GREAT LAKES FISHERY COMMISSION

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Otolith Marking Techniques for the Early Life History Stages of Lake Trout

by:

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INTRODUCTION

The research reported herein is in response to a need expressed by the Great Lakes Fishery Commission. The Commission is planning a major stocking program and study of lake trout, Salvelinus namaycush. This study will require that fish be marked as eggs (embryos), alevins and fry; that marks be "encodable" to later identify multiple groups of fish stocked at various life stages and/or localities; that the marking technique be easily and quickly applied to large numbers of fish (ie. hundreds of thousands to millions); and that the marks be persistent and distinguishable over time periods comparable to the life span of lake trout (in excess of 20 years). Some method of otolith marking was identified as the approach which would be most likely to meet all of these requirements.

The otoliths (in particular the sagittae and lapilli) are the first calcified structures to appear in unhatched or embryonic stages of lake trout (Fig. 1). Once formed, the early otolith growth structures remain intact and unchanged for the entire life of the fish. In other words, otolith microstructural features formed during a lake trout's egg, alevin and fry stages persist throughout the life of the fish and may be viewed and analyzed in fish of any age. Because the otoliths are the only permanent and persistent structures present in the very earliest life history stages, they offer the only possibility for producing an endogenous and unique mark; ie., one which does not depend upon the insertion or attachment of some artificial tag. To date, the smallest salmonids marked with coded

wire tags are emergent pink salmon (approx. 30mm in length and 250mg in mass; Thrower and Smoker, 1984). This technique requires handling of individual fish, and may have significant effects on mortality when applied to extremely small fish (for comparison, newly hatched lake trout are approximately half the length and 0.4 the mass of the smallest wire-tagged salmon). Micro-wire tags are not applicable to marking embryos or the young alevins of lake trout. Other traditional marking procedures such as fin clipping, tatooing, and dying (Wydoski and Emery, 1983) would almost certainly prove to be either too emphemeral, difficult, time consuming, or potentially damaging to the fish, and therefore of little practical value in the proposed application. Thus, otolith marking appears to be the most reasonable approach to meet the specific needs expressed by the G.L.F.C.

Otolith marking for larval and juvenile fishes has been successfully accomplished by several techniques in a variety of fishes (Brothers, 1978, 1981, and unpublished; Victor, 1982; Tanaka et al., 1981; Campana & Neilsen, 1982; Taubert and Coble, 1977; Mugiya and Muramatsu, 1982; Hettler, 1984; Schmitt, 1984 Tsukamoto, 1985 and many others). These observations and experiments were often performed during studies of otolith growth or as part of laboratory work to validate the occurrence of daily growth increments, and not to produce marked fish for release into the wild. Some otolith marking in adults has been used to mark fish for release and subsequent recapture (Lanzing and Hynd, 1966; Jones and Bedford, 1968; Blackler, 1974; Wild and Foreman, 1980; Helfman, Bozeman and Brothers, unpublished), however these fish also carried externally visible tags; the otolith work was still geared to age validation studies.

My experience with otolith microstructure studies on the early life history of many species, including trout, char, and salmon, suggested at least four possible approaches to marking the otoliths of lake trout embryos, alevins and fry. The various methods would require different treatments to produce the mark, and more significantly, very different modes of viewing or analysis to reveal the marks. The proposed methods fell into the following categories:

I. Microstructural patterns produced by environmental temperature manipulations. Although light and feeding manipulations are possibilities, my observations on salmonids demonstrate that temperature cues produce the most reliable and striking results (Brothers, 1981: see also Wilson and Larkin, 1980; Campana, 1983; and Volk et al. 1984; Campana and Neilson, 1985). The types of marks caused by temperature manipulations are clearly distinguishable with light microscopy. Such analysis would require the least critical, quickest and most economical mark "recovery", particularly in comparison to the chemically-based mark discussed below. The only preparation needed would be otolith grinding (or possibly sectioning) to view the microstructure formed during the earlier marking stages. I anticipated that temperature marking of lake trout otoliths would prove to be the most practical and effective of the proposed methods.

II. Chemical markers. Included in this category are methods which utilize chemicals to either alter "normal" otolith growth patterns, or which become directly incorporated in the otolith structure.

A. Tetracycline. This compound is a well known (Weber and Ridgway, 1962, 1967; Choate, 1964; Kobayashi et al., 1964) marker for

calcified structures (including otoliths) in fish. For larger fish it is generally injected. There has also been success by adding the compound to food, or by immersion, especially for young marine fishes (Lanzing and Hynd, 1966; Hettler, 1984; Schmitt, 1984). Immersion would be the only practical batch procedure for life stages too young to feed or too small to inject, however, there is very little published work on immersion marking of young freshwater fish (Tsukamoto, 1985). For fry, marking may be achieved by feeding, immersion, or both. Viewing of a tetracycline-marked otolith, particularly if the specimen is from an adult fish, requires grinding of the otolith and microscopic examination of a fluorescent band with U.V. illumination. An incident illumination arrangement gives superior results. Tetracycline marks are known to be somewhat light-labile, however good marks are known to persist for at least several years in internal structures such as bones and otoliths (Blackler, 1974).

B. Acetazolamide marking. Successful marking of the otoliths of young fishes has been obtained with the carbonic anhydrase inhibitor, acetazolamide (Mugiya, 1977; Mugiya & Muramatsu, 1982; and R. Radtke and W. Haake, pers. comm.). The mark produced has been described as a growth disruption or interruption; an abrupt cessation of aragonite crystallite deposition, delineated by a thin protein layer. The mark can be seen with the light microscope, however confirmation and optimal viewing requires the use of the scanning electron microscope (SEM). The only normally occurring growth interruption in the otoliths of very young salmonids is sometimes

produced at hatching. Preparation for SEM viewing would involve grinding, polishing, etching with an acidic agent, and coating (Brothers et al., 1976; Brothers & McFarland, 1981; Haake et al., 1981). Embedding of the otoliths will also be required for small specimens. To my knowledge, acetazolamide has only been administered by injection, however it is water soluble and immersion would be a possible exposure route.

C. Trace element marking. Sagittae are almost entirely composed of calcium carbonate (as aragonite) and a fibrous protein called otolin (ref. cited in Brothers, 1984; Carlstrom, 1963; Degens et al., 1969; Pannella, 1980). The remaining fraction (usually less than 1% by weight) is made up of water and a variety of naturally occurring trace elements which are incorporated or substituted into the otolith structure and apparently remain intact for the life of the fish. The trace element content of the otoliths of wild fish has been used as natural markers of stock origin or even age (Gauldie et al., 1980; Bennett et al., 1982; Papadopoulou et al., 1978, 1980; D. Martin, pers. comm.). Analysis has been by atomic absorption spectrometry and the electron microprobe. In addition, Sr/Ca ratios have been examined as possible indicators of the temperature of carbonate formation in invertebrate skeletons and otoliths (Schneider and Smith, 1982; Radtke, 1984). Recently, several workers have attempted to utilize laboratory introduced trace elements to mark the otoliths and other tissues of fishes. Marking has been by addition to the diet and by immersion (Muncy and D'silva, 1981; Calaprice and Calaprice, 1970; Miller, 1963; Trefethen and Novotny, 1963; Anonymous, 1974; Ophel and Judd, 1968; Yamada et al. 1979; D. Martin, pers.

comm.; R.J. Muncy et al., in prep., and H. Poston, pers. comm.). Although much of the work was not performed on salmonid fishes, the concept and general procedures should be applicable to the lake trout situation. Possible elements of interest include cooper, zinc, fluoride, strontium and various rare earth elements. Analysis of single or perhaps multiple exposures and resulting deposition layers would involve use of the electron microprobe and x-ray spectrometry in conjunction with a SEM. Marks should appear as discrete bands superimposed over the SEM image when the probe is used in the elemental "map" mode. Preparation for this analysis includes grinding, polishing, etching, coating and then x-ray spectral analysis with counting periods of several minutes per specimen. It will be a relatively expensive and time consuming procedure.

In summary, the objective of this study was to develop methods and techniques of marking otoliths in the early life stages of lake trout, for fish to be used in stocking experiments in the Great Lakes.

Materials and Methods

All observations and experiments were performed on eggs, alevins and fry of hatchery reared lake trout, *Salvelinus namaycush*. Fish were of the Marquette/Superior stock and were obtained at the eyed egg stage from two U.S. Fish and Wildlife sources; the Tunison Laboratory of Fish Nutrition (Cortland, N.Y.) and the Jordan River National Fish Hatchery (Elmira, Mich.). Eggs were stripped and fertilized on 11 October and 26 November 1984; eyed eggs were obtained on 16 November

and 9 January respectively. The Jordan River fish exhibited a very high percentage (ca.70%) of teratological deformities, particularly twinning (Lynn, 1938). The cause of these anomalies was not determined, but probably the result of some shock during early development (L. Wubbels, pers. comm.). Twinned trout which showed moderately typical development rates (at least to late alevin stages) were usable for otolith observations. They had a double complement of otoliths and responses to treatments were comparable to those of normal, healthy individuals. All fish were maintained with the assistance of the staff and facilities of the Tunison Laboratory. With the exception of fish being experimentally manipulated, most fish were held in a large wet laboratory supplied with a continuous flow of spring water and carefully controlled air temperature and lighting conditions. The experimental protocol was to initially set up 30 groups with about 100 eggs in a standard hatching jar. The 6.5 liter plexiglass jars, received water inflow from below a 2mm mesh stainless steel screen bottom at a rate of 2-2 liters/min. Water exited through a screened outlet near the top. The groups were either used in their entirety as experimentals and controls or they were split into smaller treatment groups. Subsequent addition of the later Jordan River fish also added a number of treatment groups. A total of 85 lots of fish were created, separately maintained, and monitored during the course of the research. Some were held in the jars for the entire five month period and served as controls and "stock" sources for new experiments. Others were subject to single or multiple exposures to temperature treatments and/or chemical markers.

Except when experiencing specific experimental treatments, all fish were maintained under uniform conditions. Water temperature in the spring water fed open system varied less than 0.1° C and was essentially constant at 9°C. It was unaffected by both interior and outside ambient air temperatures. Lighting for all treatments, both for the open system "holding" condition and experimentals was 14L:10D with lights on at 0600. Commencing with swim up, jar-held fish were fed 5 times a day with a progressively graded series of ASD-120 prepared diet (Zeigler Bros.). Fish held in temperature controlled closed systems (see below) were fed 2 or 3 times daily to reduce the accumulation of excess food and resultant deterioration of water quality. Dead or moribund eggs and fish were culled and tallied on a daily to semi-daily schedule. Jars, screened cylinders and holding tanks were cleaned at least every 3 to 4 days. Nylon screen cylinders (2mm mesh; 20cm high by 15cm diameter) were used to hold experimental groups being subjected to chemical immersion marking and temperature manipulations in closed-system tanks and aquaria.

Temperature manipulations were carried out by means of holding fish in the screened cylinders which were suspended in large (650l) holding tanks equipped with constant aeration, aggitation, and refrigerator and heater units controlled by timers and thermostats. For constant temperature conditions, controllers maintained temperatures within a 0.1°C range or a 1.8°C range, depending on the controller employed. Fluctuating temperature regimes, either on a diel cycle or some other specified pattern, were created by sequentially turning cooling or heating elements (150 to 1000 watts) on and off. A standard diel cycle is illustrated in Fig. 2.

Temperatures were continuously monitored with an electronic thermister and strip chart recorder (Y.S.I. and Hewlett-Packard) and an immersible battery powered temperature recorder (Ryan Instrument Company).

Additional otolith marking experiments were performed with fish being immersed in various chemical solutions. (See Table 1) In all cases the fish were held for periods of hours to days in 37.5 liter aerated aquaria that were either maintained at a constant 9° C or in some cases were experiencing a 12:12 heating/cooling cycle with the lower and upper temperatures being 10.5° and 16.5° C. Fish were generally held suspended in the screen cylinders, however these were not used for the larger fry; in these cases the fish were allowed to swim freely throughout the aquaria.

