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FLUORESCENT CHEMICAL MARKING OF WALLEYE LARVAE WITH A SELECTED LITERATURE REVIEW OF SIMILAR INVESTIGATIONS[1]

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Abstract.--A review of techniques that have been, or could be used to mark walleye, especially larval fish, was conducted and summarized in this report. Exposure to a tetracycline antibiotic (or similar fluorescent compound) was identified as the most likely means of mass-marking larval walleye.

Three separate trials were conducted with walleye larvae to test how oxytetracycline hydrochloride would perform as a mass-marking medium. Larvae were treated in Big Redd incubators containing 250 mg/L or 350 mg/L solution of oxytetracycline hydrochloride. External examination of larvae 1-5 d post-treatment revealed fluorescent marks to be present in all developing bones of the skull. Initial fluorescence at the larval stage is encouraging, but evidence for long term mark retention in walleye marked with tetracycline needs further evaluation.

Walleye larvae were similarly mass marked using a 125 mg/L solution of calcein. Fish sampled soon after treatment had poor incorporation of calcein into bony structures and little visible external fluorescence of those bones.

INTRODUCTION

Propagation and stocking of larval or juvenile walleye *Stizostedion vitreum vitreum* is one of the largest expenditures in Minnesota's Section of Fisheries budget. Because of the small size at which walleye are stocked (6-200 mm), application of marks for future identification is labor intensive and often difficult. Consequently, a comprehensive evaluation of walleye stocking in Minnesota, commensurate with the size of the program, has not been accomplished.

Walleye stocking is often successful in lakes where native walleye populations are nonexistent. Winterkill lakes, including natural ponds used for juvenile production, are examples of this. However, introductions represent a relatively small proportion of Minnesota's total stocking program. Stocking success in lakes with existing walleye populations, the most common stocking

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scenario in Minnesota, is not adequately known. This is particularly true for lakes that have natural walleye reproduction, or where the status of natural reproduction is uncertain. The actual contribution to these populations from stocking is unknown.

Previous attempts to evaluate walleye stocking success in Minnesota involved either fin-clipping juvenile fish, or manipulating stocking schedules to show presence or absence of year-classes in relation to stocking. There are several problems associated with each procedure. The practical size at which an individual fish can be fin-clipped is limited, and clips may adversely affect survival. Also, fin regeneration complicates recognition by inexperienced observers thus obscuring long term evaluations. In manipulating stocking schedules, the recommended schedule may not be followed for a variety of reasons such as availability of fish for a specified stocking. Interpretation of schedule manipulation requires aging of fish, and errors associated with aging may be significant, thus resulting in erroneous conclusions. Further, a variety of statistical analyses are needed to evaluate this approach, and the long term monitoring (8+ years) needed is unlikely to be achieved.

Current mass-marking methods for larval and juvenile fish include a wide array of techniques from fin-clipping to biochemical analyses. As with current methods, future marking techniques need to meet five criteria (Everhart and Youngs 1981): 1) ease and assurance of detection or mark identification; 2) high mark retention; 3) rapid application; 4) identification of separate groups of fish; and 5) minimal effect on the fishes' behavior and well-being.

The objectives of this study were to review fish marking methods that are available, search for new techniques for use on small walleye in field situations, and to test selected marking methods on a trial basis. Based on our literature review, marking a calcified structure with tetracycline appeared to be the most likely method to mark small walleye.

LITERATURE REVIEW

Although literature reviews and bibliographies on marking and tagging have been published previously (Moring and Fay 1984; Emery and Wydowski 1987; Chart and Bergersen 1988; Ebbers et al. 1988; McFarlane et al. 1990; Moring 1990; Parker et al. 1990), the scope of our review was limited to walleye marking techniques, especially those deemed suitable for larval and juvenile walleye. There has been little change in walleye marking techniques until the last decade (Table 1). In addition, there are only two published attempts to mark larval walleye (Gibson and Cuff 1967; Muncy and D'Silva 1981), and little attempt to mark juveniles other than by mutilation or spray-marking.

Biochemical and Chemical

There have been attempts to identify fish stocks by biochemical analyses and treatment with trace elements. Lapi and Mulligan (1981) successfully identified separate stocks of sockeye salmon *Oncorhynchus nerka* by the chemical composition of scales. Many authors have used trace elements or rare earths to mark fish. Muncy and D'Silva (1981) attempted to mark walleye eggs and larvae by immersion of water-hardened eggs in several rare compound solutions, but the mark was masked as the fish grew. Brothers (1985) failed to mark lake trout *Salvelinus namaycush* otoliths after immersion of eggs, alevins, and larvae in terbium, europium, and strontium solutions. Coho salmon *Oncorhynchus kisutch* fed strontium were successfully identified as adults when the scale core was examined (Yamada and Mulligan 1982). In a second study, newly-hatched salmon immersed in a strontium solution incorporated the element into their developing bones (Yamada and Mulligan 1987). Thorotrast injected into 4-6 cm trout at 1 g per fish was detected by X-ray 2.5 months after administration and did not

Table 1. Selected literature representing various mass marking methods that have been applied to walleye.

