloves) and be difficult to extricate. Add additional coats as needed.

5. Clutch is ready for injection when sampled embryos show a peri-vitelline space between the embryonic tissue and the vitelline membrane (2-4 days after egg-laying).



6. Dechorionate single clutch in weak bleach solution for 5-7 minutes. Swirling bleach gently will aid in separating chorion from embryos.

7. Wash embryos repeatedly with distilled water.

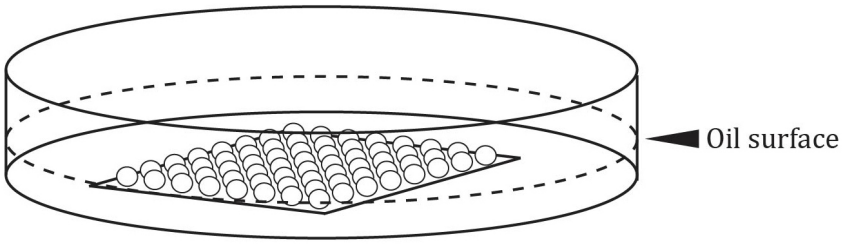
8. Gently transfer embryos to agar dish. Pipette away as much liquid as possible and remove residual water with paper tissue.

9. Working quickly, arrange embryos in 8 × 8 grid using a worm pick or other fine tool. Place coverslip (sticky side down) onto square of embryos and apply light pressure. Eggs will adhere to cover slip.

10. Rest coverslip (with eggs pointing up) in a dry area for 25 min at room temperature. Excess dehydration will decrease embryo survivorship.

11. Repeat Steps 9-10 until all embryos of the clutch are on coverslips in 8 × 8 grids.

12. Place each cover slip (with eggs pointing up) into a small petri dish and slowly add halocarbon-700 oil until eggs are just covered with oil surface.



dsRNA injection

1. Couple microinjector with micromanipulator.

2. Dilute dsRNA for target gene to 3.5-4 μg/μL. Keep on ice.

3. Add 1/20 volume of phenol red or food grade dye to dsRNA. Mix by flicking tube and spinning down. When finished, keep on ice.

4. Load capillary with a capillary-filling pipette tip.

5. Break needle with fine forceps under oil and adjust pressure as needed. Breaking point is empirically determined, but needle should be stout (due to resilience of *P. opilio* vitelline membrane) and needle tip should be beveled for best effect.

6. Bring petri dish with oil-covered eggs into view with needle. Keep needle tip height at approximately half of egg height.

7. Insert needle into egg's peri-vitelline space, avoiding contact with embryo. Peri-vitelline fluid should enter needle or escape into oil or both. Inject dsRNA slowly into peri-vitelline space until egg resists further injection.

8. Withdraw needle slowly. Some of injected material will escape due to internal pressure of embryo.

9. **Inject extra dsRNA into oil just outside needle contact site right before withdrawing needle.** Because eggs of Eupnoi harvestmen grow slightly (become turgid) during development by absorption of moisture outside the egg, the dsRNA droplet outside the egg will be absorbed over several hours, compensating for the dsRNA that escapes out of the peri-vitelline space.

10. Hereafter, move petri dish with eggs very slowly for 2-4 days. Droplets of dsRNA outside of eggs must remain in contact with egg surface as long as possible. If food-grade dye is used to visualize injection, the yolks of injected embryos will turn the color of the dye, indicating degree of dsRNA uptake.

11. After injections are complete, keep oil-covered eggs in a larger petri dish and surrounded by soaked cotton at room temperature for 1 hr.

12. Place large petri dish containing smaller petri dish in 28 °C incubator. Monitor as needed for continuation of experiment.

Retrieving embryos

1. When embryos attain desired stage of development, pour off as much oil as possible.

2. Wash several times for 4-5 minutes with heptane on platform shaker to remove adhesive. Eggs should roll back and forth when free from adhesive.

3. Wash repeatedly with water. Continue normal fixation of embryos as described by Sharma et al. (2012a).

Troubleshooting

Problem: Cannot find *P. opilio* during collections.
Solutions:

a.) Check distribution records of *P. opilio* at your research site. Museum collections and locality records will be helpful in identifying collecting sites.
b.) Check season of *P. opilio* at your research site. In northeastern USA, the season is from mid-May to late August. There will be some variation in seasonality throughout its range, e.g., in Europe, Japan, or New Zealand. *P. opilio* overwinters as eggs and juveniles, but these are difficult to locate out of season.

Problem: Females of *P. opilio* will not lay eggs.

a.) Check season of *P. opilio* at your research site.
b.) Feed more generously. A trio of large females can consume most of a cricket of 1-1.5 cm length per day.
c.) Keep container humid, make sure drinking water is available in the form of soaked cotton.

Problem: Females of *P. opilio* die rapidly.

a.) Keep container clean of debris and remains of consumed arthropods.
b.) Check for fungus or mite parasites. Discard infected *P. opilio*.
c.) Collect more females. As *P. opilio* is a seasonal species, females continuously lay large clutches of eggs and die routinely throughout the season.

Problem: Clutches die from dehydration.
Solutions:

a.) Moisten Whatman paper more frequently.
b.) Place containers of water at the bottom of the incubator to add humidity.

Problem: Clutches die from fungal attack.
Solutions:

a.) Moisten Whatman paper less frequently.
b.) Check clutches daily, removing fungus-infested eggs when they appear.
c.) Keep eggs separated or evenly spaced to limit spread of fungus from ruptured and decaying eggs.

Problem: Embryo collapses after dechorionation, during washes with water.
Solutions:

a.) Embryo may have been dead or punctured during transfer. When transferring, do not grasp eggs with forceps, but wet tips of forceps and touch side of eggs. Eggs will adhere to forceps during transfer to Whatman paper.
b.) Embryo may be too young. Prior to 2-3 days of development, the vitelline membrane is not fully rigid and will rupture in hypotonic solution (seen as yolk slowly escaping when washed into distilled water). Test with 3-5 embryos at a time to see if dechorionation is successful. If not, wait 18 hours.

Problem: High embryo mortality.
Solutions:

a.) Use the healthiest clutches from large females, early in the season. Smaller females will lay smaller clutches (and with smaller eggs in the clutch) with higher intrinsic clutch mortality. Toward the end of the season, clutch quality declines rapidly.
b.) Use finer needles. The finer the needle, the more difficult it is to insert tip into the vitelline membrane. Keep the tip beveled and find the right balance between needle width and rigidity.
c.) If injection is displacing embryo (i.e., parts of embryo escaping into oil, needle tip touches embryo), inject more slowly and add more dsRNA in the droplet outside the injection site than inside the peri-vitelline space.

Problem: Contents of egg flood needle during initial insertion.
Solutions:

a.) If contents entering the needle are limited, continue injection and displace contents back into egg.
b.) If contents entering the needle are voluminous, withdraw needle to surface of oil, expel contents, and reinsert needle at same angle and site into the embryo. Continue injection.

Problem: Low penetrance of RNAi phenotypes.
Solutions:

a.) Increase concentration of dsRNA.
b.) Increase volume of injected dsRNA.

Problem: Injected clutches develop asynchronously.
Solutions:

a.) This is a consequence of variable uptake in dsRNA (embryos that uptake more dsRNA develop more slowly). Measure and standardize injected quantity of dsRNA, including droplet outside of egg.
b.) Fix individual embryos at desired stage, rather than the entire clutch at once. To remove selected eggs from cover slip, use forceps to grasp and pull out underlying adhesive. Place egg and attached adhesive into heptane and wash briefly on platform shaker. Then wash with water. Continue fixation as before.

Acknowledgements

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References

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