Eggs, alevins and fry were also injected with the chemicals used in the immersion experiments. Injection controls received doses of 13.2 ppt NaCl in distilled water. Injection volumes varied between 1 and 5ul, depending on fish size and the required dosage. Needles were 26 gauge and were used with a microliter syringe mounted in a repeating dispenser. This arrangement allowed for fast and consistent injections of the extremely small volumes required. For eggs and alevins, the injections were intravitelline. Fluid was often seen to leak out of eggs upon removal of the needle. Fry received intraperitoneal injections. Alevins and fry were handled and physically restrained by grasping them in a folded piece of nylon screen (2mm mesh); this also allowed for accurate localization of the injection through the mesh. Mortality attributable to handling and

TABLE 1
CHEMICAL TREATMENTS

COMPOUND OR SOLUTION	METHOD OF EXPOSURE	RANGE OF CONC. USED	USED IN COMBINATION WITH	PURPOSE
Oxytetracycline hydrochloride (both liquid and powdered, Terramycin)	Injection Immersion Diet	50-100 mg/kg 250-1000 ppm 0.2-1% of feed	NaCl soln. DMSO, sea salt food	otolith marker
Dimethyl sulfoxide (DMSO)	Immersion	0-10%	tetracycline, $TbCl_2$	to accelerate uptake of markers
Acetazolamide sodium	Injection	100mg/kg	NaCl soln.	otolith marker
Sodium chloride (in distilled water)	Injection	1.3%	all injections	saline medium for injections
Strontium chloride	Injection Immersion	100mg/kg 150-300ppm	NaCl soln. DMSO	otolith marker
Terbium chloride hydrate	Injection Immersion	100mg/kg 150 ppm	NaCl soln. DMSO	otolith marker
Europium chloride	Immersion	150ppm	—	otolith marker
Sea Salt (artificial)	Immersion	0.6‰	tetracycline	to accelerate uptake of markers

stress and the injection procedure was minimal (averaging less than 2%).

A third method of chemical exposure, dietary, was only attempted for tetracycline marking of fry. In this case a standard diet pellet was prepared with tetracycline added (0.2% or 1% by weight). Fish were fed on the regular schedule while confined in the open system jars.

Throughout the course of the research, representative fish samples were taken of both controls and experimentally manipulated fish. Groups of fish were generally maintained for at least two weeks after a marking procedure before the entire group was fixed for later otolith examination. Many groups, including some controls were kept alive for the entire duration of the project. All fish were fixed and preserved in 95% ethanol. Otoliths of embryos, alevins and fry were removed with fine forceps and needles with the aid of a dissecting microscope. Sagittae were always removed, while lapilli and asterisci were examined less regularly. (Unless stated otherwise, all future use of "otoliths" refers to sagittae). Air dried otoliths were placed on glass microscope slides and covered with a drop of immersion or mineral oil. The latter was found to be preferable due to its less acidic pH. Coverslips were only used for some of the smaller embryonic and alevin samples. Otoliths were examined with four techniques, each requiring somewhat different preparation and instrumentation. Preparation also varied with the size of the fish; for example, the otoliths of larger fry required grinding in order to clearly observe internal structures with the light microscope.

Otolith marks produced by temperature manipulations and acetazolamide exposures were observed with the aid of a compound light microscope equipped with a rotatable polarized light source, oil immersion objective (100x), and video imaging system. The latter was not an absolute requirement, however it does serve to further increase magnification and electronically enhance contrast and brightness. Internal structures in whole, unground otoliths of embryos, alevins and small fry (eg. less than about 30mm SL) could be easily viewed with this equipment. Features nearest the margin of the otolith are clearest with the otolith oriented with the internal (sulcul) face up. Structures nearer to the center are best observed with the otoliths flipped over, especially for the larger fry used in this study. For critical examination of the otoliths of fry, grinding was necessary. This was accomplished by first embedding the otolith in a clear epoxy compound (Hyasol) and then grinding the otolith to the mid-sagittal plane, using mechanical grinding wheels and a series of carboundum grit papers (240, 160) and then diamond polishing compounds (9um, 3um, 1um) on felt polishing cloths. The ground and polished otoliths were then microscopically examined after covering the surface with oil (immersion or mineral).

Fluorescent marks produced by incorporation of tetracycline in the otoliths were observed with an incident illumination system on a compound light microscope. The excitatory wavelengths employed were limited by a combination of BG-12, B (500nm) and U (400nm) filters. Autofluorescence was reduced by a 530 nm barrier filter. Preparation of these samples was as described above. Whole fish, both alive and ethanol fixed were also examined under U.V. illumination (with a hand

held light source) to test for the presence of fluorescent marks in other calcified structures (fin rays, teeth, head bones). Fluorescence in the yolk was also found to be a useful indicator of injection success in eggs and alevins.

Observation of internal microstructural features with the scanning electron microscope (SEM) requires that samples be embedded, ground, polished, acid etched and then coated with a thin (~100Å) layer of a conductive material such as a gold-palladium alloy. Grinding and polishing was described above. The etching solution, supplied by Dr. Joanne Ballarino (Cornell University) consisted of 5.5% EGTA, 2% SDS, 0.4M NaOH and 0.1M phosphate buffer. This solution appeared to give superior results to 0.1 or 0.01N HCl which was also used. These solutions were brushed on the prepared samples; etching times averaged 1 to 2 minutes after which the embedded otoliths were rinsed in distilled water, air dried and glued (colloidal silver paint) to SEM stubs, and then sputter-coated with gold-palladium. Two SEMS were used, an AMR-1000A and a JEOL VSM-T200.

Elemental analysis of otoliths was done on samples prepared as for SEM analysis, except they were vacuum evaporated coated with a thin carbon film instead of the gold-palladium. An energy dispersive spectrophotometer (EDS) x-ray detector and computerized analysis system (Tracor-Northern) on the AMR SEM was used for this work. When operated in the map mode, specific elements of interest can be localized and visualized over the SEM secondary electron image. "Marks" produced by the incorporation of strontium, terbium, and europium were searched for in this manner.

RESULTS AND DISCUSSION

Mortality in controls and most experimental groups was comparable and moderate up to and through the time of hatching (average, 24%). This rate was also comparable to that experienced by fish of the same brood and maintained under the standard "hatchery" conditions (Dale Hedrick, Tunison Lab, pers. comm.). Post-hatch mortality in jar-reared controls and most of the experimentals averaged approximately 5 to 10% to the end of the succeeding four month period. Injections, temperature manipulations and chemical immersions did not produce significant mortality increases with the following exceptions. On one occasion high sustained temperatures (18°C for two days) plus elevated nitrite levels (0.78 ppm) from uneaten food produced substantial mortality and sublethal effects (eg. loss of equilibrium) for most of the alevins and fry in one of the closed-system tanks. Some of these fish eventually recovered, however this was clearly a highly stressful event (as also indicated in the resulting otolith growth pattern). Subsequently, rations were reduced and the tank was cleaned every two days. Another clear cut-source of mortality was immersion experiments with DMSO concentrations exceeding 5%. Synergistic effects of the combination with tetracycline cannot be excluded for this response, however, the tetracycline alone, in comparable or higher concentrations was found to produce no significant mortality. Approximately 25% fry mortality occurred after two hours at a concentration of 10% DMSO (plus 500ppm tetracycline at 9° C). Twenty four hour exposure to 7.5% DMSO (plus 500ppm tetracycline) resulted in total mortality or morbidity. The only other noticeable effect of the experimental manipulations was the

expected retarded growth and developmental rate exhibited by fish in certain closed-system temperature treatment experiments. Some of manipulations involved short or extended exposures to relatively constant "cold" water of 2° to 6° C, while others had the fish experiencing diel fluctuations with the mean daily temperature below the 9° C of the controls. Under all such circumstances fish exhibited development and growth rate responses that were related to cumulative temperature exposures or "degree days" (Balon, 1980). This response was not quantified in the present study. Other factors would also complicate the response, including differences in water quality and movement, and the rate of feeding in the closed-system tanks versus the open-system control jars.

The otoliths (sagittae and lapilli) appeared in the otic vesicles of embryos just prior to the "eyed" stage, ie. when the eyes become pigmented and visible without the aid of a microscope. This corresponds to the end of Balon's (1980) $E^3 6$ stage, and is attained about 20-30 days post fertilization if lake trout are reared at about 9° C. The first calcified otolith elements are a series of separate, optically dense primordia and their surrounding translucent "cores" (each 5-20um in diameter, Fig. 1). The presence of calcification can be determined by their birefringience when viewed with double (crossed) polarization illumination. The separate elements quickly consolidate into singular sagittae and lapilli once incremental growth is initiated (Figs. 1 and 3). It was at this stage that the earliest marking experiments were started. In controls, growth increments formed prior to hatching were comparatively weakly expressed and somewhat irregular or uneven in width and/or contrast. At or close to

the time of hatching, many fish developed one to three more prominent growth increments; characterized by optically dense and discrete protein-rich subunits (Fig. 4).

As defined in this study, a growth increment (microstructural) is a bipartite structure composed of two subunits or zones. One subunit appears as more optically dense or opaque in transmitted light. The other can be termed more translucent in comparison (Fig. 5). With preparation for the SEM (etching), the opaque microstructural zones are more deeply etched and appear as "valleys", with the intervening "ridges" corresponding to the translucent microstructural zones. It is generally agreed that the opaque zones are relatively enriched in proteinaceous matrix, where as calcium carbonate (aragonite) dominates the checmical composition of the translucent zones. In other words, these zones may be characterized as matrix-dominant versus calcium-dominant. Alex Wild (I.A.T.T.C., pers. comm.) has suggested using the terms M-zone and C-zone, respectively, and I will adopt this nomenclature here. It should be noted that these terms are synonomous in usage (but <u>not</u> structural implication) to "discontinuous zone" and "incremental zone" as defined by Mugiya et al. (1981). Finally, observations during this research confirmed earlier reports (eg. Brothers, 1981; Campana & Neilson, 1985) that the M-zone is formed at night and/or during periods of depressed (declining) water temperatures and the C-zone is deposited during daylight hours or when water temperatures are elevated (rising). Strong temperature cues will generally mask the effects of photoperiod.

Temperature Experiments

Eggs (embryos), alevins and fry were subjected to a wide variety of temperature regimes, including continuous exposure to constant 9 C, constant 6°C, diel temperature cycles, and intermittent exposure to rising, falling "low" (1°C), and "high" (17°C) temperatures. Otolith microstructure and daily increment deposition (validated by comparing known periods of exposure to a particular condition with counts of characteristic growth increments) varied in three primary ways as a function of the thermal environment. First, there appeared to be an inverse relation between gross optical density (ie. at low magnification) and temperature, particularly for the constant temperature exposures. In other words, those portions of the otolith produced under lower temperatures (constant or average) were more optically dense when viewed with transmitted light. Presumably this is a function of a relative increase in the proportion of otolith matrix (otolin) in these areas. This response appeared to be caused by several, non-mutually exclusive processes: at lower temperatures there is:

(a) a decrease in the width of the C-zones of increments, resulting in an overall decrease in the cumulative area of this more translucent material. The effect is analogous to the appearance of a series of more closely spaced black lines or concentric rings on a white background, as compared to a series of equivalent lines which are more widely spaced.

(b) an increase in the proportion of a matrix in the C-zones. (the white background becomes grey)

(c) an increase in the width of the M-zones (the black lines get wider)

For fish held at constant temperatures, otolith increments were more difficult to distinguish and less contrasty, compared to those in fish exposed to diel fluctuations (Fig. 6). Contrast refers to the relative opacity and translucency of the M-zones anc C-zones.

A second class of temperature effects results from exposure to long-term (more than 4 days) gradually rising or falling temperatures and results in deposition of more translucent or opaque material respectively. Daily growth increments were either absent or very faint under these conditions. Such treatments, although generally producing characteristic wide zones or "marks" were not nearly as effective in creating obvious and unmistakable marks as shorter-term temperature cycles, ie. those changing over periods of 6 hours to two days.

The third class of temperature effects is seen as alterations of the width of growth increments. Many groups of fish were exposed to diel temperature cycles, as described and illustrated earlier (Fig.1). For a typical treatment, with a range of 10° C and a heat/cool cycle of 12:12 or 10:14 hours, daily growth increments exhibited very high contrast between the M-zone and C-zone (Fig. 6). There was an exact correspondence between the number of M-zones and the number of daily temperature drops or "thermal nights" experienced by a fish. This was true whether the fish were on a typical diel cycle, synchronized with the light cycle, or one which was out of phase by 6, 12 or 18 hours. Similarly, if fish experienced thermal nights every 36, 48, 60, 72 or

96 hours, they produced prominent M-zones only at these times. These observations suggest several methods to distinctively mark the otoliths of lake trout:

(a) By shifting fish from a constant thermal environment (eg. 9° C) to a diel temperature cycle for a known number of days and then back into constant 9°C. The result is a known number of high contrast daily growth increments (Fig. 7).

