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Tetracycline as a fluorescent shell-marker in the abalone *Haliotis iris*

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Abstract. Ageing of abalone by counting rings laid down internally in the shell has presented problems of validation. Three methods of tetracycline marking were tested in *Haliotis iris*. Juvenile (< 70 mm shell length) and adult (> 115 mm) abalone were injected intramuscularly with tetracycline hydrochloride, injected with oxytetracycline, or immersed in tetracycline hydrochloride. After treatment, shells were cut sagitally and examined under ultraviolet light using a stereomicroscope. In treatments where juveniles were injected with tetracycline hydrochloride or oxytetracycline hydrochloride at dosages ranging from 20 to 600 mg per kg body weight, no fluorescent marks were visible from treatments ≤ 80 mg/kg, but 83% of juveniles treated with greater dosages retained a visible mark. In treatments where juveniles were immersed in seawater solutions of tetracycline hydrochloride at five concentrations ranging from 200 to 1000 mg per litre of seawater and sampled at 5 h intervals for periods ranging from 5 to 40 h, all showed clear fluorescent markings. Shells of adult abalone injected with tetracycline hydrochloride at four dosages ranging from 200 to 800 mg/kg all showed clearly visible marks 18 d post-treatment. Abalone injected at dosages of 600 and 800 mg/kg exhibited tissue fluorescence around the injection site 2 wk after treatment. Adults immersed for 48 h at four concentrations ranging from 200 to 800 mg/l produced marks comparable to those of injected adults. Abalone were clearly stressed by some treatments. Only 50% of adults injected at 200 mg/kg were able to right themselves within 10 min, while all those injected at higher concentrations either were incapable of righting themselves after treatment or were extremely sluggish. All immersed adults quickly righted themselves. These results show that both injection and immersion are effective in marking abalone, but that immersion is less stressful to them.

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Introduction

Many species of abalone have distinct rings within their shells, but it is not clear whether these rings are laid down annually. Because an independent method of assessing ages in fished populations provides a powerful tool in fisheries management, it is important to verify whether there is periodicity in the laying down of rings and whether this is consistent among different populations (Beamish and McFarlane 1983, Summerfelt and Hall 1987).

Many studies on ageing fish (Daiber 1960, Weber and Ridgway 1962, 1967), sea urchins (Ebert 1985, 1988, Gage 1991) and squid (Lipinski 1986, Jackson 1990) have used tetracycline to provide a reference mark against which subsequent deposition of rings can be gauged. Studies on molluscs such as abalone, however, have tended to use shifts in size-frequency modes and assessments of growth rates to verify the annual deposition of rings (Forster 1967, Kojima et al. 1977, Hayashi 1980, Kim and Cheung 1985, Prince et al. 1988). These techniques can leave considerable doubt about the degree of variability of ring deposition within and among populations.

The assessment of ring deposition in the New Zealand abalone (locally called paua), *Haliotis iris*, has been problematic. Sectioned paua shells distinctly show alternating bands of translucent calcium carbonate (nacre) and a narrower dark-brown protein (conchiolin). Some studies suggest that these rings are annual and, consequently, fisheries-management strategies have been partially based on age estimations of paua in different populations (Murray and Akroyd 1984, Murray 1986). Schiel and Breen (1991), however, found that growth rates based on ring counts, assuming rings were annual, greatly underestimated actual growth rates in two populations. They were unable to show any periodicity in ring deposition in the data available to them. Because *H. iris* comprises a major fishery in New Zealand (Schiel 1992), it is important to validate growth-ring deposition to determine if an independent and general method of ageing is available to fisheries biologists.

Murray (1986) attempted to validate ring deposition in *Haliotis iris* by scribing a notch across the posterior ventral surface. Nacre was quickly deposited, forming a scar that served as a reference point for later ring-deposition analysis. Notching, however, disturbs the abalone and physically damages the shell, which could affect normal growth and ring deposition (Sakai 1960, Clark 1974, Fritz and Haven 1983, Peterson et al. 1983).

