

## **Calcein (SE- MARK®) Clinical Field Trials - INAD 10-987**

### **Year 2007 Annual Summary Report on the Use of Calcein (SE- MARK®) in Field Efficacy Trials**

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#### **Summary**

Marking agents such as calcein and oxytetracycline are routinely used in fisheries programs to mark otoliths and other calcified tissue (i.e., fins, rays, and scales) in fish as a way to monitor fish propagation programs. The U.S. Food and Drug Administration has authorized the use of Calcein (SE- MARK®; CAL) under the Compassionate Investigational New Animal Drug (INAD) Exemption #10-987 for the purpose of gathering efficacy data to support a new animal drug approval. In calendar year 2007 (CY07) the efficacy of CAL to mark calcified tissue was evaluated in 21 trials involving approximately 0.30 million fish/mussels. Trials were conducted at nine different hatcheries, including four U.S. Fish and Wildlife Service fish hatcheries, four state hatcheries, and one tribal hatchery during this period. The compassionate study protocol under which treatments were administered allowed the investigator to use CAL at a dosage of either: (1) 125 - 250 mg/L calcein for 1 - 6 h, or (2) 2.5 - 5.0 g/L calcein for 1 - 7 min with a pre-treatment of 1 - 5% solution of non-iodized salt for about 3.5 min. Efficacy was based on whether or not a "readable" mark could be seen in the calcified tissue of a subsample of treated fish/mussel. Overall, results from the treatment trials

conducted in CY07 indicated that approximately 90% of trials appeared efficacious, while 10% of the trials were ineffective.

## **Introduction**

Calcein is an effective and convenient marking agent for use on early life stages of fish. Large numbers of fish can be marked simultaneously by simple exposure to a calcein solution for concentration dependant durations that could last from several minutes to several hours. In many cases, immersion marking is one of the only practical means of permanently marking large numbers of small fish for the purpose of evaluating fishery management strategies. In general, marking is accomplished by immersing very young fish in a bath containing either 1) 125 - 250 mg/L calcein for 1 - 6 h; or 2) 2.5 - 5.0 g/L for 1 - 7 min. A pre-treatment exposure of fish to 1 - 5% solution of non-iodized salt for about 3.5 min is recommended to facilitate the osmotic transfer of calcein across fish tissue membranes and into calcified tissues.

The overall objective for using calcein as a marking agent under this INAD was to develop clinical field efficacy data for non-intrusive marking of fish larvae or very young fish prior to, or shortly after, initiation of feeding, and to observe the marks on live fish to evaluate whether they are “readable.” An advantage of immersion mass marking fish of such a small size is that these fish cannot be marked by fin clip or by using other conventional tagging procedures. Fish marked at early life stages are not available for human consumption until they have grown to a much larger size, which in virtually all

cases requires at least a year or more of additional growth. Except for threatened and endangered species and research fish destroyed after use, calcein treatment under INAD 10-987 authorizes that no fish larger than 2 grams in size may be marked with calcein. Therefore, Investigators using calcein under this INAD may be able to successfully mark fish for stocking with high confidence that calcein absorbed in fish tissues will not pose a human health concern.

### **Purpose**

The purpose of this report is to summarize the results of CY07 CAL field efficacy studies conducted under INAD #10-987. Furthermore, it is expected that data from these trials will be used to enhance the existing CAL database that has been established from previous years studies for the purpose of developing an appropriate label claim for the use of CAL in aquaculture.

### **Facilities, Materials, and Methods**

#### **1. Facilities**

A total of 21 CAL efficacy trials were conducted at nine fish culture facilities during CY07, including four U.S. Fish and Wildlife Service fish hatchery, four state hatcheries, and one tribal hatchery. Mean water temperature during all trials was 65.4 °F, and water temperature during treatments at the various testing facilities ranged from 35.0 to 76.8°F.

## 2. CAL used in trials

All CAL used during the reporting period was SE- MARK<sup>®</sup>, which is a commercial liquid product supplied by Western Chemical, Inc., Ferndale, Washington. The strength of SE- MARK<sup>®</sup> is a pH buffered, 1.0% solution of calcein. Western Chemical's SE- MARK<sup>®</sup> is the only form of calcein available for use under INAD #10-987.

