Aquaculture 298 (2009) 162–167

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Anesthetic effect of eugenol and menthol on handling stress in *Macrobrachium rosenbergii*

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ARTICLE INFO

Article history:
Received 27 May 2009
Received in revised form 5 September 2009
Accepted 15 October 2009

Keywords:
Eugenol
Menthol
Handling
Stress
M. rosenbergii

ABSTRACT

A combination of eugenol and menthol was tested for mitigating handling stress of adult *Macrobrachium rosenbergii*. Prawns were first exposed to four different doses (100, 200, 400 and 800 μL L^{-1}) of eugenol and menthol; both individually and in combination. The dose at which induction and recovery time less than 30 min was regarded as the critical dose. It was demonstrated that 200 μL L^{-1} dose was sufficient for induction, recovery and significantly ($p<0.05$) lower oxygen consumption rate. Further, metabolic responses [glucose, lactate dehydrogenase (LDH), malate dehydrogenase (MDH), alanine amino transferase (ALT) of *M. rosenbergii* were higher when exposed to 30 min handling stress alone (T_1), over (T_2) when exposed to stress in the presence of the anesthetic mixture. Lower levels of glycogen and acetylcholine esterase (AchE)] were observed in T_1 over T_2, indicating secondary metabolic responses to cope up with the stress due to handling. A control (without handling stress and sampling without anesthetic exposure) was maintained to compare the effect of handling stress. In order to ensure its margin of safety, accumulation and clearance of the anesthetic residues were tested from the muscle of *M. rosenbergii*, which indicated that eugenol and menthol accumulate in tissues of *M. rosenbergii*. However, 24 h purging protocol cleared the residues completely from the tissues of *M. rosenbergii.* Overall results indicate that eugenol and menthol based anesthetic formulation is safe and effective for handling of adult *M. rosenbergii.*

1. Introduction

Capture, handling, crowding, confinement and transport are all components of aquaculture, which influence the physiological stress response (Barton et al., 2000; Davis, 2004; Jentoft et al., 2005) in various fishes and shellfishes. It is generally accepted that the animal respond to these stressors, by a phenomenon called general adaptation syndrome (GAS) (Barton et al., 2000). In crustaceans, the primary effect of GAS involves the increased activity of crustacean hyperglycemic hormone under stressful conditions resulting in secondary changes (elevated glucose levels and changes in metabolic enzymes) (Jørgensen et al., 2002) ultimately result in tertiary effects, which are manifested as gross changes in physiological performance of the whole animal (Thompson et al., 1993). Different strategies have been proposed as being potentially beneficial for reducing the physiological effects of stress in prawns during handling, of which anesthetic agents assume importance. Of the different anesthetics approved by USFDA for aquaculture, tricane methane sulfonate (MS222) is not effective in many crustaceans (Coyle et al., 2004). There are no reports available on the use of carbon dioxide for anaesthetizing crustaceans. However, cold anaesthetization using chilled saw dust has been found efficient in *Macrobrachium rosenbergii* for short-term transportation (Salin, 2005). MS222 is reported to cause occupational hazard (retinopathy) to the users and has a 21-day withdrawal period before the product can be consumed (Smith et al., 1999). Recently, there has been a growing interest in using eugenol/clove oil as an anesthetic for aquaculture use (Anderson et al., 1997; Stone and Tostin, 1999). Aqui S, an anesthetic, comprised primarily of iso-eugenol, has been registered for aquaculture (Stehly and Gingerich, 1999; Davidson et al., 2000). Clove oil and Aqui S were found to induce anesthesia efficiently at lower concentrations than quinaldine in the freshwater prawn, *M. rosenbergii* (Coyle et al., 2005). Eugenol is miscible in water at temperature below 16 °C (Soto and Burhanuddin, 1995). At higher temperatures, eugenol appears as beads at the bottom of water column (immiscible), owing to its high relative density. The second anesthetic in our study, menthol, derived from peppermint, *Mentha arvensis* has been used as a local anesthetic for humans and often masks disagreeable tastes of drugs, chewing gums and candies. Our observation indicates that menthol is partially miscible in water and forms a scum over the water surface when mixed in water, which may affect the diffusion of atmospheric oxygen in water (unpublished data), if added alone, owing to its low relative density. Menthol is reported to induce mild anesthesia of flat oyster, *Ostrea edulis*, when applied at the rate 2% (Culloty and Mulcahy, 1992).

Glucose continues to increase for a longer period after the onset of stress (Strange, 1980) to cope up with the energy demand due to stress

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doi:10.1016/j.aquaculture.2009.10.020

(Barton and Schreck, 1987; Vijayan and Moon, 1994). Hyperglycemia during stress is under the influence of crustacean hyperglycemic hormone in crustaceans. However, the assay of glucose is simpler over crustacean hyperglycemic hormone in *M. rosenbergii*. One of our earlier studies established various responses to monitor the effect of stress viz., circulating glucose, glycogen, metabolic enzymes in crustaceans (Manush et al., 2005). In the present investigation, the effectiveness of the mixture of eugenol and menthol was tested by (1) finding the critical dose for induction and recovery and the difference in oxygen consumption rate of both individual components and combinational formulation (2) assessing the metabolic enzyme profiles of *M. rosenbergii* to handling in the presence of eugenol, menthol and its combination (3) assessing the accumulation and clearance of anesthetic formulation in muscle of *M. rosenbergii*.

2. Materials and methods

2.1. Experimental animals

Inter-molt adult *M. rosenbergii* (mean weight ± S.E = 32 g ± 2.1) were used for all the experiments without regard to gender. Prawns cultured at Bhivandi farm, Mumbai were brought in aerated open containers to Wet Laboratory, Central Institute of Fisheries Education, Mumbai and held in fibre glass reinforced plastic pools (500 L capacity) for 15 days at ambient water temperature (30 °C). During this period, prawns were fed with supplementary feed (25% crude protein) before being used in experiments.