Application	Age	Author
	Biochemical and Chemical	
Immersion	Egg, Larval	Muncy and D'Silva 1981
	Dyes and Pigments	
Reflective granules	Juvenile	Scidmore 1961
Vital stains	Larval	Gibson and Cuff 1967
Fluorescent pigment	Adult	Imler 1974
Fluorescent pigment	Juvenile	Christianson 1975
Fluorescent pigment	Juvenile	Bandow 1987
	Genetics	
Electrophoresis	Adult	Vely 1970
Electrophoresis	Adult	Ulrikson and Laarman 1971
Electrophoresis	Adult	Clayton, et al. 1974
Electrophoresis	Juvenile, Adult	Ward and Clayton 1975
Electrophoresis	Adult	Murphy 1981
Electrophoresis	Adult	Murphy et al. 1983
Electrophoresis	Adult	Terre and Murphy 1984
Electrophoresis	Adult	Murphy and Lee 1986
Electrophoresis	Adult	Waltner 1988
Electrophoresis	?	Billington and Hebert 1990
4Electrophoresis	Adult	McInerny et al., in press
	Meristics and Morphometrics	
Morphological analysis	Adult	Murphy and Lee 1986
Morphological analysis	Adult	Waltner 1988
	Mutilation	
Fin	Juvenile	Johnson 1953
Fin	Juvenile	Maloney 1953
Fin	Juvenile	Churchill 1963
Fin	Juvenile	Laarman 1981
Fin	Adult	Hauber 1983
Operculum	Juvenile	Madsen 1986
	Scale Morphology	
Scale shape	Adult	Jarvis et al. 1978
Scale shape	Adult	Riley and Carline 1982
	Tagging	
Tubing	Adult	Wolfert 1963
Tubing	Adult	Wolfert and Van Meter 1978
Coded wire tag	Juvenile	B. Parsons, Minnesota Department of Natural Resources, unpublished data, D-J Study 138.
Coded wire tag	Juvenile	D. Friedl, Minnesota Department of Natural Resources, file data.
	Tetracycline	
Immersion	Juvenile	Scidmore and Olson 1969
Injection	Juvenile, Adult	Babaluk and Campbell 1987
Immersion	Larval	K. Kayle, Ohio Division of Wildlife, unpublished data
Immersion	Larval	R. Schademann, Ecology and Environment, Incorporated, unpublished data

harm the fish (Hasler and Faber 1941). Michibata (1981) and Michibata and Hori (1981) fed samarium to two species of fish, and marks were detectable one to two years after application with no apparent harm to the fish. Yamada and Mulligan (1990) evaluated 19 elements as potential chemical markers for fish, and found manganese and strontium marks were detectable 2 years after application. Trace elements can be applied in many ways and to various fish sizes, but detection techniques are complex, ranging from X-ray (Muncy and D'Silva 1981) to thermal neutron bombardment and spectrometry (Michibata and Hori 1981).

Biological Stains and Pigments

Biological stains (frequently referred to as dyes, stains, and vital stains) have been used as mass-markers for a variety of fish and over a wide range of sizes. Vital stains applied through immersion of the fish results in short-term marks. Gibson and Cuff (1967) immersed 14 d old walleye in neutral red and bismark brown Y vital stains with dye retention of 5 and 7 d, respectively. Mortality from immersion dye marking may be high, depending on the species to be marked (Jessop 1973). When marking with dyes, the experimenter may be more creative. For example, Bartos and Wirtz (1984) fed dyes to female parents of several species, which resulted in offspring retaining the marks for up to 10 d.

Other small-lot dye application techniques are labor intensive and have varying retention times. Dyes have been injected into fish using jet inoculators, a hand held precision instrument for subcutaneous and intramuscular injections of medicine. Juvenile striped bass *Morone saxatilis* were successfully injected with aniline blue resulting in visible marks after 12 months (McIlwain and Christmas 1975). Pitcher and Kennedy (1977) reported that roach *Rutilus rutilus* jet-inoculated with alcian blue retained visible marks for up to 3.5 years, but marks became diffuse as the fish grew. Similar to dyes, Imler (1974) was able to successfully inject fluorescent pigments into the head region of adult walleye. Pigment retention was positively correlated with fish size, and larger walleye retained marks for three years (Imler 1974). While inoculation may prove useful as a marking method for shorter term studies, its greatest disadvantage is that marking difficulty increases as fish size decreases.

Spray-marking with fluorescent pigments was initiated by Jackson (1959). Hennick and Tyler (1970) marked 33 mm pink salmon *Oncorhynchus gorbuscha* larvae of which 76% retained the pigment for up to 31 d. White (1976) noted a time related loss of fluorescent pigment with less than 50% of spray-marked pink salmon larvae retaining a mark at 203 days. Retention of fluorescent pigments is excellent for short-term studies and somewhat varied for long-term studies (Moring and Fay 1984). When used as a long-term marker, pigment retention rates for chinook salmon *Oncorhynchus tshawytscha* varied from 50-60% (Evenson and Ewing 1985) to 63-88% (Negus et al. 1990). Nielson (1990) reported a maximum mark-retention time of 12 years by spray-marked cutthroat trout *Salmo clarki utah*.

There is evidence that male salmonids will retain less pigment than female salmonids due to sexually dimorphic skin characteristics (Evenson and Ewing 1985; Negus et al. 1990), but it is unknown if non-salmonids exhibit this potential shortcoming as well. A positive relationship between pigment retention and size of marked fish has been described by Nielson (1990). Also, retention rates will vary with the color of the pigment applied (Nielson 1990).

Bandow (1987) spray-marked several lots of juvenile walleye with fluorescent pigments whose mark varied in duration from several days to approximately 10 months. However, stress from the spraying procedure appeared to cause high mortality among the treated walleye. Christianson (1975) also observed high mortality when spray-marking juvenile walleye, but attributed part of the mortality to warm water temperatures. Scidmore (1961) found

mortality to be higher for smaller walleye than larger ones when spray-marked with small light reflecting granules. Andrews (1972) observed similar size-related mortality in fathead minnows *Pimephales promelas*, and recommended a minimum size of at least 35 mm when spray-marking with fluorescent pigments. Spray-marking has the advantage of rapid application, low cost, and potentially low marking mortality (Chart and Bergersen 1988). Mark retention and mortality, however, can vary with particle size and spray pressure (Bandow 1987), and size of marked fish (Chart and Bergersen 1988; Nielson 1990).

