

## EVALUATION OF FLUORESCENT ELASTOMER INJECTION AS A METHOD FOR MARKING SMALL FISH

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### ABSTRACT

Fluorescent elastomer injection was used to mark newly settled Hawaiian coral reef fishes (8-56 mm SL) in the laboratory and in the field. Marking success was influenced by depth of subcutaneous tag injection, anatomical location of the tag, pigmentation of the skin at that location, and investigator's experience with the technique. Visibility up to several months and little mortality was achieved with careful handling of the fish and skillful placement of the injection. In the laboratory, mortality related to marking occurred within 24 h of the procedure. Within this time, one mortality was observed for fish > 20 mm SL; fish < 20 mm SL experienced 13% mortality. Fish marked and held in the laboratory showed 100% tag visibility and retention for observation periods up to 76 d. Of 286 fish marked, released and tracked in the field, 67% were regularly resighted for observation periods up to 130 d. Of 59 fish observed in the field for at least 45 d, 36% were resighted; four were resighted at least 100 d after marking. Visibility of one tag was reduced over the first 30 d by growth of surrounding tissue. Because of small tag size and the large number of unique combinations of tag color and injection location, field identification of individuals was possible. Success in recognizing individually marked fish in the field required some observer experience. Although not necessary in this study, under low visibility conditions, resighting success could be enhanced with the use of UV-A filtered light.

Over the last decade, intense research efforts have been aimed at determining the effects of recruitment and survivorship of coral reef fishes on the structure of adult populations (Sale, 1991). However, these investigations have been hindered by difficulties in visually identifying individual juvenile fishes in situ or after recapture. This problem is evident in the absence of studies that include successful marking programs that would allow researchers to record the fate of newly settled individuals (except, see Frederick, 1997). As a result, many studies of recruitment and early life history of coral reef fishes have been based on broad assumptions about the reliability of recognizing individuals by ambiguous means under variable field conditions. A few researchers have used size differences on the order of millimeters to differentiate new settlers from older individuals (Luckhurst and Luckhurst, 1977; Talbot et al., 1978; Williams, 1983; Robertson, 1988; 1991; 1992; Stimson, 1990), whereas others have attempted to identify individuals by behavioral characteristics, including the untested assumption that newly settled juvenile fishes are sedentary (Sale et al., 1984a,b; Doherty and Sale, 1985; Victor, 1986; Doherty, 1987; Eckert, 1987; Sale and Ferrell, 1988). Some researchers have relied on variations in natural marks to distinguish individuals (Sale, 1979; Aldenhoven, 1986; Connell and Jones, 1991; Booth and Beretta, 1994). All of these means of identification limit the scope of studies by requiring that they be conducted at a reduced spatial scale in isolated sites with limited numbers of individuals (Buckley et al., 1994).

An effective and versatile method for marking newly settled coral reef fishes [typically <30 mm standard length (SL)] has not been available; most marking techniques have been developed for temperate fishery applications and larger individuals (Buckley and

Blankenship, 1990). A survey of recently tested methods for external and internal marking of juvenile fishes reveals a number of liabilities for very small sizes. Although external colored plastic streamer tags and nylon anchor tags have been used successfully by Hoelzer (1988) and Mathews (1990) on temperate juvenile fishes as small as 180 mm total length (TL), this method would be inappropriate for tagging juvenile tropical reef fishes which can be less than one-tenth this size. Similarly, mutilation by cold-branding or fin clipping produces a recognizable scar, but could cause significant biological stress and mortality for very small fishes. Although otoliths of juveniles and larvae can be marked successfully (Hettler, 1984; Volk et al., 1990; Schroder et al., 1995), marked individuals cannot be identified visually in situ and must be recovered and sacrificed for identification. Likewise, coded wire tags and PIT (passive integrated transponder) tags have been successfully used on small fishes (>35 mm TL), but they do not allow for visual in situ recognition (Prentice et al., 1990; Bergman et al., 1992; Peterson and Key, 1992). The coded visible implant (VI) tag, developed to improve upon the visual recognition limitations of the coded wire tag, has limitations related to the size, placement, retention, and in situ visibility of the tag (Haw et al., 1990; Buckley et al., 1994). Injection of dyes or paints just beneath the skin produces a visible colored mark, but with limited retention time and high mortality in marking very small size classes (Forrester, 1990; Wellington, 1992). Buckley et al. (1994) have shown successful use of the visible implant fluorescent (VIF) monofilament tag in scorpaenids as small as 30 mm TL. Beukers et al. (1995) used the (VIF) monofilament tag successfully on pomacentrids as small as 11 mm SL, but the in situ feasibility of the injection method, which required aseptic conditions and a binocular microscope, was not tested.