(b) By shifting fish back and forth between the constant temperature environment and the cycling diel regime. Each shift should last for at least 3 days. The result is a series of high constrast increments (whose number depends on the length of each exposure to the cycle regime) alternating with bands of very weak constrast increments (again the width of these bands is dependent upon the length of exposure to the constant temperature). Such alternate treatments can be varied to produce a wide variety of distinctively marked otoliths (Fig. 8).

(c) The most complicated and variable marks can be produced by controlling the relative lengths of the heating and cooling portions of the temperature cycle. A longer heating period may result in a higher ultimate temperature as well as a longer exposure to rising or elevated temperatures. These variable length heating periods may be interspersed between a standard 12 hour cooling period. A simple experiment was performed in which the diel cycle was modified to produce the temperature pattern illustrated in Fig 9. The resultant otolith response gives doubly wide C-zones during the two 24 hour heating periods. This type of response, where the width of the C-zone varies with the duration of the warming period was developed to

produce a digitally coded marking system. The "dots," "dashes," and "spaces" of the International Morse Code were produced as follows:

A heating/cooling cycle of 6:12 yielded a narrow C-zone or "dot"

A 12:12 cycle produced an intermediate width C-zone or "space"

A 24:12 cycle produced a wide C-zone or "dash"

Fig. 10 shows some examples of coded otolith marks, including "LT" for lake trout and "GLFC".

Temperature marking proved to be a simple yet highly reliable and effective method to produce unique marks in the otoliths of lake trout at all stages of development from embryos to fry. Viewing the marks only requires mid-sagittal sectioning or grinding and examination with the light microscope. Responses in the lapilli were found to be similar to those seen in the sagittae, so there are four copies of the mark in each fish, two in the sagittae and two in the lapilli (Fig. 11). Temperature related alteration of the developmental rate can be avoided by controlling the diel range so that the mean temperature approximates any desired value.

Acetazolamide

Embryos, alevins and fry were injected with acetazolamide (100mg/kg), a carbonic anhydrase inhibitor. As a result of this treatment, embryos appeared to suffer elevated mortality rates at about the time of hatching. The otolith mark produced in alevins and fry was characterized by two or three markedly narrower and lower contrast daily increments (for fish in diel temperature treatments), or simply a broad translucent zone (fish in constant temperature). Increments formed subsequent to this mark continued to show gradually wider and

higher contrast increments for a week or more, when recovery was nearly complete (Fig 12). Immersion experiments were not attempted since the marking response to injection was not consistent and was difficult to distinguish, especially for fish held in constant temperatures. The mark was also not unique in that very similar microstructural patterns could be produced by temperature manipulations or perhaps natural perturbations.

Tetracycline

Twenty-five treatment groups received some sort of exposure to tetracycline, either by injection, immersion, or as a dietary additive. Tetracycline injections (50 and 100mg/kg) in alevins and fry always produced fluorescent marks in the otoliths. The marks corresponded to one to three days of growth and were relatively faint and sometimes difficult to distinguish. Whole fish, either alive or ethanol fixed, also showed externally visible fluorescence when viewed with U.V. illumination. The most strongly fluorescing areas were fin rays (especially the caudal and dorsal fin), various head bones (especially dorsally, the operculum-hyomandibular joint, and the mandibulars), and teeth. For alevins, the yolk was also strongly fluorescent, however, this was also the site of injection. Fluorescence persisted in the yolk until it was completely absorbed. Externally visible fluorescence gradually diminished and disappeared as the fish grew. Its presence after treatment was a good indicator of the success of otolith marking.

Many immersion experiments on eggs, alevins and fry failed to produce discernible fluorescent marks in the otoliths. This was true

whether or not DMSO was also used. Immersion in a concentration of 500 to 1000ppm tetracycline for two to three days (with or without DMSO) did produce marked otoliths (Fig 13) in alevins and fry, however they were invariably fainter marks than observed in injected fish. Embryos did not pick up the tetracycline, although the egg membrane was observed to fluoresce. The effect of elevated salt concentration (6% sea salt) was not clear-cut, but appeared to slightly enhance uptake in alevins and fry.

Fish exposed to tetracycline via dietary addition did not show either distinct external fluorescence or marked otoliths.

In general, tetracycline exposure appears to be an inferior marking technique when compared with temperature marking. Otolith preparation for viewing is similar to that needed for temperature marks, however incident light, fluorescence microscopy is also required. The marks themselves can be faint and difficult to distinguish and the number of unique marking patterns possible is much more limited. The only practical marking procedure, immersion, is limited to alevins and fry and must be carried out in a closed system for a couple of days. Treatment-related mortality may be significant under some conditions. Finally, the long term permanency of tetracycline marks is still somewhat uncertain, although the best evidence suggests that marks will remain detectable for at least five years.

Rare Elements

Eggs, alevins and fry were exposed to three "rare" elements, terbium, europium and strontium, via injection and immersion (see Table 1).

Injection treatments were administered as "controls" to produce an otolith mark from a known dosage to be compared with the results of immersion treatments. Otoliths were prepared as described earlier (see Materials and Methods) and were examined with an SEM in three ways; secondary electron imagery (SEI) backscattered electron imagery (BEI) and energy dispersive x-ray spectroscopy (EDS).

Dissolution of terbium chloride hydrate and europium chloride salts in the hatchery water produced a "milky" precipitate. The composition of the precipitate was not determined. Its formation was apparently due to a reaction with some component of the spring water since a precipitate failed to appear when the salts were added to de-ionized water. Further tests eliminated calcium, sulfate and fluorine as possible reactive substances. Due to this precipitate formation, it is probable that most of the terbium and europium was taken out of solution and very little was available for take-up by the fish. No mortality resulted from immersion exposures of two days. Otolith examination did not reveal detectable amounts of terbium or europium in either immersion or injection treatments. More sensitive methods of detection are required, such as wavelength dispersive x-ray spectroscopy (WDS), or ion microscopy. Other analytical procedures would require destruction of the otolith and/or could not visualize a "mark".

Strontium was identified as a strong candidate for an otolith marker due to its known high uptake and deposition in calcified structures (eg. bone and otoliths) in salmonids (Podoliak and McCormick, 1966; Podoliak, 1967; Yamada et al. 1979) and other fish and invertebrates (Ophel and Judd, 1969; Hurley et al., 1984). Both injection and immersion treatments produced strontium-rich bands in the otoliths. EDS spectral analysis displayed three distinct energy peaks characteristic for strontium (1.80, 14.14 and 15.83 KeV). Exposure to 150ppm $SrCl_2$ for up to four days did not produce a clearcut mark (with EDS sampling times of 4 minutes), however a concentration of 300ppm resulted in the appearance of a discrete band when the EDS system was used in a mapping mode (Fig. 14). Maps were made by counting x-ray pulses in a narrow region of interest around 14.14 KeV, the energy of the K_α emission for strontium. A two day exposure resulted in a stronger and wider strontium band. SEI images gave a very slight indication of the presence of strontium, due to a combination of somewhat different etching properties plus a higher yield of secondary elections. BEI images gave the most detailed view of strontium rich-increments (Fig. 15). Backscattered electrons come from a shallower depth and narrower area than the x-rays, resulting in a much higher resolution picture. The higher atomic mass of strontium is indicated by a higher yield of backscattered electrons. In Fig. 16, the time course of the immersion exposure, uptake and incorporation can be clearly seen. The first band was a midday to midday 24 hour immersion. The bright areas correspond to portions of C-zones and the dark line is an M-zone. The difference in widths of the two C-zone portions may be interpreted to demonstrate a slight lag in uptake and then a residual deposition of strontium after the fish

were transferred out of the strontium enriched water. A similar response is indicated in the 52 hour strontium exposure represented in the second band (midday to mid afternoon).

A chemical marking system could employ variable duration exposures and intervening periods to produce a series of unique marks in otoliths. The concentrations of strontium chloride used here did not cause any mortality or other detectable detrimental effects. Limited observations on embryos did not show significant take up, however more work is needed to confirm this. The technique could be used to mass mark alevins and fry, but the fish must be maintained in a closed system during treatment. The marks produced should be permanent (ie. for the life of the fish), however preparation and "viewing" procedures are time consuming and require highly specialized and expensive instrumentation.

Conclusions

Although results of the study demonstrate the feasibility of several possible marking procedures to be used for the early life stages of lake trout, application of the criteria presented earlier (page 1) show that only one, temperature marking, can be practically applied to a large-scale stocking program. Specific conclusions include the following:

1. Temperature manipulations can be used to produce a very large variety of unique and easily visible marks in the otoliths of embryos, alevins and fry. This technique is easily applied to mass-marking in either open or closed rearing systems. Procedures for preparation and viewing of the mark are the easiest and simplest of all the marking techniques examined.

2. Tetracycline marking of alevins and fry is possible, however it requires maintenance of fish in a closed system. Increased mortality may result from the treatment conditions. The marks are weakly expressed; are of limited variety and require more specialized viewing conditions. Long term persistence of the marks is not certain.

3. Acetazolamide marking by injection produced a mark qualitatively similar to those which can be induced by temperature manipulations, but it does not offer the remarkable control of the latter method. Application by immersion was not attempted.

4. Immersion treatment of alevins and fry with strontium (as $SrCl_2$) will produce a discrete "mark" after one or two days of exposure in a closed system. The "mark" is a band or bands of strontium enriched material which is analyzed by x-ray spectroscopy or BEI and SEM. Preparation and viewing is relatively difficult, expensive and requires specialized equipment.

Recommendations

Given the requirements of the marking program being planned by the GLFC, temperature manipulations are clearly the method of choice to mark otoliths. Control of water temperature may be accomplished in closed systems by employing carefully monitored heating and cooling units. For the large volume of eggs and fish involved, an open system may be preferable. Such a system may switch or mix alternate warm and cool water sources. If a flow-through system is used, every attempt should be made to keep the water well mixed and uniform as it contacts the eggs or fish. A natural light cycle may be maintained during all

treatments, regardless of alterations of "thermal day length." Feeding rates may have to be adjusted to compensate for temperature-related changes in metabolic rate and activity. Water temperatures during maintenance periods before and after marking should be as constant as possible over the short-term, although a gradual change over several weeks will have no significant impact on the marking process. During temperature treatments, the difference between the warm and cool water extremes (ΔT) should be at least 5°C and preferably closer to 10°C to optimize the contrast between the C- and M-zones. Manipulations may be of three basic types or combinations to produce characteristic marks capable of distinguishing many groups of fish.

(1) exposure to a diel temperature cycle for a number of days; a minimum of four or five is best

(2) intermittent exposure to a diel temperature cycle and constant temperature. Each period should be at least three days for unambiguous results

(3) variation of the duration of warming and cooling periods away from the 12:12 diel cycle to produce variable width C-zones. Alternatives of six or 24 hours of heating gives good results.

Treatments may start as early as the eyed egg stage and may be performed at any time thereafter. It is best to avoid marking during the hatching period since naturally produced "hatching" marks could be a source of confusion. The success of marking procedures should be monitored a few days after a treatment by examining the otoliths of several fish. These samples will also serve as reference material for

future comparison with recaptured individuals. Temperature marking can provide an infinitely variable and exact method to mark large numbers of young lake trout.

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FIGURES

The following abbreviations are used in figure legends for micrographs:

P = photomicrograph
PV = photomicrograph from a video monitor
SEM = scanning electron microscope
SEI = secondary electron image
BEI = back scattered electron image
E-MAP= elemental map mode, x-ray analysis

The legend on some SEM micrographs is read as follows: scale bar length in um; accelerating voltage in KV; working distance in mm; a four digit reference number.

Magnificatins on other micrographs are as indicated by the scale bars.

All otoliths are sagittae unless otherwise indicated.

