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Effective fluorochrome marking of juvenile sea cucumbers for sea ranching and restocking

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ABSTRACT

Dermal spicules (or 'ossicles') of cultured sea cucumbers can be fluorescently marked with tetracycline and calcein for sea ranching and restocking but optimal immersion conditions are unknown. Lethal and non-lethal effects, and the efficacy of marking spicules in juvenile sandfish (*Holothuria scabra*), were examined under different immersion conditions. Fluorescence brightness and the proportion of marked spicules generally increased with concentration and duration of immersion. Frequency of burial (an indicator of stress) in sandfish increased with both fluorochromes at concentrations above 50 mg L^{-1}. Growth in the two weeks post-marking was unaffected at immersion concentrations of 50 and 100 mg L^{-1} compared to controls, but appeared inhibited by immersion in solutions of 200 and 400 mg L^{-1} of tetracycline or calcein. Sequential marking by tetracycline (yellow) and calcein (green), in either order, showed that calcein was deposited in a higher proportion of spicules. Three other fluorochromes with disparate colors, alizarin complexone, calcein blue and xylenol orange, also marked sandfish spicules and expanded the variety of dichromic combinations. Both tetracycline and calcein fluoresced more brightly when juveniles were marked at 26 or 30 °C than at 21 °C, and this low temperature appears also to reduce the proportion of spicules marked by tetracycline. Our findings show that seawater temperature should be regulated for *ex situ* immersion marking. The behavioral and biological sensitivities of sandfish demand care in administering the fluorochromes. Fluorochrome immersion at 100 mg L^{-1} for 24 h at ≥26 °C provides a practical compromise between minimizing the fitness of released juveniles and ensuring the efficacy of the markers for studies on the growth and survival of sea cucumbers stocked in the wild.

1. Introduction

Marking of skeletal body parts of marine animals with fluorochromes, such as tetracycline and calcein, can provide a valuable tool for mark-recapture studies. In recent decades, both fluorochromes have been used extensively for marking marine animals, including fish, mollusks, echinoderms and nemertean worms (Stricker, 1985; Stricker et al., 1985; Wilson et al., 1987; Day et al., 1995; Stewart, 1996; Kaehler and McQuaid, 1999; Purcell and Simutoga, 2008). However, the optimal methods to administer fluorochromes, and their efficacy at marking, can vary among taxa (Hernaman et al., 2000). The chemicals can also be toxic under certain conditions (e.g., Pirker and Schiel, 1993; Brooks et al., 1994). Therefore, the development of protocols for administering fluorochrome marks should aim to balance the need to induce a robust mark (or 'stain') with the potential toxicity to the animals.

Studies have long used fluorochromes to mark zones of bone growth and remodeling (Harris, 1960; Suzuki and Mathews, 1966). They are chelating agents, deposited at calcification sites in animals. Fluorochromes, like tetracycline and calcein, are commonly used to study growth in marine animals. The artificial, fluorescent marks can enable marked and released animals to be distinguished from wild conspecifics (Kayle, 1992; Lamare and Mladenov, 2000; Purcell and Simutoga, 2008). Advantages over other types of markers or tags (e.g. physical or molecular) lie in their relatively benign effects, low cost and ease of administration.

Fluorochromes can be injected into animals, added to bath solutions for immersion of whole animals or added to feeds. Immersion marking is the method preferred for small life stages. Regardless of the application method, poor choice of marking methodology can limit marking efficacy. Low concentrations of fluorochromes can result in weak marking (Pirker and Schiel, 1993; Day et al., 1995; Vigliola, 1997; Kaehler and McQuaid, 1999; Heasman et al., 2003), while concentrations that are too high incur unnecessary costs — especially important when marking *en masse*. Tetracycline and calcein have been employed for immersion-marking invertebrates at concentrations ranging from 1 to 1000 mg L^{-1}, and durations from 2 h to two weeks (Stricker, 1985;

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Stricker et al., 1985; Pirker and Schiel, 1993; Day et al., 1995; Moran, 2000; Lamare and Mladenov, 2000; Heasman et al., 2003).

Toxicity of fluorochromes has been shown in marine animals if administered at high concentration or for long durations (Wilson et al., 1987; Pirker and Schiel, 1993; Brooks et al., 1994; Bumguardner and King, 1996; Mohler, 1997). Fluorochromes can also be lethal at certain immersion temperatures (Brooks et al., 1994). Sub-lethal toxicity may cause abnormal behavior, suppress growth or make the animals susceptible to disease. Effects of fluorochrome toxicity can therefore seriously undermine the post-release survival of hatchery-produced juveniles in sea ranching or restocking programs.

We previously showed that immersion marking in fluorochrome solutions stains the spicules of juvenile sea cucumbers (Purcell et al., 2006a). The marks lasted one year, providing a tool for mark-recapture experiments (Purcell and Simutoga, 2008), e.g., in field studies on population biology, migration, and in assessing the success of sea ranching or restocking. Sea cucumbers are valuable commodities for export to Asian markets, but overfishing has left many localities without breeding populations (Battaglene and Bell, 2004; Hasan, 2005; Kinch et al., 2008; Choo, 2008). Releases of hatchery-produced juveniles could restock breeding populations in depleted fisheries or be used for sea ranching (Purcell, 2004; Bell et al., 2005). However, if fluorochromes are to be used to identify released animals then optimal conditions for marking spicules and reducing toxicity to the animals should be determined.