Here I report the first results of marking newly settled coral reef fishes using the VIF elastomer tag in the laboratory and in the field with a comparison of success between size classes. Administered as a subcutaneous liquid injection, the medical grade elastomer hardens to an inert flexible substance visible through the skin. The resulting tag promises long retention times, few biological side effects, and good in situ visibility.

#### MATERIALS AND METHODS

**LABORATORY.** — All fish used to test the fluorescent elastomer tag were collected with hand nets from natural and artificial reefs in Hanalei Bay, Kauai, between 9 February and 28 August, 1994. The fluorescent elastomer (provided by Northwest Marine Technology, Inc.) was tested using two colors: "rocket red" and "arc yellow". The elastomer was injected with a standard 0.5 cc insulin syringe. As many as 12 anatomical locations were tested for some species to examine the effect of tag location on mortality, tag retention, and tag visibility (Fig. 1). Fish were marked either on the right or left side; a few individuals were marked in two locations on one side with a single color.

Thirty-seven fish of 10 species (six families) were used in laboratory marking experiments conducted from 18 February to 11 June 1994. Individuals ranged in size from 10 to 56 mm SL at the time of marking (Table 1). Including those assigned as controls, fish were held for at least 5 d before marking; only those that fed consistently and appeared to recover from handling and transport trauma were used in the experiments. Fifteen unmarked individuals were held and observed for 23 d and up to 71 d as a measure of mortality unrelated to marking. For the laboratory marking procedure, fish were rapidly anaesthetized in a sea water solution of MS-222 (80 mg/l tricaine methanesulfonate) until they exhibited a total loss of reflex. Using extreme care in handling (including the use of rubber gloves to reduce abrasion), fish were measured, injected, and placed in an

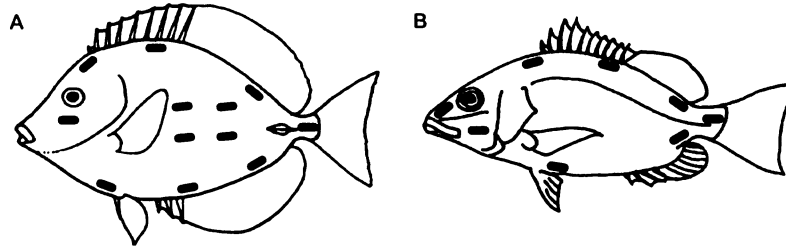


Figure 1. Elastomer injection locations shown on juveniles of the Acanthuridae (A) and Lutjanidae (B).

isolation tank until their recovery. To determine mortality, tag retention and tag visibility, each individual was observed daily for a minimum of 24 d and up to 76 d for some.

FIELD. — Field experiments were conducted from 27 June to 30 August 1994 to assess the feasibility of in situ marking and tag visibility. A total of 286 individuals of seven species (five families) ranging from 8 mm to 55 mm SL (Table 1) were marked in the field and released at 32 artificial reefs in Hanalei Bay. The artificial reefs were arranged in a circular cluster 100 m in diameter on sand substrate and were isolated by at least 350 m from the nearest natural reef. Marking was performed underwater while the fish were held in a hand net. Trauma to the fish marked in this manner appeared to be considerably less than when they were brought to the surface and anesthetized for marking. Each individual of a species was uniquely marked by various combinations of color and mark location. To observe mortality related to the marking procedure, newly marked fish were held underwater for up to 2 h before being released; based on laboratory observations, signs of imminent mortality (e.g., increased reaction time and loss of equilibrium) could be detected within that time. Subsequent mortality and tag visibility were determined by field observations daily or every other day. All 32 artificial reefs at the isolated study site where the fish were released were thoroughly searched by a diver until all fish present could be identified as uniquely marked or unmarked. The frequency of observations assured that gradual extrusion of, or tissue growth over, the tags could be recognized. Previously marked individuals not found were assumed to be lost due to mortality or emigration beyond the study area. Because of extremely poor visibility caused by unusually heavy rainfall after 48 d, field observations were conducted on only two subsequent occasions, at 80 d and 130 d after release.

## RESULTS

### LABORATORY

MORTALITY. — Fifty-three percent of all mortality for laboratory marked fish occurred immediately following or within 2 h of marking. Sixty-five percent of all mortality occurred within 24 h of marking and was easily predicted from observing the individual during its recovery period (usually the first 1-2 h). After the first 24 h period, mortality among marked fish was less than, or not significantly greater than, that of the control group of unmarked fish (Fisher's Exact, one-sided alternative;  $P > 0.8$ ).