Figure 1 A. _In situ_ view of separate otolith cores in the otic capsule of an "eyed-egg."; (PV)
B. Cores connected in a later embryo; (PV)
C. After a 3 day exposure to a diel temperature cycle; (PV)

Figure 2 Typical diel temperature cycle

Figure 3 Otoliths of alevins showing daily growth increments formed in a diel temperature cycle
A. Nineteen days after temperature manipulation; fish was about 4 days past post-hatch; (PV)
B. Central region of the otolith of a wild rainbow trout, showing embryonic (E), alevin (A) and juvenile growth (J); (SEM, SEI)

Figure 4 Hatching marks and growth increments in control (constant temperature) treatment fish.
A. The marked increments indicate hatching (1) and a two day terbium treatment (2) in a diel cycling environment; (SEM, SEI)
B. Otolith from control fish, alevin; (PV)

Figure 5 Growth increments; C-zone and M-zone
A. Diel temperature cycle; (PV)
B. Diel temperature cycle; (SEM, SEI)
C. Diel temperature cycle; (SEM, SEI)

Figure 6 Comparison of daily growth increments under diel cycling temperature (d) and constant temperature (c); alevin; also compare with 4B; (PV)

Figure 7 Constant temperature marks (c) bounding an 8 day period of diel temperature cycles (d); (PV)

Figure 8 Temperature marks produced by alternating bouts of :
- A. Diel cycle and constant temperature; (SEM, SEI)
- B. As above; (PV)

Figure 9 Temperature marks produced by varying the width of C-zones
- A. temperature pattern
- B. otolith response;(PV)

Figure 10 Temperature marks produced by varying the width of C-zones; coded in Morse Code - see text for explanation
- A. "LT", •—••/— ; (PV)
- B. "GLFC", ——•/•—••/••—•/—•—• ; (PV)
- C. As above, (SEM, SEI)
- D. As above but maintained in diel cycle after the code mark;(SEM, BEI)
- E. As above but returned to constant 9 C after the code mark;(SEM, BEI)

Figure 11 Lapillus showing growth increments and temperature marking response; "LT" mark; compare with 10A; (PV)

Figure 12 Marks produced by acetazolamide exposure (100 mg/kg)
- A. sagitta;(PV)
- B. sagitta; "abnormal" translucent growth;(PV)
- C. sagitta;(SEM, SEI)
- D. asteriscus;(SEM, SEI)
- E. asteriscus;(SEM, SEI)

Figure 13 Fluorescent tetracycline mark in sagittae
- A. Injected fish (100 mg/kg); sagitta; (P)
- B. Immersion; 500 ppm tetracycline in 0.6% seawater; lapillus; (P)

Figure 14 Chemical marking with strontium; immersion experiments
- A. SEM, SEI
- B. E-MAP

Figure 15 Strontium marking; double exposure, 24 hours and 52 hours
- A. SEM, SEI
- B. SEM, BEI
- C. E-MAP

Figure 16 Double strontium exposure; 24 hours and 52 hours; see text for explanation;(SEM, BEI)

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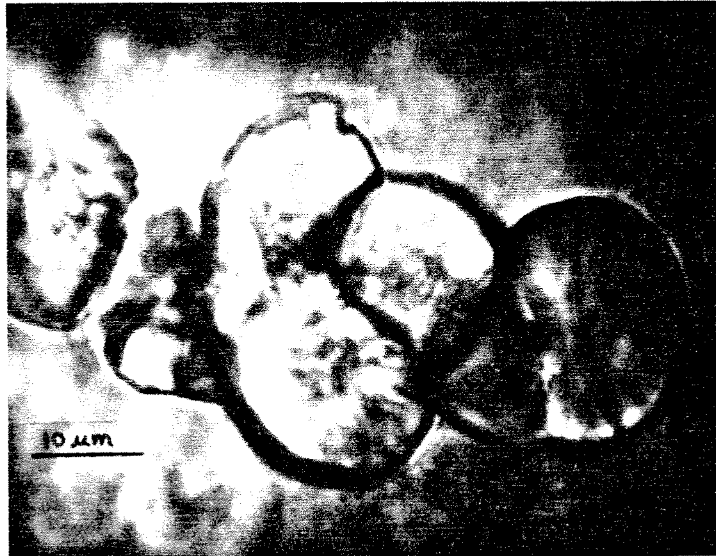
Larry Wubbels of the Jordan River National Fish Hatchery aided by supplying additional lake trout eggs. I thank Elizabeth Lawson and M. Parthasarathy for their assistance in the Cornell Biological Sciences EM facility. John Wanagel and Steve Ruoff also generously donated SEM time.

Finally, I'd like to thank Deborah Schoch for her assistance in typing the manuscript.

A.

B.



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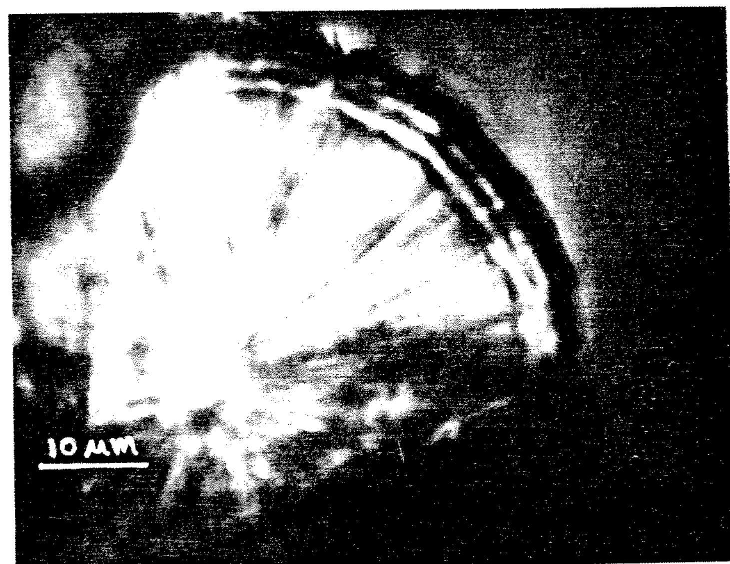

FIGURE 1

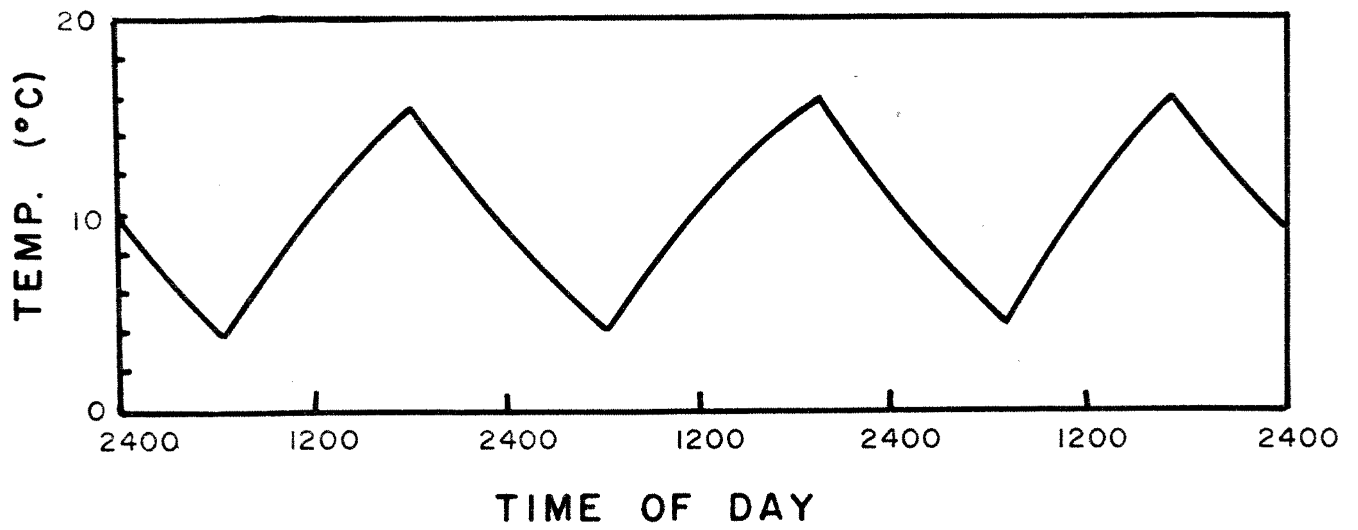

FIGURE 2

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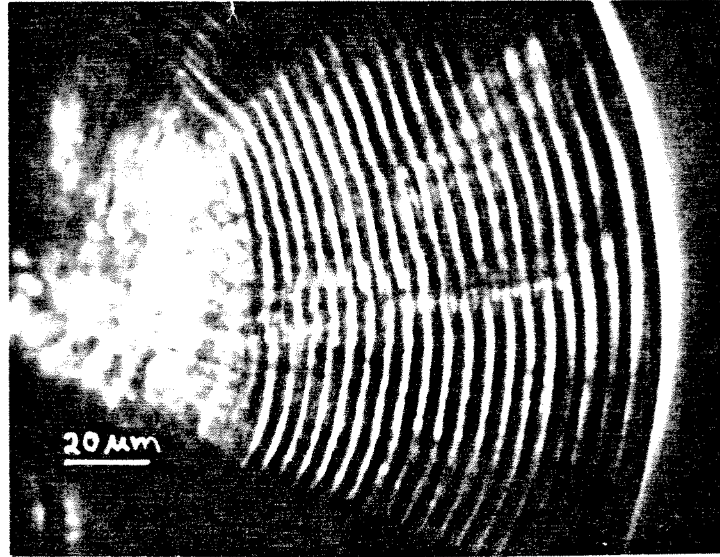

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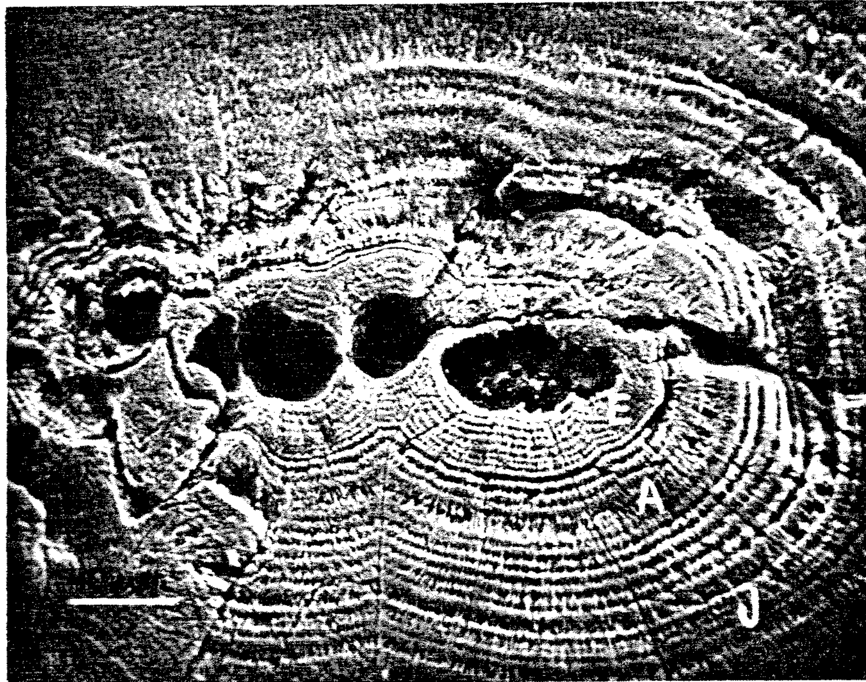

FIGURE 3

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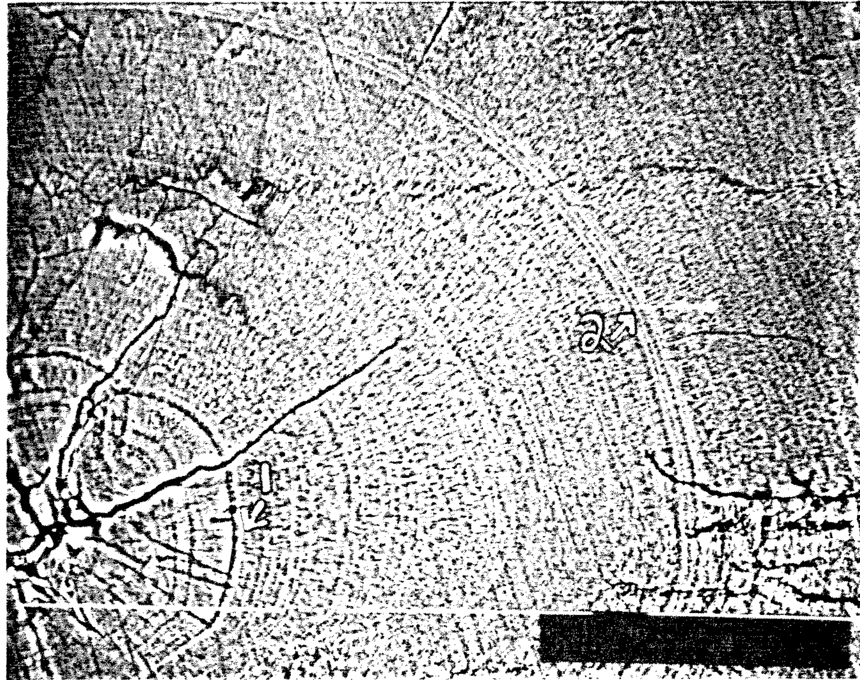