In this study, we examine protocols for administering tetracycline and calcein as markers for juvenile sea cucumbers. We focus on the most economically valuable of tropical species, the 'sandfish' *Holothuria scabra* Jaeger 1833, which is well suited to restocking and sea ranching (Battaglene and Bell, 1999, 2004). Experiments in tanks compared the efficacy of marking spicules and both lethal and non-lethal effects among different concentrations, durations and temperatures of immersion marking. Juvenile sandfish bury in sediments for longer periods when stressed (Purcell et al., 2006b; S. Purcell, unpublished), so we used both growth and the frequency of burial in sand as non-lethal indicators of marking toxicity. Further, we examined the potential of immersing individuals in tetracycline and calcein sequentially to give two marks of different color. Dichromic sequential marking could allow sea cucumbers to be distinguished by a fluorochrome combination. Lastly, we conducted preliminary investigations of three additional fluorochromes, alizarin complexone, calcein blue and xylenol orange, to see if these would also mark spicules and potentially provide many dichromic marking combinations.

2. Materials and methods

2.1. Production of juveniles

Sandfish juveniles were cultured in 2003–2004 in New Caledonia using methods described by Battaglene (1999). They originated from 5 spawnings of multiple broodstock, comprising at least 23 males and 9 females. A standardized protocol (Purcell and Eeckhaut, 2005) applied to 100 randomly selected individuals showed that the animals were healthy and free of obvious disease. For each of the experiments below, sandfish juveniles were held overnight in a bare tank prior to immersion marking.

2.2. Experiment 1: immersion concentration and duration

In this three-factor (fluorochrome, concentration, duration) experiment, 243 sandfish juveniles, averaging 4 g in weight (range 2.6–5.8 g), were divided into 81 groups of three individuals. After weighing to ±0.1 g, each group was enclosed in a 150 mL plastic vial, covered with nylon mesh to allow water exchange. Nine vials, each holding a group of three juveniles, were placed immediately and randomly into each of nine 10-L buckets of aerated sea water at 28 °C.

The buckets were placed in dark, insulated containers. Stock solutions of tetracycline (free base) and calcein were made to 2 g per 100 mL sea water, and buffered to pH 6. The solutions were added to each of four buckets to achieve immersion concentrations of 50, 100, 200 and 400 mg L^{-1} of each fluorochrome separately. The ninth bucket of seawater, with no fluorochrome, was used for nine juvenile control groups; three juveniles in each of three vials for the three holding durations. Ideally, each of the 81 groups could have been held in a separate bucket or container for immersion, but aerating and insulating that many separate containers was logistically preclusive. The identical buckets were treated identically and we found no significant concentration × fluorochrome interactions (see Results; Figs. 1–3), which could have otherwise corroborated a confounding 'bucket effect'.

Each group of juveniles was removed from the buckets after 1, 5 or 24 h and placed, by pre-determined random arrangement, into one of 81 mesh holding chambers (20 cm diameter, 20 cm high, with a solid-tray bottom) suspended half-way in the seawater of a raceway tank. Each holding chamber had a 3-cm layer of preconditioned sand. Each day, the juveniles were fed with Algamac™ and powdered shrimp feed (portioned from a slurry providing 0.03 g of each per release chamber) and observed for injuries, evisceration and death. On day 5, sandfish juveniles on the surface of sediments in each holding chamber were counted at 14:00 h, when they usually have emerged from sediments. After 13 days, sediments were emptied from the release chambers and, on day 14, each group of sandfish was weighed, as before, to ±0.1 g.

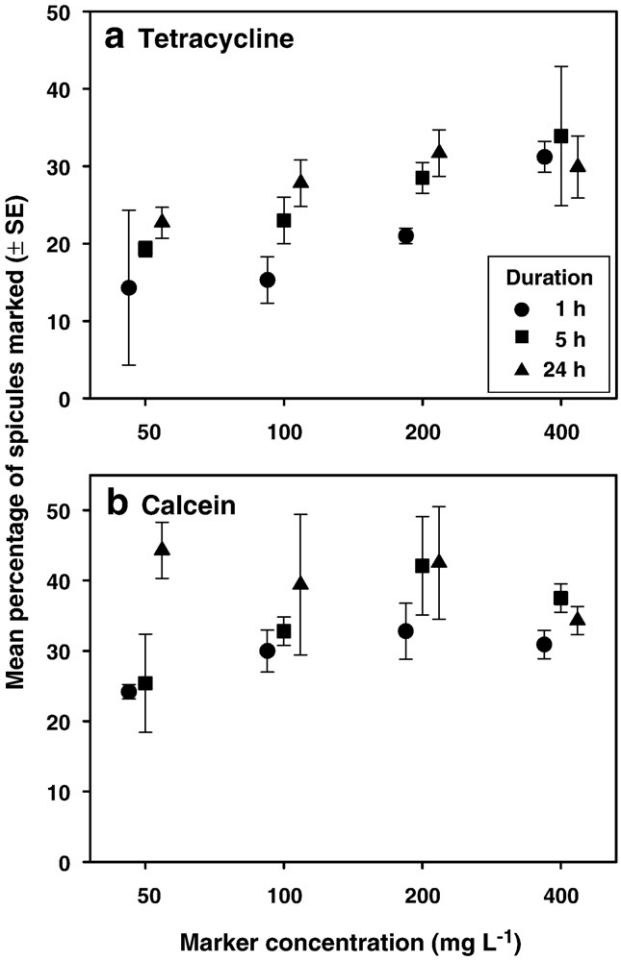

Fig. 1. Plots of mean (±SE) percentage of spicules in dermal samples marked by tetracycline (a) and calcein (b) at four different fluorochrome concentrations and three durations. Means are from the averages of duplicate fields of view of mounted spicules from one individual from each of three group replicates (i.e. $n = 3$).