The use of tetracycline to provide a datum mark may circumvent potential problems associated with shell notching. Antibiotics of the tetracycline group may be introduced to an animal via injection, immersion, or adding to feed. These antibiotics bind with proteins in the blood (McFarlane and Beamish 1987) and with alkaline earth metals such as calcium, and are incorporated into newly forming, mineralizing bone or shell (Nakahara 1961, Weber and Ridgway 1962, McFarlane and Beamish 1987, Wilson et al. 1987). The intensity of marking depends upon how rapidly the animal is calcifying. Once deposited the mark does not appear to be readily displaced (Weber and Ridgway 1962), and is visible as a yellow-gold fluorescence when exposed to ultraviolet light.

The use of tetracycline as a fluorescent marker in abalone has not been explored. The purpose of this study was to examine methods of effectively and safely incorporating tetracycline marks into the shells of juvenile and adult paua using injection and immersion techniques analogous to those used for fish.

Materials and methods

This study was done at the University of Canterbury field station in Kaikoura, New Zealand (42°25′S; 173°42′E). Four basic treatments with tetracycline-based products were used, with juvenile and adult paua being either injected or immersed (Table 1). All experiments had balanced designs, but in some cases the number of replicates varied because sufficient quantities of appropriately-sized individuals were not always available. All paua were individually tagged, using a vinyl-numbered disc attached to the shell with superglue, to ensure positive identification after treatment.

Juvenile injection

Juvenile *Haliotis iris* were collected on 7 November 1990 from the Kaikoura coast and taken immediately back to the field station, where they were placed into tanks with a well-aerated, flow-through seawater system before being treated with tetracycline.

Paua (mean shell length = 55.4 mm, SD = 17.75) were selected randomly, allocated to treatments with five juveniles per treatment (two treatments had four juveniles), and kept at the ambient seawater temperature of 16 °C. They were injected intramuscularly in the centre of the foot with either tetracycline hydrochloride (trade name Tetracycline-hydrochloride, Boehringer Mannheim NZ Limited) or oxytetracycline hydrochloride (trade name Terramycin, Pfizer, NZ). There were ten treatments and one control, in which five paua were injected with sterile seawater. The treatments were 20, 40, 80, 300, and 600 mg of either tetracycline hydrochloride (TC) or oxytetracycline hydrochloride (OTC) per kg flesh weight (i.e., meat only). Volumes injected for all treatments were dependent upon the flesh weight and the dosage desired (range 0.02 to 0.30 ml). Concentrations of 30 to 60 mg/kg are typically used in fish-tagging studies (Weber and Ridgway 1962, McFarlane and Beamish 1987, Brown and Gruber 1988). After being injected, the paua were returned to the seawater tanks and fed a mixture of drift algae collected from the area of capture.

Paua were culled either 93 d later (at dosages of 300 and 600 mg/kg) or 113 d (at all other dosages). The shell was removed from each paua and cut sagitally through the spire with a lapidary saw. A thin slice was removed along the length of the centre portion of each shell. Sections were examined within 24 h under a 15 W Phillips black light and stereomicroscope. Sections were then stored in the dark in glycerol to prevent oxidization and quenching of the fluorescent mark.

Table 1. *Haliotis iris*. Summary of treatments with tetracycline. TC: tetracycline hydrochloride; OTC: oxytetracycline hydrochloride. *n*: abalone per treatment. Dosage levels = mg per kg flesh weight for injection treatments and mg per litre seawater for immersion treatments. na: not applicable

Abalone	Treatment	(*n*)	Drug	Dosage levels	Time (h)
Juvenile	Injection	(4–5)	TC	0, 20, 40, 80, 300, 600	na
Juvenile	Injection	(4–5)	OTC	0, 20, 40, 80, 300, 600	na
Juvenile	Immersion	(5)	TC	0, 200, 400, 800, 1000	5–40
Adult	Injection	(5)	TC	0, 200, 300, 600, 800	na
Adult	Immersion	(5)	TC	0, 200, 400, 600, 800	48