Genetics

There is a growing body of literature documenting the use of electrophoretic techniques to differentiate between discrete fish stocks. Although work with salmonids has dominated the genetic literature, attempts to genetically separate walleye stocks have been increasing (Table 1). Early studies were unable to definitively separate walleye stocks from Lake Erie and several connected bodies water (Vely 1970; Ulrikson and Laarman 1971). Murphy (1981) found at least two distinct stocks of walleye present in Claytor Lake, Virginia. However, given the complexity of the stocking history of the lake, the cause of that discreteness could not be determined. Murphy and Lee (1986) and McInerny et al. (In press) noted that some genetic dissimilarity occurred among major Minnesota watersheds. In a similar study, Waltner (1988) was unable to genetically separate walleye stocks from two major drainages of South Dakota, but observed genetic differences between walleye from the Mississippi and Missouri Rivers. Murphy et al. (1983) used allele frequencies to determine the success of a Virginia reservoir walleye stocking program. Taggart and Ferguson (1986) and Seeb et al. (1986) noted the possibility of creating genetic markers on salmonids through selective breeding. Advantages of genetic tags include the lifetime presence of marks and elimination of marking stress. Unfortunately, extreme care must be taken to preserve tissue or blood samples, and extensive laboratory analysis is required.

Meristics and Morphometrics

Meristic and morphometric characteristics can be influenced by environmental variables and genetic variation among stocks (Chart and Bergersen 1988). Meristic and morphometric counts are made from actual specimens and typically are tested for groupings or similarities by using statistical techniques such as cluster or discriminant analysis. Separation of walleye stocks using meristic and morphometric characteristics has been mixed. Murphy and Lee (1986) were able to partially separate walleye stocks from three distinct drainages in Minnesota by analyzing meristic data. However, when analyzing morphometric data from the same fish, no discernible pattern was noticed in the grouping of samples from the various watersheds. In an analysis of walleye from South Dakota, Waltner (1988) identified both meristic and morphometric characteristics which explained a significant proportion of the variation among fish from varied locations in Lake Oahe, and also between two drainages in the state.

Mutilation

Historically, mutilation by fin-clipping has been the most common method of walleye marking, primarily due to the simplicity of the technique. Fin-clipping juvenile walleye was used successfully when it was necessary to identify a particular stock of fish. Laarman (1981) was able to evaluate juvenile walleye stocking success in Michigan using fin clips. Hauber (1983) used caudal fin clips with alternate year stocking to evaluate walleye stocking success in Wisconsin.

Clipping body parts and appendages of walleye appears to cause little mortality of marked fish. Walleye that were given a pelvic clip had similar survival rates to

unclipped fish (Maloney 1953). Operculum clips of juvenile walleye were retained for two to three years and initial mortality was similar to that of control fish (Madsen 1986).

Disadvantages of mutilation include variable fin regeneration, fish stress, and possible behavioral changes of marked fish. Also, water temperature is positively correlated with mortality rates of fin-clipped walleye (Johnson 1953). Perhaps the greatest disadvantage of mutilation is that it becomes increasingly difficult to mark fish as the size of individual fish decreases.

Scale Morphology

Fish scales have been widely used for stock identification, but little has been done with walleye. Scale shape was used to successfully delineate two walleye stocks from eastern Lake Erie (Jarvis et al. 1978), but the same method used in western Lake Erie was unsuccessful (Riley and Carline 1982). Henry (1961) and Tanaka et al. (1969) used various scale measurements to separate sockeye and chum *Oncorhynchus keta* salmon stocks. When using natural scale characteristics to identify stocks, there is no need to mark the fish, no handling until removing the scale, and life time retention.

Buchanan and Strawn (1970), and Craddock (1962) successfully marked smallmouth bass *Micropterus dolomieui* and sockeye salmon scales, respectively, through modified feeding rates. By adjusting feeding schedules, it is possible to create false checks or other recognizable patterns on juvenile fish. This method is limited by the ability of fish culturists to regulate the amount of available food. In addition, the juvenile fish must have developed to the point where a recognizable scale pattern can be formed. With any alterations of scale development patterns, there is always the risk of an overlap of characteristics with natural fish populations (Wydowski and Emery 1983).

Brothers (1985) used temperature manipulation to mark young lake trout by creating unique otolith increments. Marks were detectable with a compound microscope, and unique marks were possible by varying the temperature and manipulation schedule. Little preparation of the otoliths was necessary, but complex equipment was required to control temperature.

Fluorescent Chemicals

There are a variety of fluorescent chemicals that bond with calcium during bone formation and fluoresce visibly when viewed with ultraviolet light. Tetracycline and its sister compounds have been applied to fish in a variety of ways (Table 2). Tetracyclines can be detected under fluorescent light where they reflect a yellowish color. Early reports by Milch et al. (1957, 1961) described bone localization of tetracycline. The first attempt to mark fish with tetracycline was conducted by Weber and Ridgeway (1962). In 1967, Weber and Ridgeway reported that demethylchlortetracycline produced the most intense mark on fish bone of five tetracycline compounds that were tested. They used feeding, injection, and immersion to mark salmonids with injection resulting in the best marks. When applying tetracyclines by feeding, they found that potentiators such as glucosamine could increase the intensity of the fluorescent mark. Weber and Ridgeway (1967) also noted that certain bones will lose visible fluorescence with time. Swartz (1971) and Trojnar (1973) obtained long term tetracycline marks on salmonid vertebrae through feeding while Brothers (1985) found feeding to be unsuccessful in marking salmonid otoliths. Scidmore and Olson (1969) were able to successfully mark juvenile walleye by immersion in tetracycline, and marks were recognizable on the pelvic bones nine months after treatment.

Several authors have successfully marked the eggs or larvae of various species through immersion with little effect on survival or growth (Muth et al. 1988; Tsukamoto 1985, 1988). Walleye larvae 4

Table 2. Summarized procedures and results of selected literature discussing fish marking with tetracycline compounds. Concentration of tetracycline administered by oral or injection methods are reported in milligrams of chemical per kilogram of fish weight, oral experiments reflect the daily dosage. Columns under the treatment results category represent the time elapsed since treatment application and the percent of fish that were successfully marked.

