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Time-to-Full Engagement of Precopulatory Pairing in *Parhyale hawaiiensis* following Phenanthrene Exposure

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Protocol status: Working

We use this protocol and it's working



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Disclaimer

The protocol provided here is intended for educational purposes only and should be conducted in appropriate laboratory settings. The developers of this protocol are not liable for any damages or consequences arising from its use. Users should ensure compliance with relevant safety regulations and ethical guidelines when conducting experiments involving live organisms.

Abstract

This protocol describes a time-to-event assay designed to assess the impact of phenanthrene exposure on precopulatory behaviour in the tropical amphipod *Parhyale hawaiiensis*. Pairs with a prior mating history are individually exposed to graded concentrations of phenanthrene and subsequently reintroduced to quantify latency to re-engage in amplexus. Using survival analysis, this method enables the detection of sublethal, behaviourally mediated effects of contaminant exposure on reproductive function. The approach is cost-effective, reproducible, and suitable for screening chemosensory disruption in small crustaceans.

Guidelines

Experimental Design

- **Sample size:** ≥ 60 naturally precopulating pairs selected at random.
- **Parallel groups:** Five exposure groups (phenanthrene at 10, 50, 100 $\mu\text{g L}^{-1}$, solvent control) plus seawater control; minimum $n = 11$ pairs per group.
- **Blinding:** Observer blind to treatment assignment during pairing assays.
- **Environmental standardisation:** Photoperiod 12 h:12 h light:dark; salinity 30 ± 2 ppt; feed withheld 6 h prior to assay.

Procedure

1. Selection and Separation

- 1.1. Identify and record 55–60 actively engaged precopulatory pairs in culture.
- 1.2. Transfer each pair to a separate 20 mL glass vial; label as “T0” and note separation time (Day 0).
- 1.3. Maintain and feed pairs in clean seawater until natural separation occurs (typically 2–4 days).

2. Egg Shedding and Isolation

- 2.1. Immediately upon separation, transfer male (T1M) and female (T1F) partners into new labelled vials.
- 2.2. Hold females for 4 days to allow oviposition and egg release, feeding ad libitum.
- 2.3. Acclimate males and females separately for 24 h without feeding prior to exposure.

3. Exposure Phase

- 3.1. Prepare exposure vials (20 mL) for each treatment.
- 3.2. Allocate individual animals (one per vial) to treatments, ensuring balanced sex distribution.
- 3.3. Expose for 24 h; renew media twice to maintain target concentrations.
- 3.4. Monitor mortality daily; remove and record any deceased individuals (censor these in analysis).

4. Pre-Assay Handling

- 4.1. At exposure end, rinse each individual in clean seawater for 2 min to remove residual contaminants.
- 4.2. Acclimate both partners separately in 20 mL vials for 6 min at assay temperature.

5. Time-to-Full Engagement Assay

- 5.1. At “Time 0”, gently transfer the exposed male and female of each original pair into the same Petri dish.
- 5.2. Observe for up to 96 h (4 days). NB: Conduct observation every hour between 07:30 and 21:30 h (14 assessments per day). Pairs that had not re-formed amplexus during the day will be re-assessed at 07:30 h the following morning.
- 5.3. Define event as the male clasping the female in a stable amplexus posture lasting ≥ 5 min.
- 5.4. Record for each pair:
 - **Start_Time** (h post-pairing) = 0 h
 - **End_Time** = time of event occurrence or 96 h
 - **Response_Time** = End_Time – Start_Time
 - **Status** = 1 if event occurred; 0 if censored (no event by 96 h or partner mortality).
 - **Impairment Status:** 1 = latency >48 h; 0 = ≤ 48 h.

6. Data Management

- 6.1. Maintain a master spreadsheet with columns: Experiment_ID, Treatment, Pair_ID, Sex, Concentration ($\mu\text{g L}^{-1}$), Start_Time, End_Time, Response_Time (h), Status.
- 6.2. Validate data entries; double-check outliers.

7. Statistical Analysis

- 7.1. Use Kaplan–Meier survival curves to visualise latency distributions by treatment.
- 7.2. Fit Cox proportional hazards models including predictors: treatment, sex (male or female “responder”), feeding rate during exposure, and moulting frequency (if recorded).
- 7.3. Test proportional hazards assumption (e.g., Schoenfeld residuals).
- 7.4. Report hazard ratios with 95 % confidence intervals and p-values.

Critical Notes

- Ensure observer is unaware of treatment codes to mitigate bias in event determination.
- Record water quality parameters (pH, DO, temperature) at each media renewal.
- Consider video-recording assays to enable retrospective verification of event timing.
- Censoring at 96 h accommodates both delayed responders and non-responders, improving statistical power.

Materials

1. Laboratory-reared adult *Parhyale hawaiiensis* (sexed under stereomicroscope)
2. Phenanthrene stock (e.g., 5 g L⁻¹ in acetone)
3. Exposure media:
 - Phenanthrene working solutions (e.g., 1, 10, 100 µg L⁻¹)
 - Solvent control (acetone at same final concentration)
 - Seawater control
4. 100 mL beakers and 20 mL clear glass vials (labelled by timepoint and sex, e.g., T1M, T1F)
5. 70 mL glass Petri dishes
6. Aeration and temperature-controlled seawater bath (maintained at 27 ± 2°C)
7. Labelled plastic tags for individual identification
8. Pipettes: used for transferring organisms and exposure media
9. Timer/calendar for long-term observations
10. Data-logging software or spreadsheet (to record start, end, response time, status)
11. Statistical software (e.g., R) for Kaplan–Meier and Cox model fitting

Before start

Objective: To quantify the latency to complete precopulatory pairing (“full engagement”) in *P. hawaiiensis* pairs previously mated, after exposure of isolated partners to graded concentrations of phenanthrene, using a time-to-event framework with appropriate censoring.

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Protocol references

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