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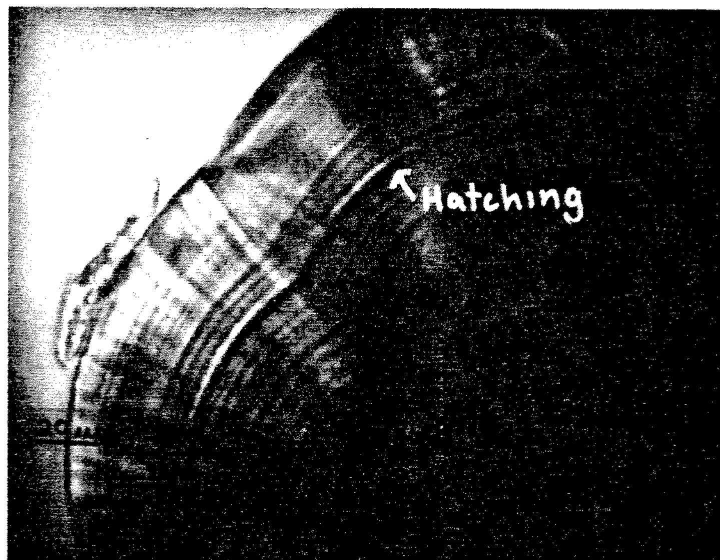

FIGURE 4

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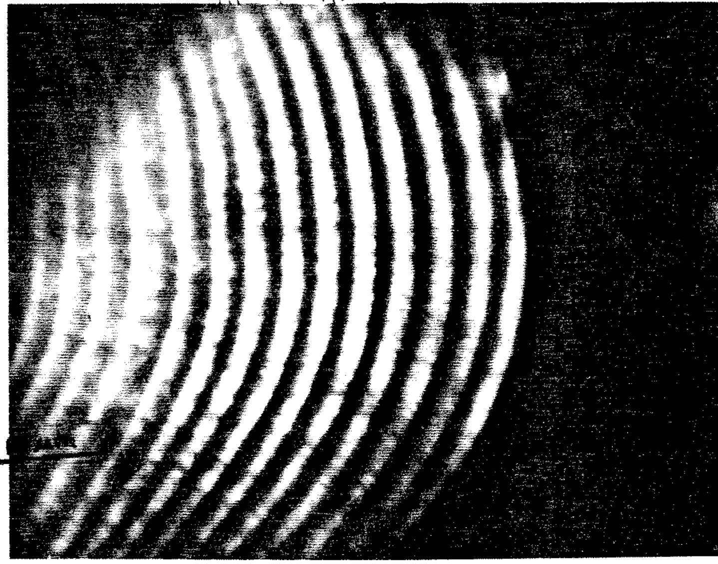

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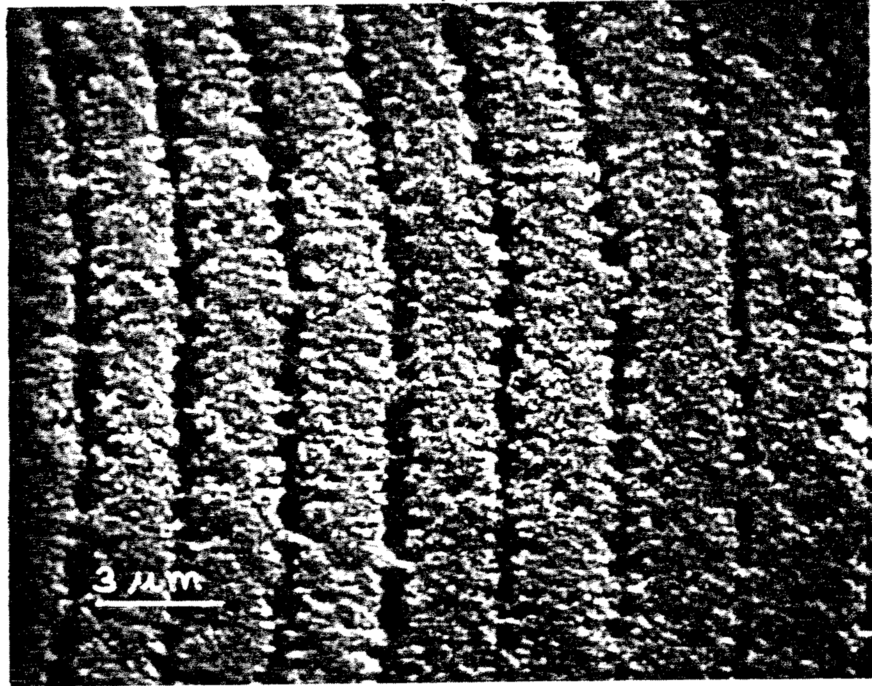


FIGURE !

c.

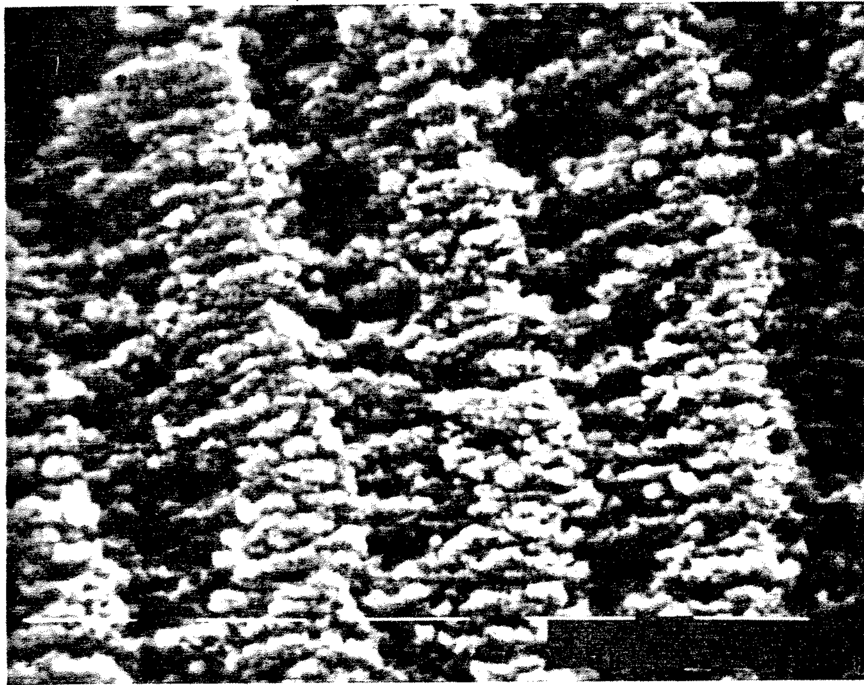


FIGURE 5

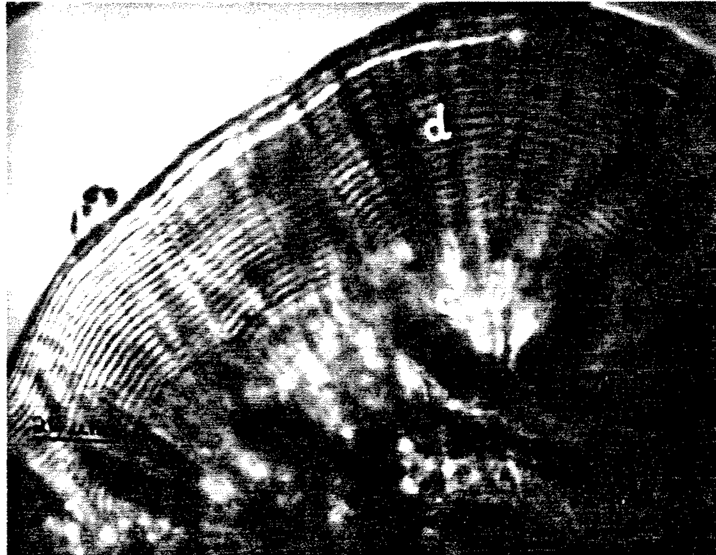


FIGURE 6

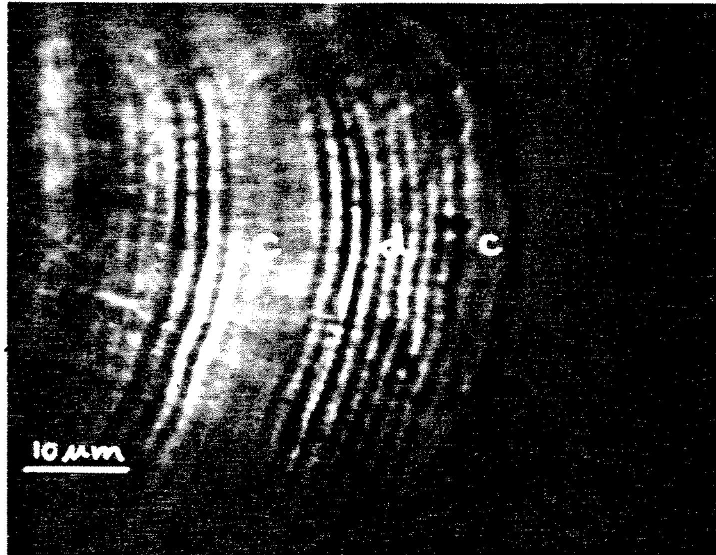
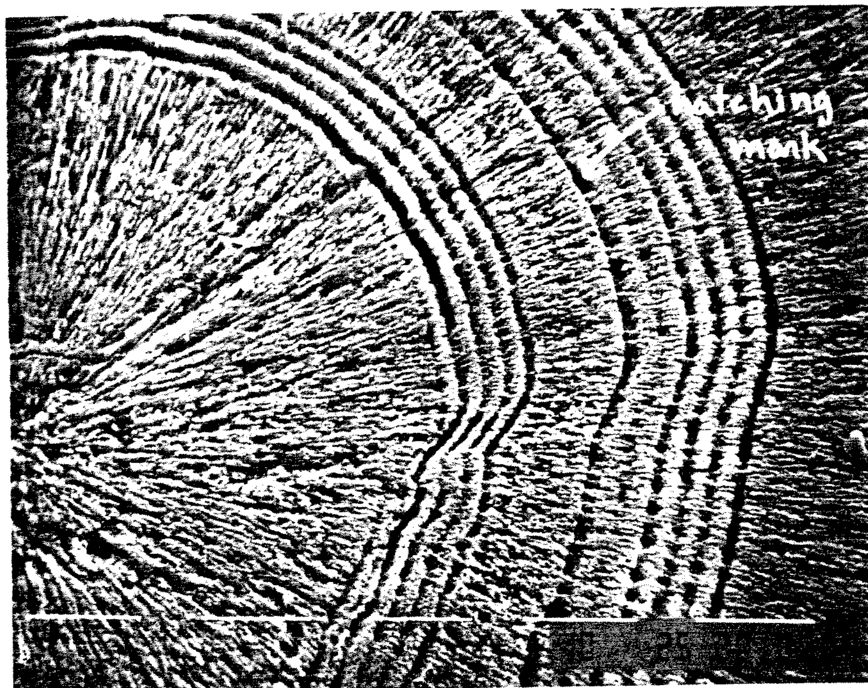


FIGURE 7

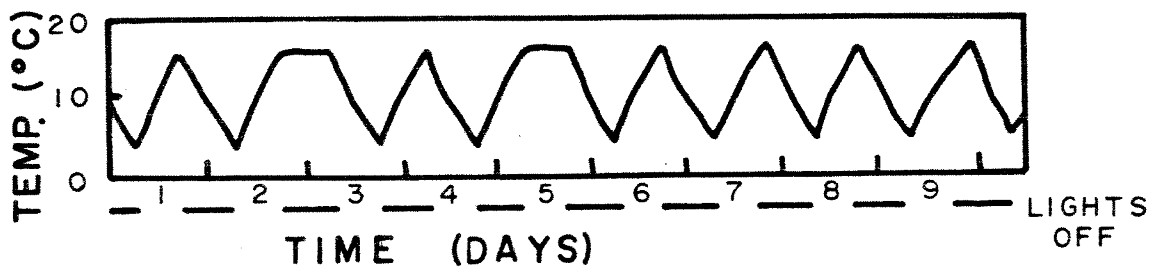
A.



B.

FIGURE 8

A.