Species	Developmental stage*	Chemical formulation**	Concentration	Treatment duration	Treatment mortality	Treatment results: Short term	Treatment results: Long term	Bones examined***	Reference
IMMERSION EXPERIMENTS									
Oncorhynchus nerka	S	CTC-H	28-39 mg/L	8-40 h	-	Several d - 0%	-	VCS	Weber & Ridgeway 1962
" "	"	"	40-99 mg/L	"	-	Several d - Variable	-	"	" " "
" "	"	"	≥100 mg/L	"	100%	-	-	"	" " "
Salvelinus fontinalis	L	OTC-H	100,500 mg/L	16 h	Negligible	? - 0%	-	USB	Choate 1964
Sillago ciliata	-	OTC-H	0.015-0.15 mg/L	4-24 h	-	? - 0%	-	USB	Lanzing & Hynd 1966
" "	-	"	15 mg/L	4-72 h	-	? - 100%	-	LIV	" " "
" "	-	"	150 mg/L	16 h	-	? - 100%	-	VCS	" " "
Paraplagusia unicolor	-	"	-	-	-	? - ≤100%	-	USB	" " "
Neoplatycephalus richardsoni	-	"	-	-	-	? - ≤100%	-	"	" " "
Urolophus testaceus	-	"	-	-	-	? - 0%	-	?	" " "
Stizostedion vitreum	J	OTC	25-300 mg/L	8-72 h	-	3-6 d - <100%	-	VCS	Scidmore & Olson 1969
" "	"	OTC[1]	25-300 mg/L	" " "	-	" " " "	-	"	" " "
" "	"	"	150 mg/L	22 h	-	-	9 mo - 100%	"	" " "
Leiostomus xanthurus	L	OTC-H[1]	100-500 mg/L	0.5-2 h	-	8 d - ≤100%	-	OTO	Hettler 1984
" "	"	OTC-H[2]	" " "	" " "	-	" " "	-	"	" "
Lagodon rhomboides	"	OTC-H[1]	" " "	" " "	-	" " "	-	"	" "
" "	"	OTC-H[2]	" " "	" " "	-	" " "	-	"	" "
Hypoatherina tropicalis	L,J	TC-H	50 mg/L	12 h	10%	-	-	OTO	Schmitt 1984
" "	"	"	250 mg/L	" "	10%	≤6 d - 100%	-	"	" "
" "	"	"	400 mg/L	" "	100%	-	-	-	" "
Spratelloides dellicatulus	L,J	TC-H	250 mg/L	" "	-	≤6 d - 100%	-	OTO	" "
Salvelinus namaycush	E	OTC-H	250-1000 mg/L	Variable	-	? - ≤100%	-	OTO	Brothers 1985, 1990
Plecoglossus altivelis	L	TC-H	100 mg/L	7 h	-	12 d - 100%	164 d - 83%	OTO	Tsukamoto 1985
" "	E	"	0-2,000 mg/L	1.5-48 h	≤100%	10 d - ≤100%	-	"	" "
" "	L	"	0-1,000 mg/L	1.5-24 h	"	3 d - ≤100%	-	"	" "
Coregonus peled	E	TC-H	600 mg/L	12 h	15%	19 d - 100%	87 d - 38%	OTO	Dabrowski & Tsukamoto 1986
" "	L	"	" "	6 h	-	8 d - 88%	68 d - 60%	"	" " "
" "	"	"	" "	3 h	-	7 d - 100%	67 d - 80%	"	" " "
" "	J	"	100 mg/L	35 h	-	22 d - 0%	-	"	" " "
" "	"	"	300 mg/L	" "	-	22 d - 100%	-	"	" " "
Alosa sapidissima	L	OTC-H[3]	50 mg/L	12 h / 4 d	13%	57 d - 98%	152 d - 82%	OTO	Lorson & Mudrak 1987
Ptychocheilus lucius	L	TC-H[1]	200 mg/L	4-36 h	Variable	7 d - 100%	77 d - 100%	OTO	Muth et al. 1988
" "	"	"	350 mg/L	" " "	"	" " "	" " "	"	" " " "
" "	"	"	500 mg/L	" " "	"	" " "	" " "	"	" " " "
Pimephales promelas	"	TC-H[1]	350 mg/L	4 h	-	" " "	-	"	" " " "

Table 2. Continued.