Juvenile immersion

Juvenile paua (mean length = 24.8 mm, SD = 12.06) were collected from the coast of Kaikoura on 3 February 1991. They were randomly allocated to treatments using 40 to 41 individuals at each dosage level. Within each treatment, 5 individuals were placed into a 2-litre aerated container of non-circulating water. These were then put into a water bath of circulating seawater to keep the temperature at a constant 16 °C. Paua were maintained in these containers with tetracycline hydrochloride concentrations of 200, 400, 600, 800 and 1000 mg/l for periods of 5 to 40 h. Control individuals were held in a 2-litre container of aerated seawater. A sample of 5 individuals was taken every 5 h from each treatment and shells were processed as for the injection treatments for observation on fluorescence.

Adult experiments

Adult paua (mean length = 121 mm, SD = 10.43) were caught during March 1991, transported back to the laboratory, and held in the seawater system with ample food. The injection and immersion experiments were done simultaneously, beginning on 5 April. Because the tetracycline hydrochloride and oxytetracycline hydrochloride results were similar in the experiments with juveniles, only tetracycline hydrochloride was used in the experiments with adults. The relative stress to individuals in both treatments was assessed as the time it took paua to right themselves after being placed upside down immediately after exposure to a treatment.

Nineteen adults were injected (range 0.32 to 1.43 ml) with tetracycline at concentrations of 200, 300, 600, and 800 mg/kg flesh weight and held at the ambient water temperature of 15 °C, with plenty of food available. As in the juvenile experiments, control individuals at each concentration were injected with an equivalent amount of sterile seawater. Paua were maintained in laboratory tanks for 18 d, after which they were removed and their shells sectioned for observations on fluorescence.

Adult immersion treatments were done in the same manner as for juveniles. Five paua were placed into each of five 12-litre containers with aerated seawater. The containers were water-bathed

with ambient seawater of 15 °C. One container contained only seawater, while the others contained tetracycline concentrations of 200, 400, 600, or 800 mg/l seawater. Based on the results of the juvenile immersion experiments, where paua did not appear to be stressed, it was decided to keep adults immersed in tetracycline treatments for 2 d. Paua were then returned to normal seawater tanks and kept for another 16 d before their shells were sectioned and examined.

Because the injection and immersion experiments with adults were carried out simultaneously, a comparison could be made of the stresses imposed on the paua by the two marking methods. An indication of stress was measured as the time it took an individual to right itself when placed ventral side up immediately after exposure to a treatment. Other studies have noted the sluggish behaviour of abalone stressed by transport or damage, which is especially evident as a slow righting response (Tegner and Butler 1985, Tegner et al. 1989). In the present study, the time limit for righting was arbitrarily set at 10 min because paua taking longer than this were usually incapable of righting themselves.

Results

Juvenile injection

No mortalities of juvenile *Haliotis iris* occurred during the experiment (Table 2), although there was some local tissue damage around the point of injection. This was no longer visible 2 wk after treatment. Paua injected at dosages of 20, 40 and 80 mg/kg flesh weight showed no discernible fluorescent marks. However, in 83% of the individuals treated at 300 and 600 mg/kg, fluorescence was seen as a narrow brilliant yellow-gold band (Fig. 1). This was less intense at 300 mg/kg, and the best results were obtained at 600 mg/kg.

Injections with TC and OTC resulted in equally intense fluorescence. The fluorescent mark was particularly noticeable at the anterior margin of the shell, presumably the area of most active calcification. Control paua showed no detectable fluorescence.

Juvenile immersion

Juveniles held in TC concentrations of 200 to 600 mg/l for <5 h produced poor or no fluorescent marks (Table 3). All paua in all concentrations of tetracycline produced a discernible fluorescent mark when immersed for >5 h. Most individuals displayed good fluorescent marks from 25 h onwards. However, the best results were at 600 and 800 mg/l for 30 to 40 h, for which 27 out of 31 abalone (87%) showed good fluorescent marks. The intensity of shell fluorescence appeared to increase with the length of time the paua were held in solution.