Initial (24-h) mortality due to marking was significantly greater (Fisher's Exact, one-sided alternative;  $P = 0.001$ ) in early laboratory trials conducted in February than in subsequent trials conducted April-June, presumably due to the investigator's inexperience

Table 1. Number and sizes of fishes marked in the laboratory and in the field.

LABORATORY		n	Standard Length (mm)				
			median	min.	max.		
Experimental	Acanthuridae	<i>Acanthurus blochii</i>	1	38			
	Apogonidae	<i>Apogon coccineus</i>	2	36	35	37	
		<i>Apogon kallopterus</i>	1	22			
	Chaetodontidae	<i>Apogon sp.</i>	7	14	10	17	
		<i>Chaetodon miliaris</i>	1	28			
		<i>Heniochus diphreutes</i>	1	48			
	Kuhliidae	<i>Kuhlia sandvicensis</i>	5	31	29	56	
	Labridae	<i>Thalassoma duperrey</i>	1	38			
	Pomacentridae	<i>Chromis ovalis</i>	10	16	13	18	
		<i>Dascyllus albisella</i>	8	13	11	14	
	Control	Kuhliidae	<i>Kuhlia sandvicensis</i>	4	30	28	33
		Labridae	<i>Halichoeres ornatissimus</i>	1	28		
		Lutjanidae	<i>Lutjanus kasmira</i>	1	38		
		Pomacentridae	<i>Chromis ovalis</i>	3	10	8	16
<i>Dascyllus albisella</i>			5	11	8	13	
Serranidae	<i>Epinephelus quernus</i>	1	27				
FIELD	Acanthuridae	<i>Acanthurus blochii</i>	96	36	22	42	
	Apogonidae	<i>Apogon sp.</i>	4	11	9	24	
	Chaetodontidae	<i>Chaetodon miliaris</i>	14	26	22	29	
		<i>Heniochus diphreutes</i>	93	36	26	55	
	Lutjanidae	<i>Lutjanus kasmira</i>	65	32	20	52	
	Pomacentridae	<i>Chromis ovalis</i>	1	12			
		<i>Dascyllus albisella</i>	13	18	8	21	

with the procedure. Marking mortality decreased over time despite a decrease in the average size of fish that were being marked. In fact, after accounting for this "learning curve", there was no significant difference between initial mortality of individuals marked April-June and that of the control group (Fisher's Exact, one-sided alternative;  $P = 0.347$ ).

However, there was no control for differences in size and species of fish marked during the study, and testing for statistical differences in marking mortality between species was not feasible due to low and uneven sample sizes. But based on qualitative observations, size was more important than species in determining marking mortality. Because a species effect may confound the change in laboratory mortality over time, two measures of mortality are given. Maximum mortality was calculated with a total count of 37 and included all fish marked in the laboratory. Minimum mortality included only the final 22 individuals marked in the laboratory April-June. Minimum and maximum cumulative percent mortality of marked and unmarked fish were calculated for two size classes at four time periods after marking (Table 2). Fish <20 mm SL experienced greater mortality than those >20 mm SL. For both size classes, most mortality occurred shortly after marking.

Table 2. Cumulative percent mortality over four time intervals for two size classes of fishes marked in the laboratory and in the field. Maximum laboratory mortality was calculated using all fish marked February-June; minimum laboratory mortality was calculated using only fish marked April-June. Laboratory mortality estimates at 48 h are equal to 24 h estimates. Field mortality includes emigration. For field mortality at 28 d for fishes SL > 20 mm, n = 120.

		LABORATORY			FIELD
		unmarked	minimum	maximum	
Size < 20 mm SL	2 h	0.00	0.07	0.32	0.13
	48 h	0.00	0.13	0.40	0.56
	7 d	0.17	0.13	0.44	0.88
	28 d	0.33	0.20	0.50	0.94
		n=6	n=15	n=25	n=16
Size > 20 mm SL	2 h	0.00	0.00	0.08	0.00
	48 h	0.00	0.00	0.08	0.31
	7 d	0.00	0.00	0.08	0.51
	28 d	0.25	0.33	0.30	0.73
		n=8	n=7	n=12	n=270

**TAG RETENTION.** — One individual (*Kuhlia sandvicensis*), marked early in laboratory experiments, shed its tag after 45 d. Tag retention in the laboratory for all other fish was 100%. Because the elastic polymer takes approximately 24 h to harden after being injected, extrusion of the material occurred if the fish was very active during this period. However, the excess material always detached itself with no obvious detrimental effect to the fish or the tag.