Species	Developmental stage*	Chemical formulation**	Concentration	Treatment duration	Treatment mortality	Treatment results: Short term	Treatment results: Long term	Bones examined***	Reference
Coregonus lavaretus	L	TC	200-800 mg/L	1-17 h	Variable	6 d - ≤100%	-	OTO	Nagiec et al. 1988
" "	"	"	" " "	2 h	-	12 d - 100%	275 d - 67%	"	" " " "
Coregonus peled	"	TC	400 mg/L	15 h	-	-	176 d - 98%	OTO	" " " "
Plecoglossus altivelis	E	TC-H	12.5-1,000 mg/L	27 h	Variable	5 d - ≤100%	-	OTO	Tsukamoto 1988
Ptychocheilus lucius	E	TC-H[1]	200,350 mg/L	12-36 h	Variable	0 d - 100%	15 d - 100%	OTO	Muth & Nesler 1989
" "	L	"	" " "	12-24 h	"	" " - "	" "	"	" " "
Tautogolabrus adspersus	J	TC-H	500 mg/L	2 h	15%	13-19 d - 100%	-	OTO	Gleason and Recksiek 1990
Stizostedion vitreum	L	TC[1]	200 mg/L	24 h	-	-	6 mo - 70%	MAX	R. Schademann, unpub. data
Alosa sapidissima	L	OTC[4]	25 mg/L	12 h / 4 d	10-27%	79 d - ≥80%	158 d - ≥7%	OTO	Hendricks et al. (in press)
" "	"	"	50 mg/L	12 h / 4 d	6-36%	49 d - 98%	168 d - 94%	OTO	" " " " "
" "	"	"	200 mg/L	6 h	4-27%	54 d - 100%	192 d - 100%	OTO	" " " " "
" "	"	"	400 mg/L	6 h	18%	-	101 d - 100%	OTO	" " " " "
" "	"	TC-H[2]	200 mg/L	6 h	2-34%	107 d - 100%	178 d - ≤100%	OTO	" " " " "
ORAL EXPERIMENTS									
Oncorhynchus gairdneri	S	CTC-H[1]	10-40 mg/kg	5 d	0%	Several d - 0%	-	USB	Weber & Ridgeway 1962
Oncorhynchus nerka	"	"	" " "	" "	"	" "	" -	"	" " "
Oncorhynchus tshawytscha	"	OTC[2]	1,600 mg/kg	" "	"	Several d - 50%	-	VCS	" " "
" "	"	OTC-H[4]	130 mg/kg	7 d	"	Several d - 100%	-	"	" " "
Salvelinus fontinalis	L	OTC-H	250-3,200 mg/kg	2.5-12 d	-	3-36 d - ≤100%	-	VCS	Choate 1964
Oncorhynchus nerka	L	CTC	25-1,000 mg/kg	2-10 d	-	? - ≤100%	-	VCS	Weber & Ridgeway 1967
" "	J	CTC[1]	250-300 mg/kg	1 Feeding	-	? - 100%	-	"	" " "
" "	J	OTC[3]	2,000 mg/kg	4 d	-	2 mo - 100%	3 y - ≤100%	"	" " "
Oncorhynchus kisutch	J	"	500 mg/kg	2 d	-	196 d - 94%	1.5 y - ≤100%	"	" " "
Oncorhynchus gairdneri	L	TC-H	100 mg/kg	4,8 d	-	1 d - ≥92%	-	VCS	Trojnar 1973
" "	"	"	400 mg/kg	" "	-	1 d - ≥87%	-	"	" "
" "	"	"	700 mg/kg	" "	-	1 d - ≥88%	-	"	" "
" "	"	"	700 mg/kg	9 d	-	15 d - 93%	365 d - 100%	"	" "
Salmo salar	J	OTC-H[4]	250 mg/kg	15 d	-	7 d - 100%	11 mo - ≤100%	VCS	Odense & Logan 1974
" "	"	OTC-H	660 mg/kg	9 d	-	-	4 mo - ≤100%	"	" " "
" "	"	"	1.3% wet wt feed	14 d	-	-	11 mo - 0%	"	" " "
" "	A	"	" " " "	-	-	-	23 mo - ≤100%	"	" " "
Coregonus lavaretus	J	TC	1,200-15,000 mg/kg	9 d	-	9 d - 100%	-	VCS	Nagiec et al. 1983
Oncorhynchus keta	L	OTC	2 g/454 g feed	7 d	-	23 d - 100%	2 y - 50-100%	VRT	Bilton 1986
" "	"	"	" " " "	14 d	-	7 d - 100%	2 y - ≥94%	"	" "
" "	"	"	" " " "	21 d	-	9 d - 100%	2 y - 100%	"	" "
Oncorhynchus nerka	J	OTC	4.5% wet wt feed	40 d	-	≤54 d - 100%	9 mo - 91%	HOM	Koenings et al. 1986
" "	"	"	" " " "	60 d	-	≤56 d - 100%	9 mo - 100%	"	" " " "
Salvelinus namaycush	E,L	OTC-H	0.2-1.0% feed	-	-	? - ≤100%	-	OTO	Brothers 1985
Esox lucius x E. masquinongy	J	OTC-H[4]	500 mg/kg	6,12 d	-	? - ≤100%	-	?	Wahl & Stein 1987
Alosa sapidissima	L	OTC	3,6,9 g/454 g feed	7,14 d	-	102 d - 0%	158 d - 0%	OTO	Hendricks et al. (In press)
" "	"	OTC[3]	6 g/454 g feed	7 d	-	-	168 d - 76%	OTO	" " " " "
" "	"	OTC[3]	40 g/454 g feed	3 d	-	126 d - 100%	178 d - 95%	OTO	" " " " "
" "	"	TC-H[3]	40 g/454 g feed	3 d	-	101 d - 100%	210 d - 100%	OTO	" " " " "

Table 2. Continued.