## 3. Drug dosages

According to the protocol, Investigators were able to treat fish/mussel with the following treatment regimens: (1) 125 - 250 mg/L calcein for 1 - 6 h (fish/mussels), or (2) 2.5 - 5.0 g/L calcein for 1 - 7 min with a pre-treatment of 1 - 5% solution of non-iodized salt for about 3.5 min (fish only). During the reporting period, Investigators treated fish/mussel with 125 - 250 mg/L calcein for 1 - 6 h in two trials; and with 2.5 - 5.0 g/L calcein for 1 - 7 min with a pre-treatment of 0 - 5% solution of non-iodized salt for 1.5 - 4.5 min in 14 trials.

**Study Protocol Deviation:** Treatment regimen administered in the remaining five trials deviated from the protocol. In one trial, the Investigator treated mussels with 250 mg/L calcein for 12 hrs. Please note that the mussels are a non-edible food source and will not enter the human food chain. In the remaining four trials, June suckers and channel catfish were treated with 50 g/L calcein for 4 min with a pre-treatment of 0 - 1.5% solution of non-iodized salt for 4.0 min. Please note

that these fish were used for a pilot target animal safety study and all test fish were destroyed after the study period.

## **Fish Species**

### **1. Species of fish treated**

A total of 11 different fish species and mussels were treated during CY07, including four species of salmonids, six non-salmonids fish species, and one mussel species. Mean weight of treated fish was 1.42 g (excluding the Atlantic salmon – see protocol deviation), and fish size ranged in weight from 0.10 - 90.7 g.

**Study Protocol Deviation:** Mean weight of treated fish exceeded 2.0 g in five trials. In one trial involving Atlantic salmon, the Investigator noted that the treated fish weighed 90.7 g. However, regulatory discretion for this deviation was granted by CVM on May 2, 2007. Please note that there is no legal harvest permitted for the Atlantic salmon. In four trials involving channel catfish, the Investigator noted that the average weight was 3.8 g. Please note that all of the treated catfish were euthanized at the end of these trials.

The following fish/mussel species were treated during CY07:

### **Salmonids**

Atlantic salmon *Salmo salar*

lake trout *Salvelinus namaycush*

rainbow trout *Oncorhynchus mykiss*

sockeye salmon *O. nerka*

### **Non-salmonids**

channel catfish *Ictalurus punctatus*

fathead minnow *Pimephales promelas*

June sucker *Chasmistes liorus*

largemouth bass *Micropterus salmoides*

pallid sturgeon *Scaphirhynchus albus*

walleye *Sander vitreus*

### **Mussels**

fatmucket *Lampsilis siliquoidea*

## **2. Marking**

Fish/mussels were treated with CAL to provide a mark in calcified tissue such as otolith, skeletal tissue, fin rays, scales, or mussel shell for the purpose of identifying hatchery-stock fish/mussel in the wild.

## **Data Collected**

### **1. Efficacy of marking procedure**

A sub-sample of fish/mussel from the test population were collected and evaluated for efficacy of the marking procedure and mark retention data, as well as morbidity and mortality related to the marking procedure.

### **2. Effect of treatment on treated fish**

Study Investigators were encouraged to include general observations on the effect of treatment on fish behavior and response to routine culture/management activities (i.e. feeding activity, level of stress, or negative fish behavior).

## **Discussion of Study Results**

**1. Summary results on the efficacy of CAL for marking fish/mussel** - Efficacy was based on whether or not a “readable” mark could be seen on calcified tissue from a subsample of treated fish/mussel. (Note: A summary of the individual CAL studies conducted during CY07 under INAD #10-987 in which trials appeared to be efficacious are presented in Table 1; Table 2 provides a summary of all trials in which treatments were ineffective; Table 3 describes the treatment regimens used and fish/mussel species tested; and Table 4 lists all treatment trials conducted during this reporting period).

### **A. Efficacy of CAL at 250 mg/L for 6 h**

Mussels and June suckers were exposed to 250 mg/L CAL for 4.25 - 6 h in two trials (Table 1). The Investigator noted that mussels were checked for a CAL mark at 300 days post-treatment and all checked mussels had a dimly visible dull green mark (i.e. rated as poor). The trial involving the June suckers was an experimental test to duplicate study number 10-987-05-001 where most of the fish died. Fish were approximately the same size, and dose/duration and temperature were also duplicated. Only one mortality was noted throughout the entire study. A clearly visible green mark (i.e. rated as good) was visible on the checked fish five days post-treatment. CAL treatment appeared to be effective in both trials.