**TAG VISIBILITY.** — Tags of all fish remained visible for the duration of the laboratory trials, after which the marked fish were released. After losses from mortality and early release, 18 fish were observed in the laboratory for at least 23 d, 11 fish for at least 51 d, two for at least 66 d, and one for 76 d.

#### FIELD

**MORTALITY.** — Cumulative percent mortality of marked fish was calculated for two size classes at four time periods after marking (Table 2). After the 2 h observation period, marking mortality could not be distinguished from mortality due to predation or emigration beyond the census site. Within the 2 h observation period before release, mortality was 13% for fish <20 mm SL. No mortality was observed for fish >20 mm SL. Given that only one death caused by marking was observed in the laboratory for this size class, mortality estimates after the observation period are likely to overestimate mortality caused by the marking procedure.

A statistical comparison of mortality between unmarked, laboratory marked (all trials occurred April-June), and field marked individuals was feasible for *Dascyllus albisella*. One mortality occurred in a group of eight marked *D. albisella* held in the laboratory, while four of eleven field marked individuals were lost in the first 24 h after marking. These values are not significantly different from the control group (Fisher's Exact, one-sided;  $P = 0.62$  (lab) and  $P = 0.18$  (field)).

**TAG VISIBILITY.** — Long-term field monitoring of the pomacentrids and apogonids was not possible due to high mortality (including possible emigration from the study area). All but two individuals in this group (<20 mm SL) were lost within the first week (Table 2). Few individuals of this size class were marked, so it is not clear what fraction of the losses resulted from the marking method. Because no other individuals of these species were present at the time the losses were noted, it is unlikely that these losses were due to tag failure. The last observed individual (*D. albisella*) was sighted up to 34 d when tissue growth over the tag prevented positive identification.

Individuals of four larger species (>20 mm SL) (one lutjanid, two chaetodontids and one acanthurid) marked in the field were resighted regularly up to the last daily observation on 30 August. After losses from mortality and/or possible emigration beyond the study area, 36% of marked fish under observation for at least 45 d (21/59) were resighted. One marked *Heniochus diphreutes* was subsequently observed 77 d after release, two were observed 100 d after release, and two were observed 130 d after release.

#### DISCUSSION

**MORTALITY.** — Skilled application of the elastomer injection was crucial for keeping mortality low, as suggested by the decrease in mortality of marked fish during the laboratory experiment. Shallow injections made into peripheral musculature near the dorsal and caudal fins or at least away from vital organs caused least mortality (Figure 1). Injection of tags in multiple locations was feasible for fish larger than 20 mm SL. Only one insertion of the syringe and a minute amount of elastomer at a location was necessary for each mark. To ensure minimal and delicate handling of the fish, field applications of the elastomer were performed in situ. This may be preferable for laboratory injections as well, although the procedure was more efficient when the fish were anaesthetized and briefly held out of water for marking.

Based on casual behavioral observations over 76 d, mortality after the initial 24 h period for laboratory marked fish appeared to result from stress (e.g., intraspecific aggression), rather than direct complications from the elastomer injection (e.g., infection in tissues surrounding the tag and muscle or nerve damage from the injection). Apparently after successful injection, the tag remains relatively compatible with the physiology of the marked fish. This observation is supported by independent histological studies performed on rainbow trout tagged with the elastomer where no cellular changes typical of inflammation or tag rejection were observed in the salmonid tissues (J. Morrison, U.S. Fish and Wildlife Service, Olympia Fish Health Center, unpublished).

**TAG VISIBILITY.** — Proper placement of the tag to ensure long-term retention and visibility also required some practice, as noted by the failure of one tag in an early laboratory trial of this study. Tag visibility was affected by subcutaneous depth and the pigmentation of the skin at that location. The most visible tags were those injected close to the surface of transparent tissue. The best injections were made parallel to but just under the skin along a straight path several millimeters long. As the needle was withdrawn, the path filled with the elastomer. For darkly pigmented tissue, the most successful method was to "tattoo" the outermost tissue layer from the inside. A shallow injection was made parallel to the surface. Angling the syringe and repeatedly bringing the needle tip close to the inner surface without breaking through allowed the elastomer to infuse the pigmented

surface layer. Deeper injections tended to gradually lose visibility due to growth of adjacent tissue. Of all the species tested, successfully marking *D. albisella* was the most difficult because of their very small size (8-12 mm SL) and dark pigmentation at settlement. Although necessary to ensure long-term visibility of the mark, the extended handling required for the "tattoo" method for dark pigmentation can be biologically stressful for these very small fish. The results of this study suggest, however, that with skill and favorable field conditions, these sizes can be successfully marked in situ with the fluorescent elastomer injection.