Species	Developmental stage*	Chemical formulation**	Concentration	Treatment duration	Treatment mortality	Treatment results: Short term	Treatment results: Long term	Bones examined***	Reference
INJECTION EXPERIMENTS									
Fundulus heteroclitus	A	TC	20 mg/kg	Daily	-	-	-	SCL	Bevelander & Goss 1962
Oncorhynchus nerka	S	CTC	25-200 mg/kg	1 Injec.	-	Several d - 100%	3 mo - 100%	VCS	Weber & Ridgeway 1962
" "	"	TC-H,OTC	100-400 mg/kg	" "	Variable	" " "	48 d - 100%	"	" " "
Carassius auratus	-	TC-H	20-400 mg/kg	1-4 Injec.	Variable	? - 100%	<8 mo - 100%	VRT,OTO,SCL	Kobayashi et al. 1964
Thmaccoyi	-	OTC-H	15 mg/kg	1 Injec.	-	<101 d - 100%	<701 d - 100%	VCS	Lanzing & Hynd 1966
Sillago ciliata	-	"	0.1-10 mg/kg	" "	-	? - 100%	-	LIV	" " "
" "	-	"	10-200 mg/kg	" "	-	63 d - 100%	-	VCS	" " "
Urolophus testaceus	-	"	50 mg/kg	" "	-	? - 0%	-	?	" " "
Gadus morhua	-	TC	0.87-1.7 mg/fish	1 Injec.	-	1 mo - ≤100%	4 mo - ≤100%	OTO,SCL	Jensen & Cummings 1967
Pseudopleuronectes americanus	-	"	" " "	" "	-	-	6 mo - ≤100%	SCL	" " "
Raja clavata	-	TC-H	50, 100 mg/kg	1 Injec.	-	-	≤3 y - 100%	VRT	Holden & Vince 1973
Thunnus albacares	J,A	OTC	125 mg/4.54 kg	1 Injec.	-	-	-	OTO	Wild & Foreman 1980
Katsuwonus pelamis	"	"	" " "	" "	-	-	-	"	" " "
Platichthys stellatus	J	OTC-H	100 mg/kg	1 Injec.	-	1 d - 100%	? - 97%	OTO	Campana & Neilson 1982
Ophiodon elongatus	J,A	OTC	100 mg/kg	1 Injec.	0%	-	2-3 y - 100%	DSL	Cass & Beamish 1983
Pristipomoides filamentosus	J	OTC	30 mg/kg	1 Injec.	0%	≤39 d - 100%	-	OTO	Ralston & Miyamoto 1983
Triakis semifasciata	-	OTC-H	25 mg/kg	1 Injec.	-	-	≤3 y - 64%	VRT	Smith 1984
Salvelinus namaycush	E,L	OTC-H	50-100 mg/kg	1 Injec.	-	? - 100%	-	OTO	Brothers 1985
Stizostedion vitreum	J,A	TC	50-100 mg/kg	1 Injec.	-	-	≤2 y - 100%	OPR	Babaluk & Campbell 1987
Squalus acanthias	-	OTC	25 mg/kg	1 Injec.	0%	-	-	DSL	McFarlane & Beamish 1987a
Anoplopoma fimbria	A	OTC-H	25-100 mg/kg	1 Injec.	≥0%	-	1-5 y - ≤100%	OTO	McFarlane & Beamish 1987b
Brevoortia tyrannus	J	OTC	13-20 mg/kg	1 Injec.	0%	11 d - 80%	-	OTO	Simoneaux & Warlen 1987
" "	"	"	0.2 mg OTC/fish	" "	-	7-14 d - 78.6%	-	"	" " "

* Developmental stages abbreviations are as follows:
E - Embryo
L - Larval
S - Smolt
J - Juvenile
A - Adult

** Reporting of the exact formulation of tetracycline used varied between authors, abbreviations are as follows:
TC - Tetracycline
TC[1] - Tetracycline and 1.5% DSMO (dimethyl sulfoxide)
TC-H - Tetracycline hydrochloride
TC-H[1] - Tetracycline hydrochloride buffered with tris buffer
TC-H[2] - Tetracycline hydrochloride buffered with potassium and sodium phosphates
TC-H[3] - Tetracycline hydrochloride mixed with glucosamine as a potentiator
CTC - Chlortetracycline
CTC[1] - Chlortetracycline mixed with glucosamine or terephthalic acid, as potentiators
CTC-H - Chlortetracycline hydrochloride
CTC-H[1] - Chlortetracycline hydrochloride mixed with carboxymethlcellulose
OTC - Oxytetracycline
OTC[1] - Oxytetracycline buffered with DSMO (dimethyl sulfoxide)
OTC[2] - Oxy tetracycline mixed with carboxymethlcellulose
OTC[3] - Oxytetracycline mixed with glucosamine as a potentiator
OTC[4] - Oxytetracycline buffered with potassium and sodium phosphates
OTC-H - Oxytetracycline hydrochloride
OTC-H[1] - Oxytetracycline hydrochloride buffered with tris buffer
OTC-H[2] - Oxytetracycline hydrochloride and DSMO (dimethyl sulfoxide), buffered with tris buffer
OTC-H[3] - Oxytetracycline hydrochloride buffered with potassium and sodium phosphates
OTC-H[4] - Oxytetracycline hydrochloride mixed with glucosamine

*** Bone abbreviations are as follows:
DSL - Dorsal spines or fin rays
HOM - Homogenized whole fish
LIV - Liver
MAX - Maxillae
OPR - Operculum
OTO - Otoliths
SCL - Scales
USB - Unspecified bones
VCS - Various calcified structures
VRT - Vertebrae or vertebral centra

Author's note: So experimental results could be concisely presented, some information presented in this table is the combined results from a series of experiments, we suggest the reader should refer to the original reference when exact details of a particular study are desired.

1. Use of company and trade names does not imply product endorsement.

and 5 d old were successfully marked by immersion in tetracycline and retained fluorescence for 6 months (R. Schademann, Ecology and Environment, Incorporated, unpublished data). Brothers (1985) immersed lake trout embryos, alevins and larvae. Alevins and larvae retained a mark while embryos did not. He also noted that tetracycline injection produced a brighter mark than immersion. Koenings et al. (1986) developed a method of quantifying tetracycline uptake after feeding sockeye salmon. This allowed the use of tetracycline to mark batches of fish uniquely, but tetracycline detection required chemical extraction.

Other chemicals, with fluorescent properties similar to the tetracyclines, have also been used to mark fish. The compounds calcein and alizarin complexone were evaluated for marking otoliths of ayu *Plecoglossus altivelis* embryos by Tsukamoto (1988). He found that alizarin produced a highly visible scarlet-pink ring, while calcein yielded a relatively poor yellowish-green mark. Conversely, calcein was found to produce a viable fluorescent mark when three species of estuarine fish were immersed in a calcein solution (Wilson et al. 1987). Hankin (1978) used calcein as a fluorescent scale marker on guppies *Poecilia reticulata*. When administered in the diet to growing fish, marks were visible under a fluorescent microscope at the conclusion of the three month experiment. Repeated application showed no effect on reproduction or growth. The advantage of this technique over tetracycline marking is the apparent retention of external marks, but there has been no further evaluation of mark longevity.

METHODS

Walleye larvae were chemically treated in Big Redd (TM)[1] incubators (Olson 1981), each of which contained three incubation tubes and manifolds for delivering water, air, and oxygen. Fish were treated in a static environment within the incubator, and fresh water flow was restored after the treatment period. Reduced air flow and an increased oxygen flow prevented excess foaming and assured adequate dissolved oxygen in the treatment incubator.