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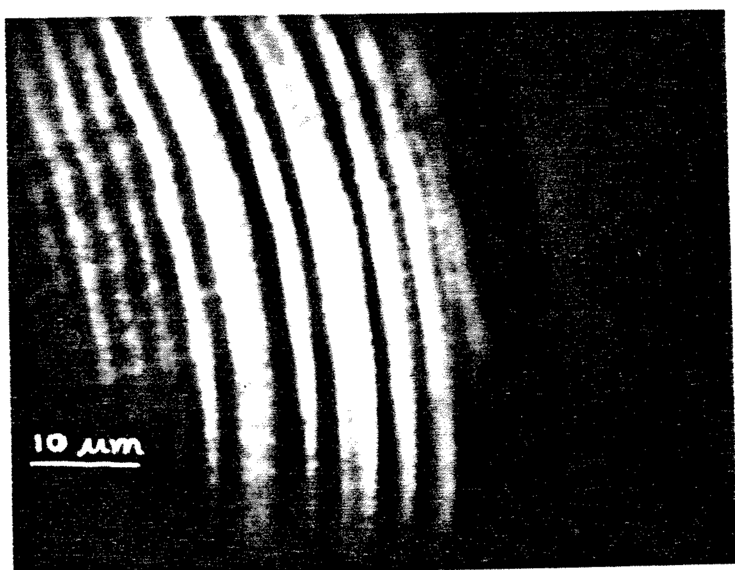

FIGURE !

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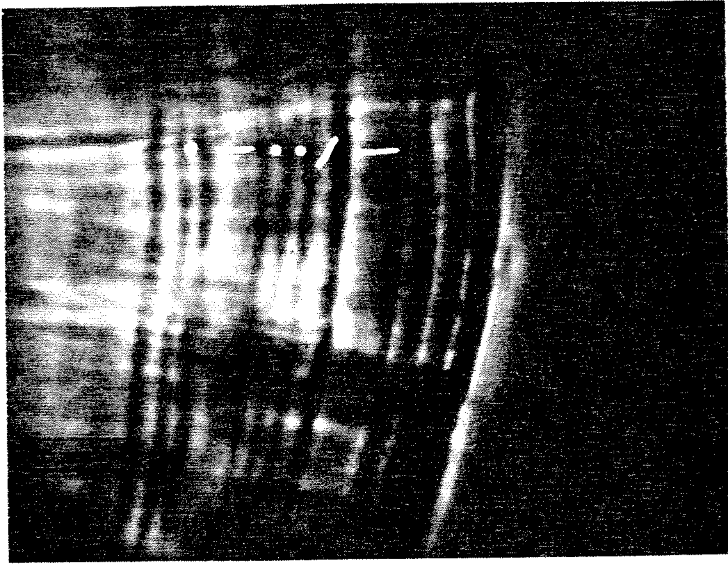

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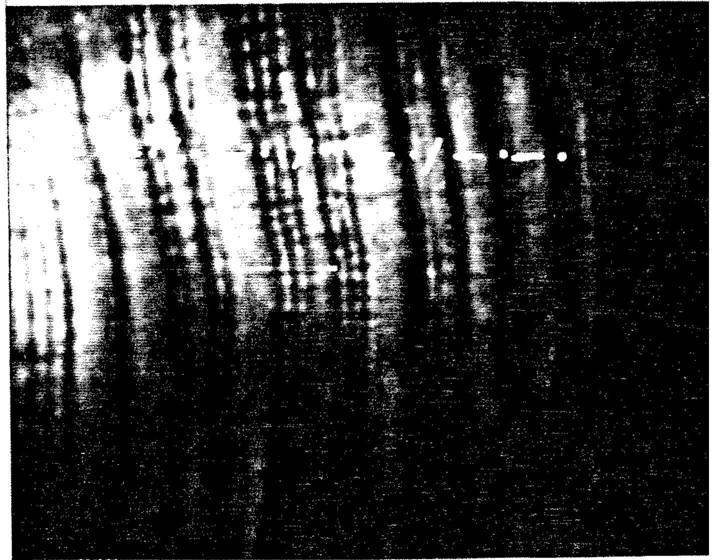

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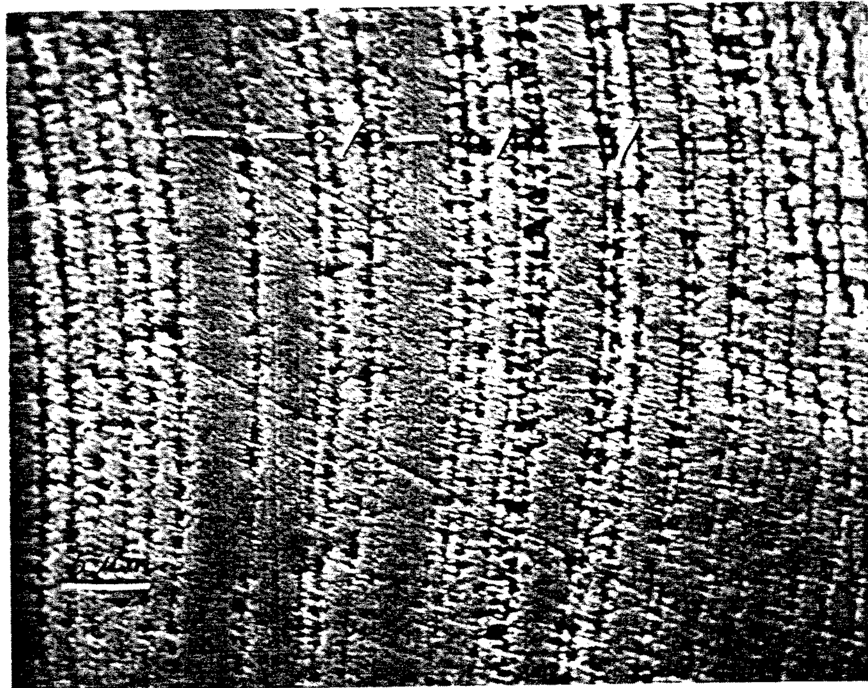
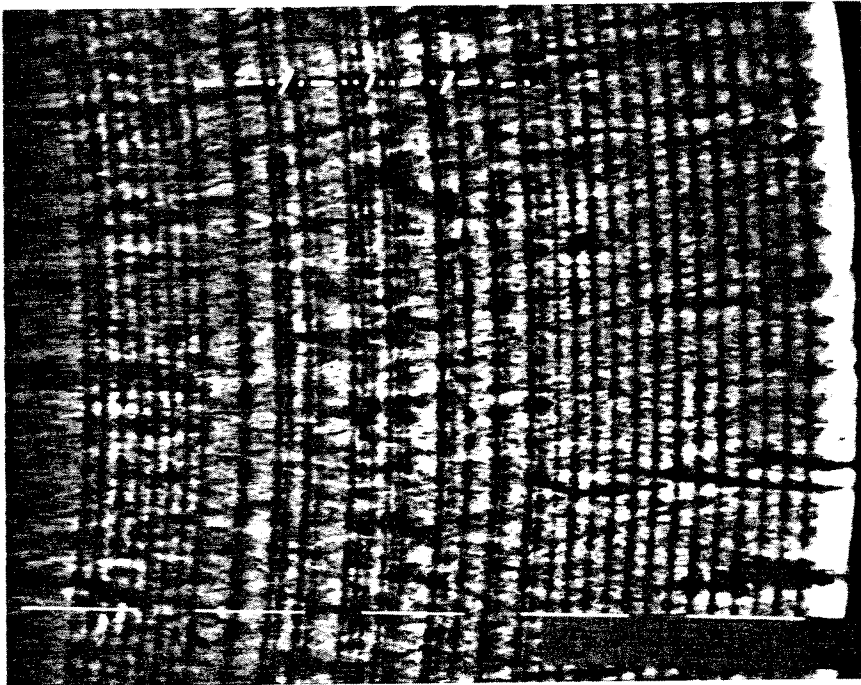


FIGURE

D.



E.

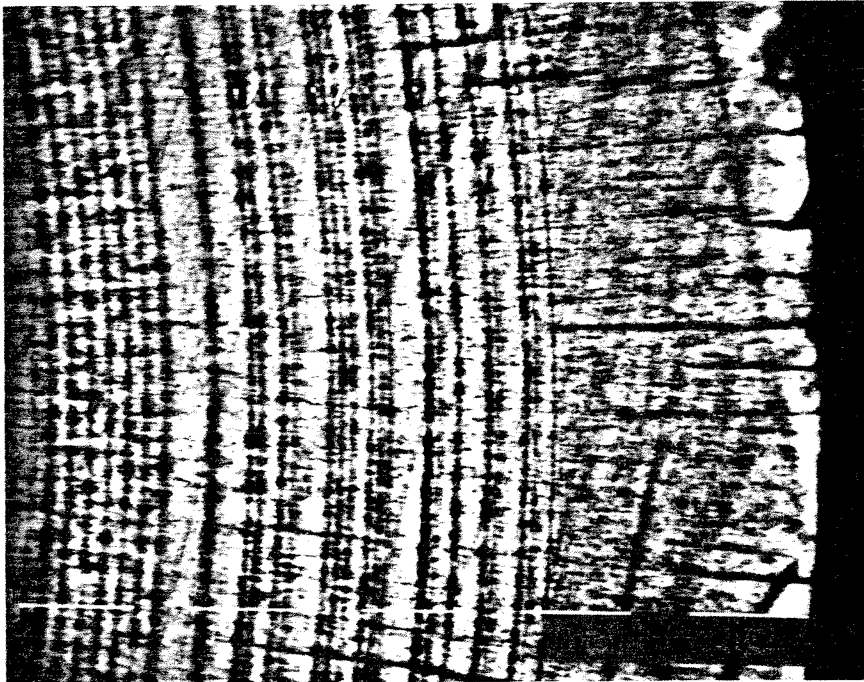


FIGURE 10

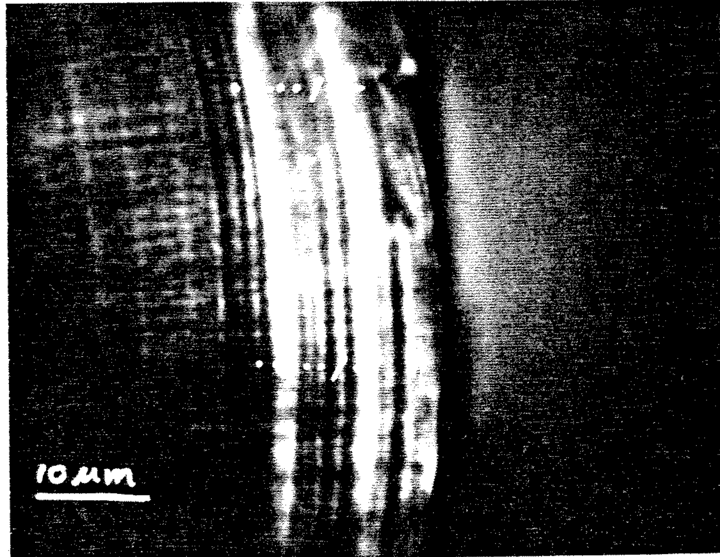
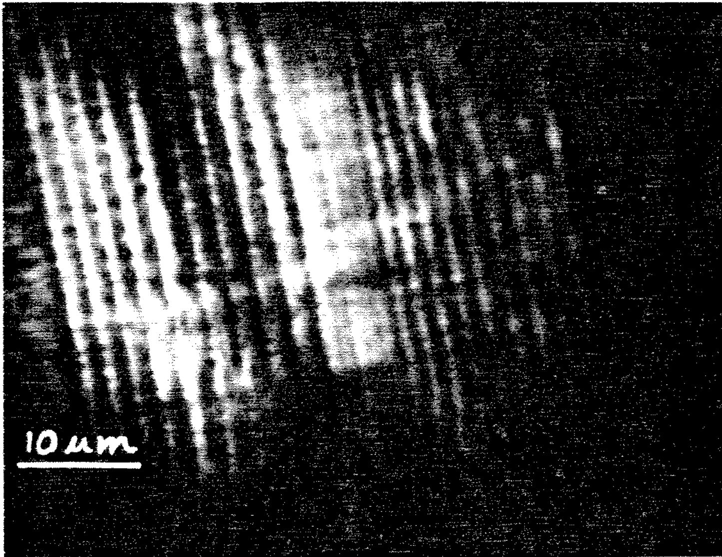


FIGURE 11

A.