The behavior of fish also influenced tag visibility. Tags in mobile or schooling fish were most visible laterally, adjacent to the dorsal, caudal, or anal fins (Fig. 1). Tags in less mobile or sedentary fish were most visible dorsally and anteriorly, in the nape, snout, or suborbital regions. Because most fish were observed from above, tags in ventral locations made resighting difficult.

Just as there was a learning curve for the marking procedure, field observers required experience to recognize marked individuals and to differentiate between the two colors of the fluorescent elastomer tags (red and yellow), which looked very similar under the attenuated light at 10 m depth. Because the tags were very small and the number of distinct marking combinations was large, observers needed a priori knowledge of the possible anatomical locations to look for tags. In this study, two colors and 12 anatomical marking locations were used to mark acanthurids. Using as few as two marks of one color for each individual provided over 150 distinct marking combinations. Many more combinations of marks and colors are possible such that using up to three marks of both colors of elastomer per individual could have allowed over 600 individuals to be distinguished.

Although the developers of the tag recommend using a light equipped with a UV-A filter to excite the fluorescent dye in the tag and increase visibility, I found that a light was not always necessary for in situ resighting. With visibility averaging 15 m during the study, the tags were readily visible to the experienced observer from about 1 m away. At 10 m depth, UV-A filtered light from a dive lamp did not noticeably enhance visibility of the tags from this distance. However, in conditions of low visibility (<4 m) when the ambient light was significantly reduced, the tags were visible from only about 0.5 m. Under these conditions, or during crepuscular or night observations, a UV-A filtered light could enhance tag visibility and help the observer distinguish tag colors. Bonneau et al. (1995) used a UV-A filtered light to identify marked juvenile bull trout (*Salvelinus confluentus*) <100 mm TL at night by diver and stream bank observer.

Ideally, a marking method should have good in situ visibility, retention time of at least a few months, and cause little or no mortality for even the smallest fish (< 10 mm SL). Currently, injection of some colored liquid (paint or dye) below the skin seems to be the preferred method for marking very small fish when *in situ* identification is desired. The greatest shortcomings of this method are limited retention time, high mortality, and untested biocompatibility of the injection liquid. Forrester (1990) found marking *D. aruanus* < 25 mm fork length (FL) with liquid latex or tattooing dye caused increased mortality, and for those that survived, the marks faded too quickly. In a subsequent experiment, Forrester (1995) reported successful use of the VI tag and acrylic paint to mark gobies (*Coryphopterus glaucofraenum*) < 25 mm SL, but did not report on tag retention or tag related mortality. Another experiment using Alcian blue stain and tattooing dyes to mark *Stegastes* spp. 12-22 mm SL showed that the marks faded within 2 wks and that mortality

was 10-15% (Wellington 1992). Riley (1966) used liquid latex successfully on plaice as small as 18 mm TL with overall mortality less than 1% and good visibility in the laboratory after 2 yrs. However, the toxic effects of none of these materials were experimentally determined.

Other researchers have successfully used the fluorescent elastomer injection on larger freshwater fishes. In a study using the fluorescent elastomer to mark bluegills (*Lepomis macrochirus*) (34-133 mm TL), researchers reported 99% retention after 6 mo in the laboratory and 98% re-identification after 70 d in experimental ponds (Dewey and Zigler, 1996). Bonneau et al. (1995) reported 100% retention after 2 mo and 99% retention after 4 mo with no mortality for juvenile bull trout (*Salvelinus confluentus* < 100 mm TL).

In this experiment, the fluorescent elastomer tag showed good in situ visibility, which could be enhanced in low light conditions using UV-A filtered light. The mark was observed in experimental fish as long as 76 d in the laboratory and 130 d in the field before observations ended, indicating that the marking technique could be applied to studies lasting several months. After skilled application of the technique (as described above), no mortality related to marking was observed for fish > 20 mm SL. For small fish 8-20 mm SL, mortality was as much as 13%. However, this estimate is based on sample sizes of ~15. With practice and field conditions favorable for in situ marking, mortality may have been reduced.

A successful method for marking very small fish for individual recognition will enhance the accuracy of survivorship estimates made by visual census, differentiating and quantifying sources of post-settlement loss, tracking post-settlement movement, and characterizing post-settlement habitat preferences (Jones, 1990). This study suggests that the fluorescent elastomer tag is a useful method for marking juvenile tropical or temperate reef fishes in situ and also allows visual identification of cohorts or individuals. Marking individuals in multiple locations with multiple colors could allow for whole cohorts of uniquely marked individuals to be monitored simultaneously during their early juvenile stages.

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