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Version 2

## Protocol for behavioural screening of shrimps (*Penaeus vannamei*) during anaesthesia method testing V.2

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**We are still developing and optimizing this protocol**

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## Disclaimer

The authors acknowledge that, by definition, an anaesthetic is a substance (chemical or physical) that induces a temporary loss of sensation or awareness. However, the use of the term 'anaesthetic' throughout the protocol may not fully align with this definition, as additional measures beyond behavioural observations are required to confirm substance as such.

## Abstract

This protocol was designed to standardize the testing of anaesthetics methods in crustaceans, specifically by assessing the different stages observed during and after the application through behavioural observations. Although it has been used to trial anaesthetics dissolved in water, it can be adapted for injected or physically applied agents. The protocol has been fine-tuned for *P. vannamei* however we think it can be used for other species of *Penaeus* spp.

## Guidelines

This protocol aims to minimize animal handling by avoiding transfer between different containers, enabling observation of undisturbed behaviour through video recordings. This approach facilitates the identification of anaesthetic stages described by de Souza (2022), based on behavioural cues. It also promotes standardization, given the wide range of physical and chemical agents previously trialled in crustaceans (Rotllant *et al.*, 2023). Establishing a protocol for anaesthesia and analgesia in decapod crustaceans, from an ethical point of view, is essential to ensure the humane handling and slaughter of experimental animals, aside from their recent recognition as sentient beings (Elwood, 2025).

## Materials

### Aquaria and volume manipulation

- Aquaria (big enough to allow the shrimp to move freely at every possible direction).
- Running seawater (before and after the method testing phase).

### Chemicals used

- When testing an anaesthetic (chemical or physical) that has been used on other or similar species, start with the working concentration found in literature (e.g. Rotllant *et al.*, 2023).
- If it is a new or unknown working substance, make sure it meets International regulatory bodies and frameworks (WHO, UNEP, ECHA, FAO).

### Other materials

- Camera for video recording
- Stopwatch
- Suction pump or tubes
- Funnel
- Blunt and smooth-tipped tweezers
- Coloured background to maximize video recording contrast

## Safety warnings

### Ethic statement

This protocol requires prior approval by the users' Institutional Animal Care and Use Committee (IACUC) or local equivalent ethics committee.

In case an abrupt/harsh response is observed when the anaesthetic is poured, remove the animal immediately and place it in other aquaria with fresh seawater. If the aggression persists, euthanize/dispatch the animal in accordance to local regulations.

## Ethics statement

This protocol requires prior approval by the users' Institutional Animal Care and Use Committee (IACUC) or local equivalent ethics committee.

## Before start

1. Mark the aquaria on the desired water levels, to make sure volume is maintained between a minimum (to avoid air exposing the shrimps) and a maximum working volume to be considered for the final anaesthetic concentration.
2. Once the working volumes are known, prepare the anaesthetics, taking into account the final volume used in the aquaria.
3. Finally, take the shrimp from the maintenance tanks and place them in individual aquaria for acclimatization (8-12 h, or Over night, ON) without feeding it.

## Experimental setup

- 1 Place the recording camera at a distance and angle to have a full view of the shrimps and their movement (Fig. 1). Make sure there is enough recording memory and battery (if the camera isn't plugged) and safe from any seawater splash.



**Fig. 1.** Experimental setup including aquaria, shrimp acclimatised and camera recording the full aquaria.

## Procedure: Behavioural screening of shrimps under anaesthesia methods

- 2 Start by recording for 5 min to check for activity and any abnormalities on the shrimp's behaviour. Later stop the inflow of seawater (Fig. 2A).

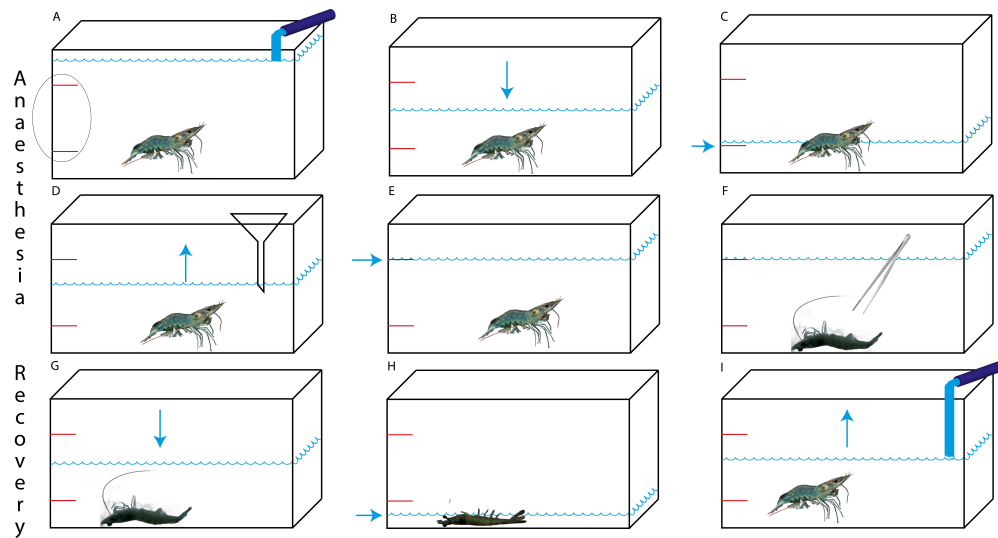


Fig. 2. Experimental protocol to evaluate potential anaesthesia induction and later recovery in the shrimp *Penaeus vannamei*. Shrimp acclimatized to aquaria with running seawater and level marks (A). Lowering seawater level through multiple tubes (B). Minimum seawater level, to avoid shrimp from being air exposed (C). Gentle pour of the anaesthetic solution through a funnel (D). Desired final volume of anaesthetic by checking the aquaria level (E). Loss of shrimp response to tactile stimuli (F). Removal of anaesthetic (G). Minimum water level to ensure the maximum anaesthetic elimination (H). Fresh seawater poured quickly but gently, reconnecting seawater influx, to let the shrimps recover (I).

- 3 Once checked, continue by lowering the seawater level in the aquaria with a tube to withdraw the water, keeping a minimum to avoid air exposure (Fig. 2B&2C).
- 4 With the help of a funnel start placing the anaesthetic solution up to the desired water level (Fig. 2D&E). This volume should contain the desired final anaesthetic concentration.
- 5 Start the stopwatch and the registration of all behaviour, both by writing and voice recording.
- 6 When the shrimp stops to move then touch them gently with a tweezer, starting with the antenna and appendices and at the end point tweeze the tail until no movement is observed and *surgical* anaesthesia is reached (Fig. 2F).
- 7 To recover from this state and distinguish from an irreversible condition, start by removing the seawater with anaesthesia from the aquaria, trying to take as much as possible (Fig. 2G&H). Keep the time running.
- 8 When the least amount of seawater is left, add gently fresh seawater (Fig. 2I) and place the incoming seawater tube back into the aquarium (allowing for overflow and constant change).



- 9 Register any behavioural changes as the shrimp returns to a behaviour similar to the one before the anaesthesia was added.

## Annotation of behaviours

- 10 Note all movements and behaviours including their orientation, spasms, body contractions, stillness, loss of balance, movement of appendices and overall colour.

## Secondary observation

- 11 Once all the registered behaviours are associated with their time of appearance and duration, it is imperative that an independent observer verifies their legitimacy. To do so, the observer should be provided with a description of the type of registered behaviours and re-watch the recording using a stopwatch, with the sound turned off.

## Protocol references

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