In the first trial, approximately 60,000 larval walleye averaging 1.5 d post-hatch were apportioned (10,000 per incubation tube) between two Big Redds. During treatment, larvae were immersed in a bath of 250 mg/L OTC-H (oxytetracycline hydrochloride - Terramycin concentrate) three times over a 10 d period (Table 3). Incubation tubes were submersed in the treatment solution for either 8 or 10 h. In the second trial, larvae were immersed in the OTC-H bath for one 8 h interval (Table 3). After the treatment period during trials one and two, 25 larvae were sampled and preserved in ethanol. In the third trial, 6,000 walleye larvae were immersed four separate times in 350 mg/L OTC-H (from a powdered formulation) for 8 h (Table 3). Twenty-five larvae were removed after each immersion and examined immediately for fluorescence.

During all trials, water temperature varied between 10.4 and 13.9° C, and dissolved oxygen always exceeded 6.2 mg/L. When necessary, treatment water was buffered using Tris buffer(Tris(Hydroxymethyl)Aminomethane) to near normal pH after the addition of OTC-H caused a reduction in pH.

The above procedures were repeated using a marking solution of 125 mg/L calcein (2',7'bis[[bis(carboxymethyl)-amino]methyl]fluorescein). During trial four, 200 larvae were immersed in the test solution for 2 h and kept at 14° C (Table 3). In the fifth trial, two tubes containing 10,000 larvae each were treated for 4 h at 12° C. Samples of larvae were removed after each calcein treatment for external examination under ultraviolet light.

All larvae samples were illuminated with a portable Blak-Ray long wave ultraviolet lamp[2] and examined externally with a stereomicroscope. Photographs of larval fish were taken with a Nikon epi-fluorescent microscope[2] equipped with V-1A filter combina-

Table 3. Summary of oxytetracycline and calcein marking experiments on walleye larvae.

Date	Treatment duration (h)	Elapsed time before sampling (h)	Number sampled	Fish age (d) when marked	Total length (mm) Mean	Total length (mm) Range	Percent of fish with external fluorescence
Oxytetracycline - 250 mg/L							
Trial #1							
4 May, 1987	8.0	12.0	25	1.5	8.2	7.5- 9.0	4.0
	10.0	10.0	25	1.5	8.2	7.0- 9.0	12.0
7 May, 1987	8.0	12.0	25	4.5	8.6	8.0-10.0	20.0
	10.0	10.0	25	4.5	8.0	7.0- 9.0	32.0
11 May, 1987	8.0	0.5	25	8.5	8.6	7.5- 9.5	84.0
	10.0	0.5	25	8.5	9.0	8.5-10.0	80.0
Trial #2							
27 May, 1987	8.0	96.0	25	14.0	9.2	9.0-10.0	60.0
Oxytetracycline - 350 mg/L							
Trial #3							
12 May, 1990	8.0	24.0	25	1.0	8.4	8.0- 9.0	12.0
14 May, 1990	8.0	24.0	25	3.0	9.4	9.0-10.0	24.0
16 May, 1990	8.0	24.0	25	5.0	9.5	8.5-10.0	100.0
18 May, 1990	8.0	20.0	25	7.0	9.7	9.5-10.5	100.0
		67.0	25	7.0	9.8	9.0-10.5	100.0
		115.0	25	7.0	9.9	9.0-10.5	100.0
Calcein - 125 mg/L							
Trial #4							
10 May, 1988	2.0	-	25	1.5	-	-	24.0
Trial #5							
13 May, 1988	4.0	-	25	1.5	-	-	56.0

tion and illuminated by a 100 watt mercury lamp. Temperature units (TU) were calculated by summing degree-days above 0° C.

RESULTS

Mortality of larvae during oxytetracycline and calcein treatments was minimal. During oxytetracycline treatments, visual observations indicated a greater mortality of larvae in treatment tubes than control tubes, and in tubes that were held in the marking solution for 10 h as opposed to 8 h. Likewise, mortality during calcein marking was minimal. The three environmental controls of the Big Redd allowed for a very successful experimental environment while helping to assure a low experimental mortality.

External fluorescent marks were most evident in the splanchnocranium and pectoral girdle of recently marked larval walleye. Fluorescent marks appeared as thin yellow lines against a dark purple background when the OTC-H had been incorporated into the bone, especially in the premaxillae, maxillae, dentary, and developing pectoral girdle bones. The largest and most brilliant marked bones were the premaxillae, maxillae, and dentary (Table 3). The quality of marking on bony parts was consistent among the sampled larvae.

Marking success varied between trials (Tables 3 and 4). Observations indicated that marking success increased with fish age, size, and as OTC-H concentration increased. Greater than 80% of larval walleye 5 d old or older successfully incorporated OTC-H into their bony parts (Table 3). An OTC solution of 250 mg/L failed to mark all treated fish, while 350 mg/L OTC-H assured 100% marking success. In our experimental environments, walleye larvae needed to be at least five d old or 9.5 mm in length, and immersed in 350 mg/L of OTC-H before a total batch mark was obtained. Key developmental characteristics we noticed at 100% marking were (trial three; third immersion): jaws and teeth were very developed, yolk sac was rapidly depleting, gill arches, and lamellae were present in most fish.

Table 4. Relative intensity of fluorescent marks in various bones of larval walleye that were immersed in tetracycline hydrochloride solutions. Mark intensities were determined from 25 larvae from each treatment and ranked 0-3 (absent-bright). Values were averaged within each sample lot to produce a batch mark intensity value.