Most paua immersed in 1000 mg/l showed good fluorescent marks, but mortality occurred during the longer immersion periods. Five of 11 paua immersed for 35 and 40 h died (45%). There was no mortality in other treatments. Predictably, none of the 39 control paua fluoresced or died.

Adult experiments

Fluorescence was detectable in all individuals injected at dosages of 200, 300, 600, and 800 mg/kg (Table 4). As for the juvenile injection experiment, however, best results were seen at dosages of 300 and 600 mg/kg. Adults still exhibited tissue fluorescence at the point of injection, deep within the foot, 18 d after injection. This was particularly noticeable at concentrations of 600 and 800 mg/kg. However, one individual died within 24 h at the 800 mg/kg dosage, and another exhibited a poor mark.

All adults immersed at concentrations of 200, 400, 600, and 800 mg/l exhibited a discernible fluorescent mark (Table 5). As for juveniles, this was particularly noticeable at the anterior margin of the shell. At the lower two concentrations, fluorescence was often absent or very faint at the posterior end of the shell. Best results

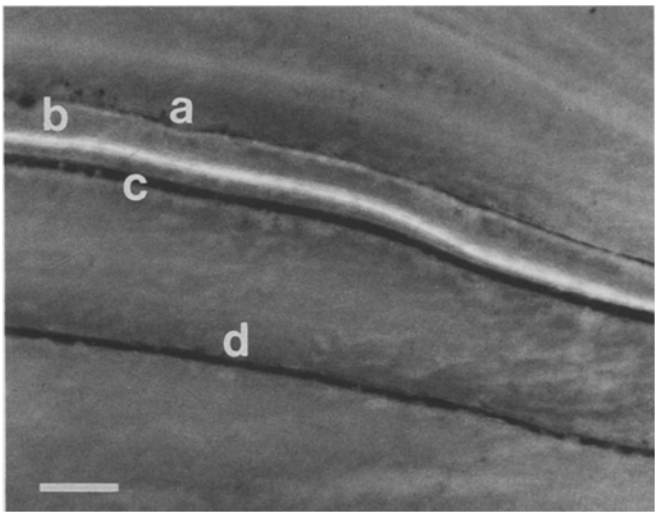

Fig. 1. *Haliotis iris*. Photomicrograph (under UV light) of posterior area of juvenile abalone shell (100×) sectioned sagitally through spire one year after injection with 600 mg/kg tetracycline. a: growth ring laid down prior to treatment; b: fluorescent mark band as result of TC treatment; c, d: growth rings laid down after TC treatment (Scale bar = 100 μm)

Table 2. *Haliotis iris*. Results of juvenile injection experiment. Number of abalone in each treatment exhibiting no fluorescence, poor or good fluorescence under UV light is indicated. Abbreviations as in Table 1

Dosage (mg/kg)	Treatment	(*n*)	Readability of mark (number of paua)			Mortality
			no mark	poor mark	good mark	
20	TC	(5)	5	0	0	0
20	OTC	(5)	5	0	0	0
40	TC	(5)	5	0	0	0
40	OTC	(5)	5	0	0	0
80	TC	(5)	5	0	0	0
80	OTC	(5)	5	0	0	0
300	TC	(5)	1	3	1	0
300	OTC	(5)	2	3	0	0
600	TC	(4)	0	0	4	0
600	OTC	(4)	0	0	4	0
Control	seawater	(5)	5	0	0	0

Table 3. *Haliotis iris*. Results of juvenile immersion experiment using tetracycline hydrochloride