### **B. Efficacy of CAL at 250 mg/L for 12 h**

Mussels were exposed to 250 mg/L CAL for 12 h in one trial (Table 1). The Investigator noted that mussels were checked for a CAL mark at 300 days post-treatment and all checked mussels had a dimly visible dull green mark (i.e. rated as poor). CAL treatment appeared to be effective in this trial.

### **C. Efficacy of CAL at 5.0 g/L for 0.45 - 6.0 min**

Fish were exposed to 2.5 - 5.0 g/L calcein for 0.45 - 6.0 min in 14 trials (Tables 1 & 2) involving Atlantic salmon, lake trout, rainbow trout, sockeye salmon, channel catfish, fathead minnows, June suckers, largemouth bass, pallid sturgeon, and walleye. The Investigators in 12 trials noted that an excellent mark was seen on

checked fish; range of time when fish were evaluated for a mark ranged from immediately after treatment to seven months post-treatment. The Investigator in two trials involving 2 and 5 day old pallid sturgeon, noted that marks were extremely difficult to see if they were present at all. Overall, results indicated that treatment appeared effective in 12 trials, while two trials involving pallid sturgeon were ineffective.

#### **D. Efficacy of CAL at 50.0 g/L for 4.0 min**

June suckers and channel catfish were exposed to 50.0 g/L calcein for 4.0 min in four trials (Table 1). The Investigator noted that an excellent mark was seen on all checked fish seven days post-treatment. This was a pilot 10X Target Animal Safety Study conducted by the Aquatic Animal Drug Approval Partnership Program at the Bozeman Fish Tech Center. All test fish were destroyed at the end of the post-treatment period. CAL treatment appeared to be effective in all trials.

## **2. Observed Toxicity**

No toxicity or adverse effects relating to CAL treatment were reported in 62% of the trials conducted in CY07. Investigators in the remaining 38% of the trials noted test fish exhibited abnormal behavior either due to the pre-treatment salt bath or with the CAL bath. In two trials involving fathead minnows, the Investigator noted that the fish seemed to take the treatment well; however, the pre-treatment salt bath elicited a "frantic" response. In two trials involving pallid sturgeon, the Investigator noted that

the fish experienced a decrease in the normal swim-up and float down behavior during the treatment. In four trials involving June suckers, the Investigator noted that fish were agitated when exposed to treatment and that they gulped at the surface and darted about the water column. Two of the four June sucker studies were conducted at 50 g/L and it was noted that high mortality was experienced during the post-treatment period.

### **Number of Treated Fish under Slaughter Authorization**

Total number of treated fish during CY07 was 302,586. The total number of treated fish to count against the slaughter authorization dated October 20, 2003 is 1,884,176; and the total number of mussels to count against the slaughter authorization dated August 26, 2004 is 240. No changes have occurred to the current CAL INAD #10-987 study protocol. Please note that an amended authorization for INAD 10-987 was submitted to CVM on May 15, 2008. This amendment requested fewer fish under the slaughter authorization and to update facility information.

### **Facility Sign-up List**

Please see “Table 5. Facilities and Names of Investigators” for facilities that signed-up to participate in the CAL INAD #10-987 during CY07. Facilities not listed in Appendix III-a of the current CAL INAD #10-987 study protocol have been highlighted.

The following facility received CAL during CY07 but never used the drug:

1. Klamath Falls FWO

### **Summary of Study Results**

CAL was used in 21 trials involving Atlantic salmon, lake trout, rainbow trout, sockeye salmon, channel catfish, fathead minnows, June suckers, largemouth bass, pallid sturgeon, walleye, and mussels during CY07. Trials involved a single bath treatment at dosages of either 1) 250 mg/L calcein for 4.25 - 12 hr; or 2) 2.5 - 50.0 g/L for 0.45 - 6.0 min with a pre-treatment of 0.5 - 5.0% solution of non-iodized salt for 1.5 - 4.5 min. Approximately 0.30 million early life stage fish/mussel were treated during this period. Water temperature during treatment ranged from 35.0 to 76.8°F. Efficacy was based on whether or not a “readable” mark could be seen in the otolith, skeletal system, or shell of a subsample of treated fish/mussel. Overall, results from the treatment trials conducted in CY07 indicated that CAL treatments appeared effective in approximately 90% of the trials, while results from the remaining 10% of the trials were ineffective. Investigators reported no evidence of toxicity or adverse effects related to CAL treatments in 62% of the trials. Although data from these trials will be considered as ancillary, trial results should provide useful corroborative data to support a future label claim for CAL. It is anticipated that additional ancillary efficacy data will continue to be collected under INAD #10-987. In future trials conducted under INAD #10-987, efforts will continue to be directed towards the generation of high quality data.