Mark intensity by OTC concentration, exposure time, and number of immersions											
	250 mg/L							350 mg/L			
	Trial #1						Trial #2	Trial #3			
	8 h			10 h			8 h	8 h			
Number of immersions	1	2	3	1	2	3	1	1	2	3	4
Mean length (mm)	8.2	8.6	8.6	8.2	8.0	9.0	9.2	8.4	9.4	9.5	9.7
Fish age when marked (d)	1.5	4.5	8.5	1.5	4.5	8.5	14.0	1.0	3.0	5.0	7.0
Percent of fish exhibiting external fluorescence	4.0	20.0	84.0	12.0	32.0	80.0	60.0	12.0	24.0	100.0	100.0
Anatomical features examined[a]											
Dentary	0	0.1	1.7	0.1	0.6	1.6	0.9	0	0.1	1.3	2.8
Teeth	0	0	0.6	0	0	1.0	0.2	0	0	0.2	1.2
Otoliths	0	0.2	1.1	0	0	1.4	0.7	0	0.1	0.8	1.0
Premaxilla	0	0.1	1.7	0.1	0.6	1.6	0.9	0	0	1.3	2.8
Maxilla	-	-	-	-	-	-	-	0	0	1.3	2.8
Pectoral[b]	-	-	-	-	-	-	-	0	0	0.8	2.0
Hyoid[b]	-	-	-	-	-	-	-	0	0	0.3	0.9
Yolk sac	-	-	-	-	-	-	-	1.1	0.2	0.6	1.4
Unidentified skull bones	0.1	0.1	0.2	0	0.2	0.2	0.2	0.1	0.2	0.2	2.8

[a] Parts of the operculum and branchial apparatus were observed to fluoresce after the last treatments in experiments 1 and 3, although no determination was made on their relative brightness.

[b] Intensity values reported here refers to the group of bones, the individual bones of these groups were not separated.

Comparatively, a smaller percentage of calcein treated fish were successfully marked, based on external examination (Table 3). The calcein had concentrated in the gastrointestinal tract, but not the bony parts of the treated larvae.

DISCUSSION

Since walleye larvae are small (< 10 mm) when they are stocked, it would be desirable to have a permanent mark that could be quickly and effectively applied before stocking. We were able to successfully mark walleye larvae with OTC-H by whole-body immersion. Initial fluorescence observed in larval walleye shortly after marking was most evident in the developing bones of the skull. We found that the premaxillae, maxillae, and the dentary fluoresced most brilliantly. These bones are among the first to ossify in the developing walleye larvae (Norden 1961; McElman and Balon 1979). Previously, 4 and 5 d old larval walleye were marked with similar results in Kansas (R. Schademann, Ecology and Environment, Incorporated, unpublished data).

With varying percentages of OTC-H marked fish among trials, several hypotheses can be drawn. First, the dosage of OTC-H was increased to 350 mg/L during the third trial. This increase in dosage, when compared to the first two trials, may have exceeded an application threshold that assured a mark on all treated larvae. A second hypothesis is that larval walleye must obtain a critical age, size, or developmental phase before a quality mark is achieved. Support for this hypothesis is especially evident in trials one and three, where the percentage of marked fish increased with both age and size. From our data we were not able to ascertain if either age, size, or developmen-

tal phase had more influence on marking success.

In trial three, our experimental larvae were compared to the developmental phases of early walleye ontogeny described by McElman and Balon (1979). They describe the third phase in the embryonic development of the walleye as the period from hatch to the start of external feeding. Calcification of the splanchnocranium begins early in the third phase, and by the the third phase in the embryonic development of the walleye as the period from hatch to the start of external feeding. Calcification of the splanchnocranium begins early in the third phase, and by the end of this phase, teeth and jaws have become calcified. Also, larvae transfer to gill respiration and experience rapid yolk depletion prior to the start of feeding. It is the later step of the third phase (Step F^2 11; McElman and Balon 1979) that best describes how developed our study fish were when 100% retention of OTC-H was first observed. Walleye size at hatch and growth rates will vary with location. We suspect that comparing fish to be marked with tetracycline to a developmental phase will prove to be better indicator of readiness for an OTC-H application than either size or age since hatch.

Since growth is directly related to water temperature, it is unknown how much water temperature variation will influence the time of OTC-H incorporation or overall marking success. McElman and Balon (1979) determined it took walleye embryos 6 d 18 h (or 101 TU) to go from hatch to external feeding. During trial three, our study fish had experienced 77 TU since hatch when a 100% mark was first achieved (third immersion), and 101 TU by the fourth immersion. During trial three, larvae were held for 114 TU post hatch before they were stocked. At this time the yolk and oil globule were nearly absorbed, and according to Balon and McElman (1979) our study fish had became larvae (Step PP^1 12). The 114 TU our study fish had experienced during the post hatch embryonic phase was similar to the 101 TU described for this phase by McElman and Balon (1979).

McElman and Balon (1979) found walleye otolith formation occurs approximately midway between fertilization and hatch. Using a StereoZoom microscope, otoliths were not visible until 3 d post-hatch during our study. It is possible that we could have observed otoliths at a younger age, and found all otoliths to fluoresce more brilliantly if we had examined them with epi-fluorescence micropsy. During this study, larval walleye otoliths were calcified and visibly fluorescent, but they never obtained the visible brilliance of the jaw bones. Otoliths from squawfish *Ptychocheilus lucius* and ayu have been successfully marked as late embryos or newly hatched larvae (Tsukamoto 1988; Muth and Nesler 1989).

Our experiments suggest that OTC-H may be more effective as a marking medium than calcein at the concentrations that were used. However, the calcein treated fish were recently hatched (1.5 d) when they were treated, and similar to the initial OTC-H treated fish, may have been too young to exhibit induced fluorescence. More experimentation is needed to determine exactly why calcein treatments were not as successful.

MANAGEMENT IMPLICATIONS

We have demonstrated the potential use OTC-H for mass-marking larval walleye, but the persistence of this mark is unknown. Development of a successful laboratory technique would allow marked larvae to be identified as juveniles or adults which would in turn make it possible to assess the effectiveness of a stocking program, success of natural reproduction, stock structure dynamics, and the economic efficiency of a stocking program.

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