2.2. Preparation of anesthetic formulation

Eugenol is denser (relative density—1.1) and menthol is lighter (relative density—0.9) in freshwater. Eugenol and menthol were diluted in ethanol such that the mixture forms an emulsion in water. Therefore, 6 mL eugenol and 1 g menthol were mixed with 3 mL ethanol and applied to the rearing water at different concentrations and determined the minimum concentration to induce anesthesia. Eugenol and menthol were diluted separately in absolute alcohol in similar proportions for assessing the individual anesthetic effects during handling of prawns.

2.3. Comparative efficacy of eugenol, menthol and combinational formulation

Ninety six prawns were maintained in four different doses (100, 200, 400 and 800 μL L^{-1}) of eugenol, menthol and its mixture for assessing induction and recovery time (eight animals per treatment). The time required for inducing anesthesia (losing sensation against disturbance by means of a glass rod is considered induced) and the time required for recovery (regaining sensation against disturbance) were monitored. In the present investigation, the minimum dose of anesthetic to induce anesthesia in *M. rosenbergii* in less than 30 min was considered as the critical dose. Oxygen consumption of another lot of 96 animals was recorded during the induction time (30 min) using eugenol, menthol and the mixture. They were kept individually in a sealed glass chamber (5 L) with 6.4 mm thick glass lid cut to cover the top portion completely. An opening in the lid fitted with a gasket to ensure an air tight seal permitted the insertion of a dissolved oxygen probe. A magnetic stir bar was used to maintain constant water circulation. A plastic mesh shield was placed over the stir bar to prevent incidental contact with the animal. The chamber was acclimatized inside the thermostatic aquarium at ambient temperature (30 °C) and maintained for 30 days before oxygen consumption experiment was carried out in the presence of increasing doses of anesthetic mixture. All the four sides of the aquarium were covered with opaque screen to minimize visual disturbances of the experimental animal. Oxygen consumption rate/h was measured by digital oxy-meter 330 (Merck, Germany) following the method described (Manush et al., 2004).

2.4. Metabolic responses of *M. rosenbergii* to handling in presence of anesthetic formulation

Prawns were maintained in two different groups (T_1 and T_2) in four pools (500 L) for each treatment in each treatment with six prawns in each pool. In T_1 group, test prawns were exposed to handling stress without any anesthetic. In T_2 group, test prawns were exposed to handling stress in the presence of anesthetic mixture at 200 μL L^{-1}. Handling stress was induced in T_1 and T_2 groups by disturbing the prawns by netting at 2 min interval for 30 min. A control group (24 prawns equally distributed in four pools) was maintained without handling stress for comparing the handling stress. Sampling was done in control group without any anesthetic. All prawns were sampled after 30 min and analyzed for haemolymph glucose, hepatopancreatic glycogen and enzyme activities.

2.5. Sample preparation

Haemolymph was collected from the prawns (six/treatment) individually for glucose estimation. Pre-weighed hepatopancreas (0.3 g) was dissected out from remaining prawns sampled (six/treatment) and dissolved in KOH (30%) and used for glycogen estimation. A part of the brain, hepatopancreas and muscle (0.5 g) were homogenized in chilled sucrose solution (0.25 M) by a mechanical tissue homogenizer and was centrifuged (3000 *g* at 4 °C for 10 min). The whole procedure was performed in ice-cold condition. Supernatant was collected and frozen (−20 °C).

2.6. Glucose and glycogen

Haemolymph glucose was estimated at 540 nm by using arsenomolybdate reagent (Nelson and Somogyi, 1965). Hepatopancreatic glycogen was estimated colourimetrically at 590 nm by treating with anthrone reagent (Hassid and Abraham, 1957).

2.7. Enzyme assay

Lactate Dehydrogenase (LDH) (L-lactate NAD oxidoreductase; E.C.1.1.1.27) was assayed using 0.1 M-phosphate buffer (pH 7.5), 0.3 mM NADH solution in 0.1 M-phosphate buffer solution. 2 mM of sodium pyruvate was used as the substrate and optical density (OD) was measured at 340 nm (Wroblewski and Ladue, 1955). Malate Dehydrogenase (MDH) (L-malate: NAD oxidoreductase; E.C.1.1.1.37) was assayed using 0.3 mM NADH solution in 0.1 M phosphate buffer solution. 1 mM of oxaloacetate solution was used as the substrate and OD was measured at 340 nm (Wroblewski and Ladue, 1955). Alanine Amino Transferase (ALT) (L-alanine 2 oxaloglutarate aminotransferase; E.C.2.6.1.2) was assayed using 0.2 M D, L-alanine and 20 mM α-ketoglutarate in 0.05 M phosphate buffer (pH 7.4) as the substrate and OD was estimated at 540 nm (Wooton, 1964). Acetylcholine Esterase (AChE) (Acetyl hydroxylase, E.C.3.1.1.7) was assayed using M/15 phosphate buffer (pH 7.2), 4 mM acetylcholine (pH 4.0) as substrate and reaction was terminated using alkaline hydroxylamine solution and read OD at 540 nm (Hestrin, 1949). Total protein content was analyzed from supernatant (Lowry et al., 1951) for calculating enzyme activities. Bovine serum albumin was used as the standard protein. Enzyme activities were optimized and were linear and the protein concentration was saturating and not inhibitory. All the enzyme activities were assayed at 37 °C. Optical density for enzyme assay was determined with a UV–VIS spectrophotometer (E-Merck, Germany).