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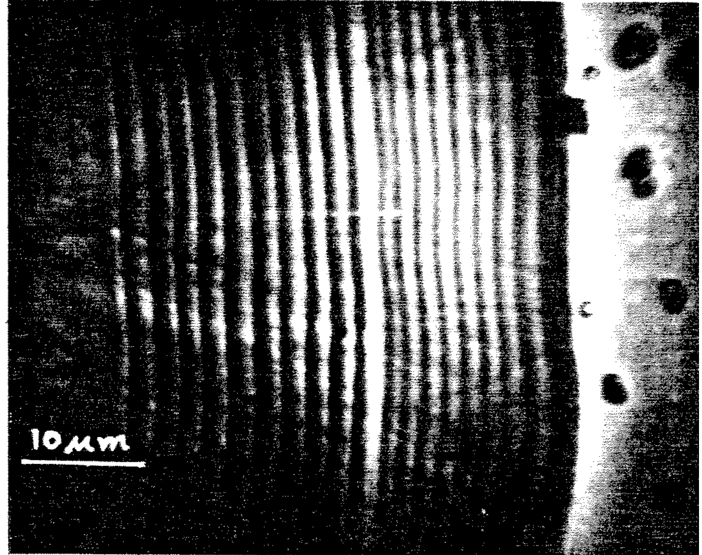


C.

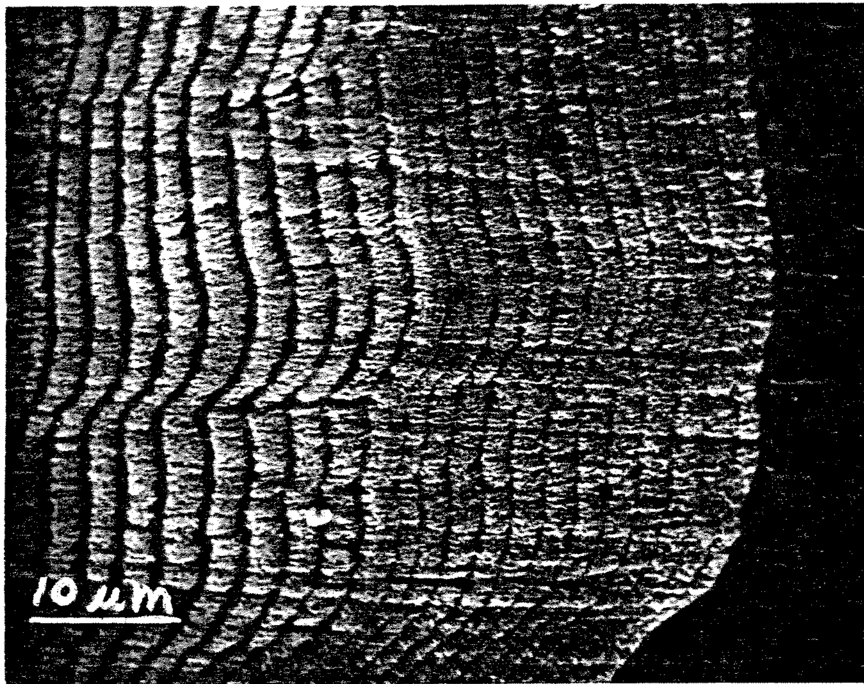
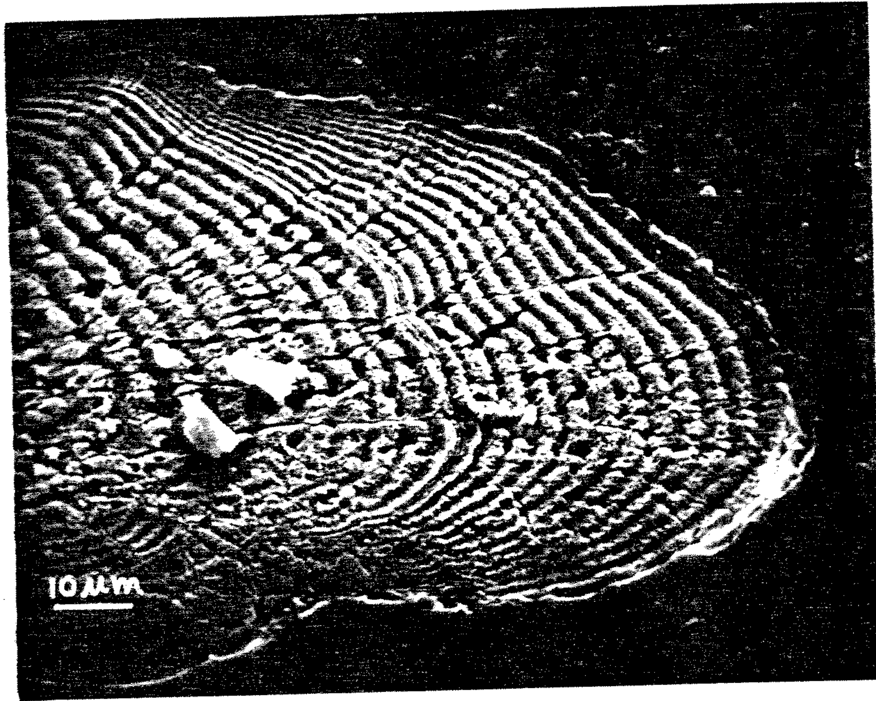


FIGURE 1

D.



E.

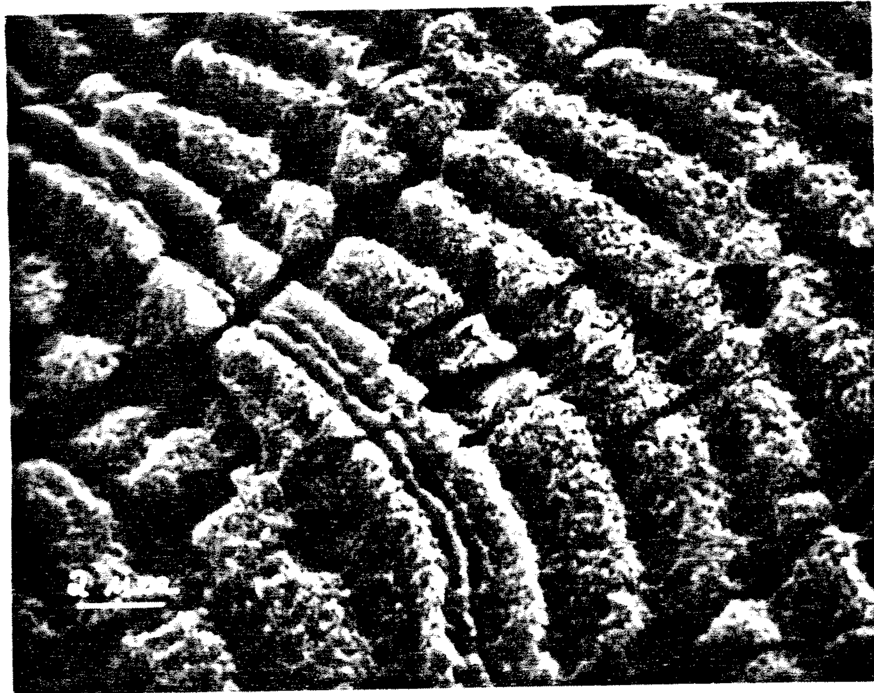


FIGURE 12

COMPLETION REPORT

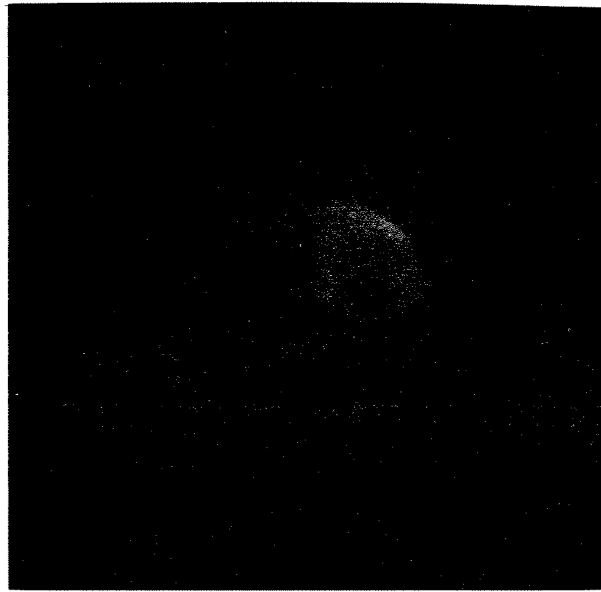
TITLE: Development of otolith marking techniques for the early life history stages of lake trout (*Salvelinus namaycush*).

SUBMITTED TO: The Great Lakes Fishery Commission

BY: Edward B. Brothers, Ph.D.
3 Sunset West
Ithaca, New York 14850
(607/347-4203)

DATE: September 20, 1985

A.



B.

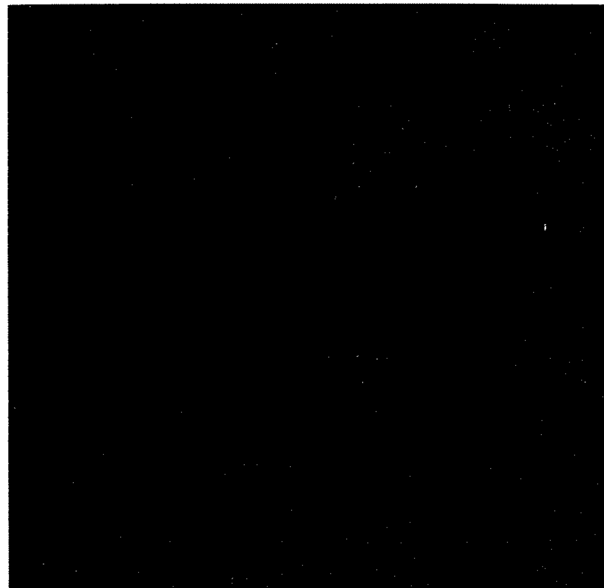
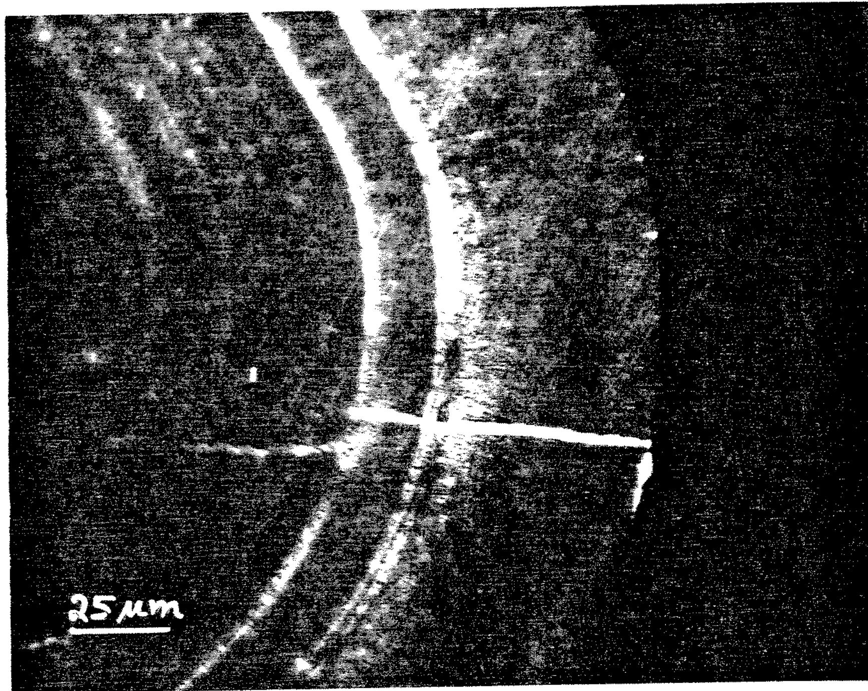


FIGURE 13

A.