TC conc (mg/l)	Treatment time (h)	(*n*)	Readability of mark (number of paua)			Mortality
			no mark	poor mark	good mark	
200	5	(5)	3	2	0	0
200	10	(5)	0	5	0	0
200	15	(5)	0	5	0	0
200	20	(5)	0	5	0	0
200	25	(5)	0	1	4	0
200	30	(5)	0	2	3	0
200	35	(5)	0	3	2	0
200	40	(5)	0	0	5	0
400	5	(5)	2	3	0	0
400	10	(5)	0	2	3	0
400	15	(5)	0	4	1	0
400	20	(5)	0	4	1	0
400	25	(5)	0	1	4	0
400	30	(5)	0	1	4	0
400	35	(5)	0	2	3	0
400	40	(5)	0	2	3	0
600	5	(5)	2	3	0	0
600	10	(5)	0	2	3	0
600	15	(5)	0	3	2	0
600	20	(5)	0	1	4	0
600	25	(5)	0	1	4	0
600	30	(5)	0	0	5	0
600	35	(5)	0	0	5	0
600	40	(5)	0	1	4	0
800	5	(5)	0	3	2	0
800	10	(5)	0	1	4	0
800	15	(5)	0	5	0	0
800	20	(5)	0	1	4	0
800	25	(5)	0	1	4	0
800	30	(5)	0	0	5	0
800	35	(5)	0	2	3	0
800	40	(6)	0	1	5	0
1 000	5	(5)	0	2	3	0
1 000	10	(5)	0	3	2	0
1 000	15	(5)	0	5	0	0
1 000	20	(5)	0	1	4	0
1 000	25	(5)	0	0	5	0
1 000	30	(5)	0	0	5	0
1 000	35	(5)	0	0	4	2
1 000	40	(6)	0	0	3	3
Controls	5–40	(39)	39	0	0	0

were seen at concentrations of 600 and 800 mg/l, for which 78% of paua showed a good fluorescent mark.

In the comparison of stresses imposed by the two marking methods, only 20% of adults injected with TC at the lowest dosage of 200 mg/kg were able to right themselves in less than 10 min. All adults injected at 300, 600 and 800 mg/kg were unable to right themselves. However, all adults in the immersion treatments righted themselves within 10 min (Table 6). These results imply that the immersion method is a less stressful method of fluorescent labelling.

The observed differences in the righting response are more an expression of stress between the injection and immersion methods than the quantity of TC used. With injection, TC must travel from the point of injection to the mantle, where it is deposited. This exposes most of the abalone internally to TC. Results from the injection method show that at greater concentrations there is increased stress. The immersion method, however, exposes the abalone externally to TC; it is postulated that the major route of TC entry here is through direct mixing with the extrapallial fluid (the thin layer of fluid between the shell and the mantle).

Table 4. *Haliotis iris*. Results of adult injection experiment using tetracycline hydrochloride

Dosage (mg/l)	Treatment	(*n*)	Readability of mark (number of paua)			Mortality
			no mark	poor mark	good mark	
200	TC	(5)	0	2	3	0
300	TC	(5)	0	0	5	0
600	TC	(4)	0	0	4	0
800	TC	(5)	0	2	3	1
Control	seawater	(16)	16	0	0	0

Table 5. *Haliotis iris*. Results of adult immersion experiment using tetracycline hydrochloride. Immersion time = 48 h

Dosage (mg/l)	Treatment	(*n*)	Readability of mark (number of paua)			Mortality
			no mark	poor mark	good mark	
200	TC	(5)	0	2	3	0
400	TC	(5)	0	2	3	0
600	TC	(4)	0	1	3	0
800	TC	(5)	0	1	4	0
Control	seawater	(5)	0	0	0	0

Discussion

These experiments show that it is relatively easy to mark *Haliotis iris* with a fluorescent band. After the administration of TC hydrochloride or OTC, either through injection (at 300 to 600 mg/kg flesh weight) or immersion (at 600 to 800 mg/l), a bright yellow-gold fluorescence was visible on and within the shell. Fluorescence was most intense in the region of active calcification (cf. Milch et al. 1957), which was at the anterior margin of the shell where growth is proportionately greatest. As found for fishes treated with TC (Weber and Ridgway 1962), fluorescence in both adult and juvenile paua was easily distinguishable from normal autofluorescence, irrespective of the method used to administer the drug.