**Table 1. Summary of CY07 Calcein (SE- MARK®) Efficacy Results - Efficacious Trials**

Hatchery	Number of Trials	Fish Species	Number of Fish	Fish Size (gm)	Treatment Duration (min)	Dose (g/L)	Salt Conc.		Temp. (°F)
							%	Duration (min)	
Bozeman FTC	1	JSK	100	0.60	4.25 hrs	0.25	-	-	65.0
Genoa NFH	1	MSL	100	-	6 hrs	0.25	-	-	70.0
	1	MSL	100	-	12 hrs	0.25	-	-	70.0
N.E. Fishery Center	1	ATS	90,000	90.70	4.0	2.5 - 5.0	1.5	4.0	45.0
Bozeman FTC	1	CCF	30	3.80	4.0	2.5 - 5.0	-	-	64.0
	1	CCF	30	3.80	4.0	2.5 - 5.0	1.5	4.0	64.0
Valley City NFH	2	FHM	6,384	1.90	2.0 - 3.0	2.5 - 5.0	3.0	1.5 - 2.0	76.8
Bozeman FTC	1	JSK	40	0.70	4.0	2.5 - 5.0	-	-	72.5
	1	JSK	40	0.70	4.0	2.5 - 5.0	0.5	4.0	72.5
University of Vermont	1	LAT	20,000	0.30	4.0	2.5 - 5.0	5.0	3.5	35.0
Manning SFH	1	LMB	31,322	1.90	4.5	2.5 - 5.0	1.2	4.5	70.0
Nampa Research Office	1	RBT	56,000	0.16	3.5	2.5 - 5.0	1.5	3.5	54.5
Hoko Falls Hatchery	1	SOS	97,600	0.22	4.0	2.5 - 5.0	4.1	4.0	48.4
Waterville SFH	1	WAE	660	0.42	4.0	2.5 - 5.0	2.5	2 - 4	71.5
Bozeman FTC	1	CCF	30	3.80	4.0	50	-	-	64.0
	1	CCF	30	3.80	4.0	50	1.5	4.0	64.0
	1	JSK	40	0.70	4.0	50	-	-	72.5
	1	JSK	40	0.70	4.0	50	0.5	4.0	72.5

**Table 2. Summary of CY07 Calcein (SE- MARK®) Efficacy Results - Ineffective Trials**

Hatchery	Number of Trials	Fish Species	Number of Fish	Fish Size (gm)	Treatment Duration	Dose (g/L)	Salt Conc.		Temp. (°F)
							%	Duration (min)	
Bozeman FTC	2	PLS	40	0.10	0.45 - 6.0	2.5 - 5.0	-	-	72.0

**Table 3. Description of Treatment Regimes Used and Fish Species Treated during CY07 Calcein (SE- MARK®) Efficacy Studies**

<b>Total Number of Fish Treated:</b>	<b>302,586</b>
Number of fish treated in effective trials	302,546
Number of fish treated in ineffective trials	40
<b>Total Number of Trials:</b>	<b>21</b>
Number of trials in which treatments were effective	19
Number of trials in which treatment results were ineffective	2
<b>Treatment Regimes Used:</b>	
250 mg/L static bath for 4.25 - 6 hr	2 trials
250 mg/L static bath for 12 hr	1 trial
2.5 - 5.0 g/L static bath for 0.45 - 6.0 min	14 trials
50.0 g/L static bath for 4 min	4 trials

Treatment Water Temperature (°F): 35.0 - 76.8

Size of Treated Fish (gm): 0.1 - 90.7

**Species Treated:**

**Salmonids**

Atlantic salmon *Salmo salar*

lake trout *Salvelinus namaycush*

rainbow trout *Oncorhynchus mykiss*

sockeye salmon *O. nerka*

**Non-salmonids**

channel catfish *Ictalurus punctatus*

fathead minnow *Pimephales promelas*

June sucker *Chasmistes liorus*

largemouth bass *Micropterus salmoides*

pallid sturgeon *Scaphirhynchus albus*

walleye *Sander vitreus*

**Mussels**

fatmucket *Lampsilis siliquoidea*