In our experiments, fluorescence persisted for at least 98 d and was not noticeably diminished after a year's storage in a dry dark area. Quenching was observed, but not to a great extent, when shells were exposed to UV light for long periods of time.

Table 6. *Haliotis iris*. Summary of marking experiments using tetracycline hydrochloride (TC) and oxytetracycline hydrochloride (OTC). na: not applicable

	Juvenile injection	Juvenile immersion	Adult injection	Adult immersion
Treatment	TC+OTC	TC	TC	TC
Amount used	20–600 mg/kg	200–1 000 mg/l	200–800 mg/kg	200–800 mg/l
Immersion time	na	5–40 h	na	48 h
Best results	300–600 mg/kg	600–800 mg/l at 30–40 h	300–600 mg/kg	600–800 mg/l
Mortality	None	40% at 35 h, 60% at 40 h at 1 000 mg/l	25% at 800 mg/kg	None
Stress	Not tested	Not tested	Only 20% righted themselves in <10 min at 200 mg/kg	All righted in <10 min at all concentrations

Immersion of paua in a solution of TC was found to be the easiest method of marking both juveniles and adults. This method avoids the inefficiencies of individually handling paua several times and appears to minimize stress to them. Because paua are held in tanks for several hours, there is also sufficient time to identify individuals that may be in a stressed condition and replace them in tagging experiments where they are returned to the field.

Injected paua produced fluorescent marks comparable to immersed individuals. One advantage of injection is that it can be done on site in the field, so that the abalone do not have to be transported back to a laboratory. It is also considerably quicker. The disadvantages, however, are that the abalone are clearly stressed by injection, which may have deleterious effects on subsequent survival. Because *Haliotis iris* that are dropped into the sea invariably land upside down, the inability to right themselves makes them vulnerable to predators such as crabs, fishes and seastars (cf. Tegner and Butler 1985, Schiel and Welden 1987, Tegner et al. 1989) and to physical damage from being tossed around in high-energy inshore conditions.

It appears that the best method of marking paua is to immerse them in concentrations of 600 to 800 mg/l for a period of at least 30 h (Table 6). They can then be removed from TC and allowed to move and feed normally in seawater tanks before replacement into the field. Higher concentrations induce mortality and are risky for ongoing growth experiments.

The ability of *Haliotis iris* to withstand high concentrations of TC and OTC with very little or no mortality was surprising, especially given the high dosages used (600 to 1000 mg/kg). These concentrations are well beyond those that cause great mortality in fishes (Weber and Ridgway 1962). Higher doses that are safe for laboratory studies, however, may cause high mortalities in the field. For example, McFarlane and Beamish (1987) found that sablefish, *Anoplopoma fimbria*, injected with OTC dosages of 25 and 100 mg/kg, survived well in the laboratory. However, only 11 and 3.6%, respectively, were recovered in field trials vs 16% for saline-injected individuals. For fishes, the optimum dosages which minimize treatment mortality while maximising the number of individuals with a useable mark may be different for field and laboratory studies. Our study implies that this may also be the case for injected *H. iris*, with mortality in the field being a likely consequence of the stress associated with injection.

Another method of fluorescent marking of invertebrates, immersion in seawater mixed with calcein, has been used in sea urchins (T. Ebert personal communication) and various molluscan larvae (Rowley personal communication). Calcein may be more benign than TC, and stable in solution (whereas TC is rapidly oxidised), while having a brighter fluorescence (Wilson et al. 1987). Although calcein is more expensive than TC, only small dosages are required to produce a fluorescent mark (Yamada 1971).

Our experiments represent only the ground work for studies in TC-labelling of abalone and perhaps other molluscs. Its advantages are low cost, simplicity, persistence, and the ease with which the mark is identified without a complicated apparatus. The method has several potential uses in studies of calcification, growth, and movement (Milch et al. 1957, Tufts 1967). Of particular importance to the fishery for *Haliotis iris*, however, is the ability to put a datum mark within the shell so that validation studies can be done on ring deposition, which may aid in determining the age structure of populations and in better modelling of the fishery.

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