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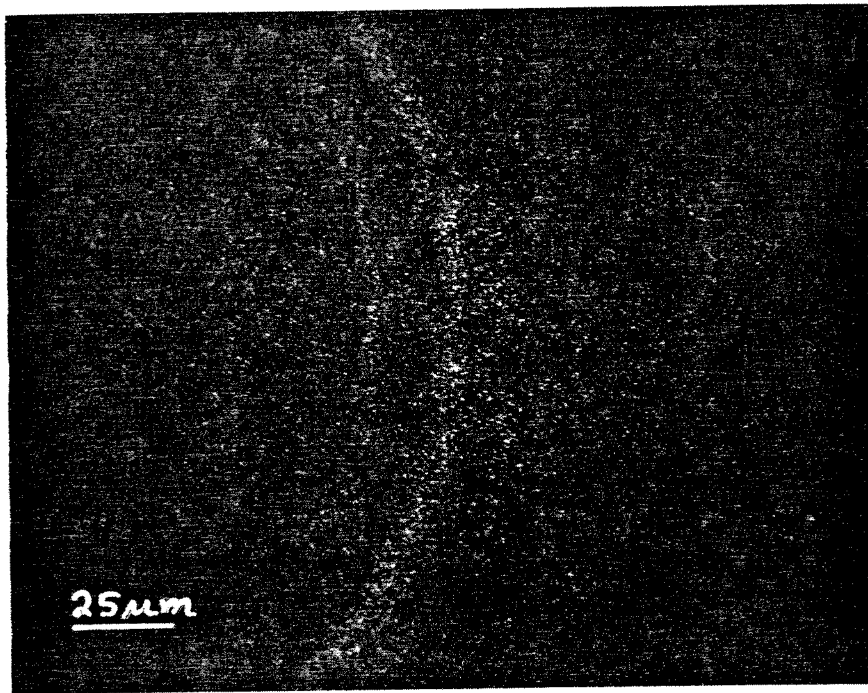
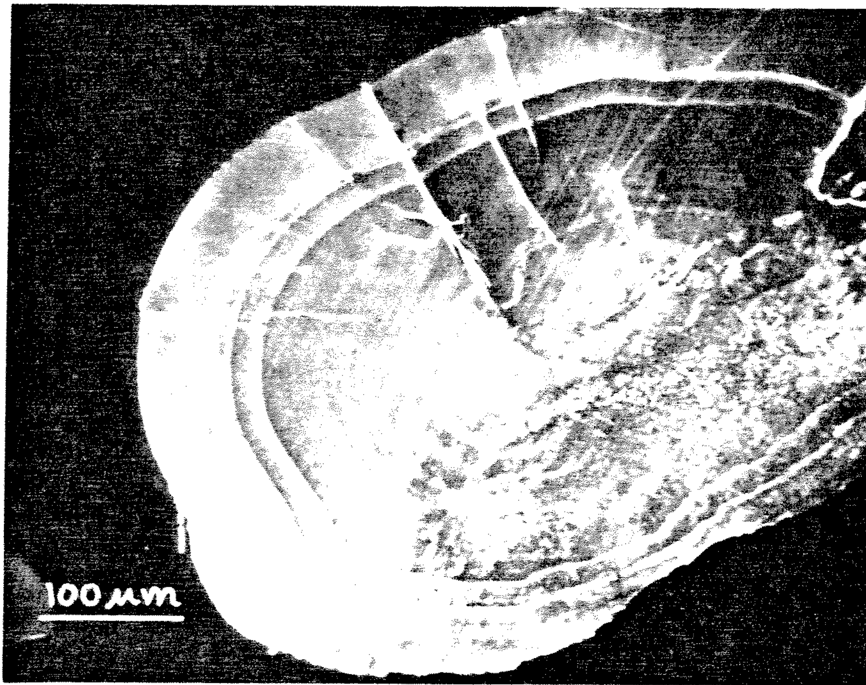


FIGURE 14

A.



B.

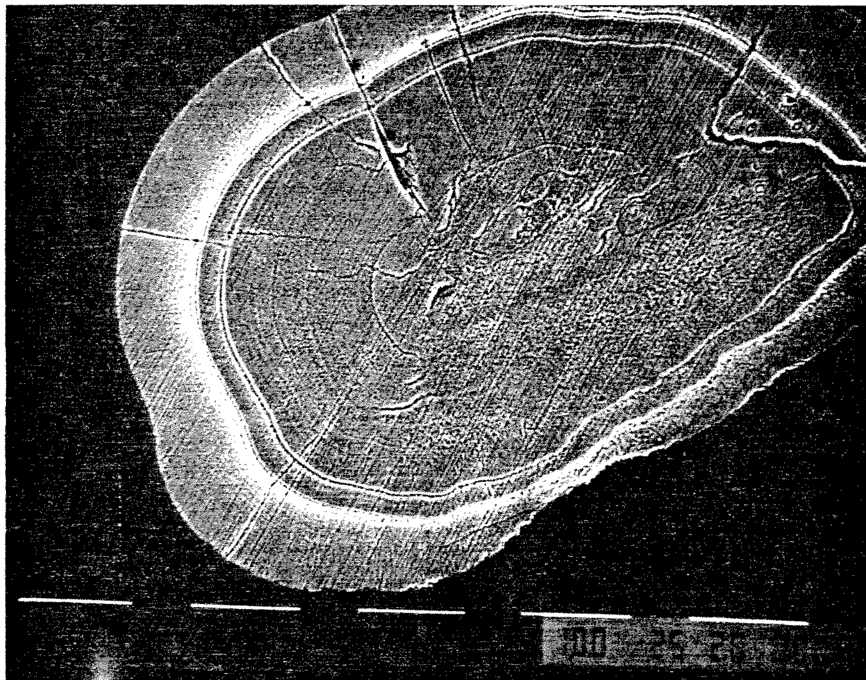


FIGURE 15

C.

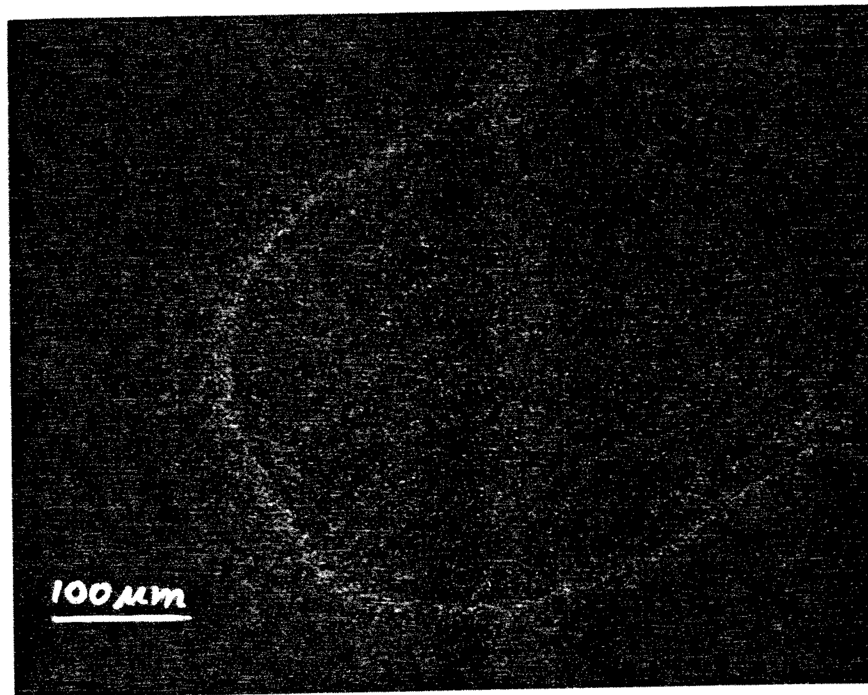


FIGURE 15

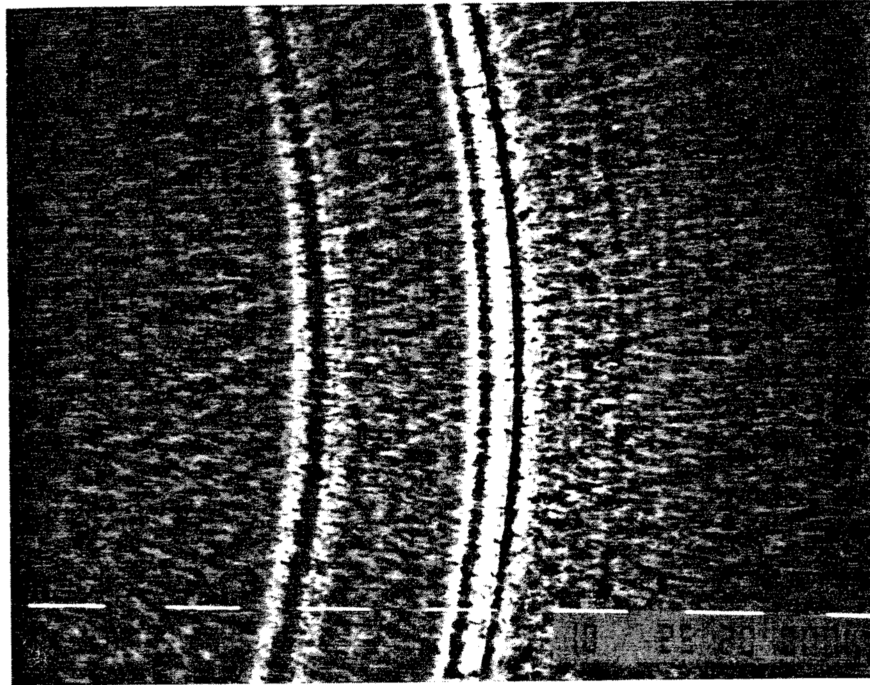


FIGURE 16

7H20 Rsch file Brothers

Great Lakes Fishery Commission

ESTABLISHED BY CONVENTION BETWEEN CANADA AND THE UNITED STATES TO IMPROVE AND PERPETUATE FISHERY RESOURCES

17 December 1984

Dr. Edward B. Brothers
3 Sunset West
Ithaca, NY 14850

Dear Dr. Brothers:

Enclosed are two original copies of a contract between the Great Lakes Fishery Commission and you, signed on behalf of the Commission by Carlos M. Fetterolf, Jr., Executive Secretary.

If you find the contract acceptable, please sign both copies and return one fully executed copy to me. The other copy may be retained for your files.

Sincerely,

Barbara S. Staples

Barbara S. Staples
Administrative Officer

rk

Enclosure

1451 Green Road • Ann Arbor, Michigan 48105 • Telephone: (313) 662-3209 / FTS 378-2077

ESTABLISHED BY CONVENTION BETWEEN CANADA AND THE UNITED STATES TO IMPROVE AND PERPETUATE FISHERY RESOURCES

CONTRACT FOR DEVELOPMENT OF OTOLITH MARKING TECHNIQUES
FOR THE EARLY LIFE HISTORY STAGES OF LAKE TROUT

This contract entered into this 13th day of December 1984 between the Great Lakes Fishery Commission (hereinafter called the Commission), and Dr. Edward B. Brothers (hereinafter called the Contractor).

1. Description of Work: The Contractor will conduct research investigations on development of otolith marking techniques for the early life history stages of lake trout, as more particularly set forth in the Contractor's letter and proposal, attached and made a part of this contract. Deviations from the Contractor's experimental design are not permissable without prior written approval from the Commission. Failure to obtain clearance for such deviations will result in forfeiture of remaining funds. It is understood and agreed that the work will be conducted under the supervision of Dr. Edward B. Brothers.

2. Total Cost: $16,325

   A. The total cost to the Commission for the full performance of this contract will not exceed the estimated amount specified. The Commission further advises the Contractor that any requests for additional funds must be reviewed by the Commission which could take several months. The Commission discourages the Contractor from seeking additional funds and reminds the Contractor that this agreement is a contract and not a grant. The Contractor shall notify the Executive Secretary in writing whenever it appears to the Contractor that the cost of completing the performance of this contract will exceed the total cost specified. The Commission shall not be obligated to reimburse the Contractor for, and the Contractor shall not be obligated to incur expenditures in the performance of the work contemplated by this contract in excess of the cost limitation unless, and until, it shall have been increased by amendment of this contract.

   B. The Commission shall pay to the Contractor as full compensation for his undertakings the total cost of $16,325. Fifty percent of the total cost shall be payable upon receipt of the signed contract and the remaining fifty percent following acceptance by the Commission of a completion report.

3. Time of Performance: 1 November 1984 to 30 June 1985

CONTRACTOR	GREAT LAKES FISHERY COMMISSION
	Carlos M. Fetterolf, Jr.
Dr. Edward B. Brothers	Carlos M. Fetterolf, Jr. Executive Secretary
	18 December 1984
Date	Date

1451 Green Road • Ann Arbor, Michigan 48105 • Telephone: (313) 662-3209 / FTS 378-2077

REQUEST FOR RESEARCH GRANT

GREAT LAKES FISHERY COMMISSION

1451 GREEN ROAD
ANN ARBOR, MICHIGAN 48105

Name and Address of Principal Investigator: Edward B. Brothers
3 Sunset West
Ithaca, NY 14850

Institution or Agency:

Private Consultant

Cooperators:

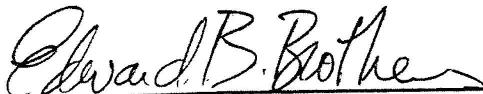
Tunison Laboratory of Fish Nutrition; U.S. Fish & Wildlife Service, Cortland, NY

Amount Requested: $16,325.00

Dates for Proposed Research: from: Oct. 1984 to June 1985

Title of Proposal: Development of otolith marking techniques for the early life history stages of lake trout (Salvelinus namaycush)

Budget *(Direct costs)*		Totals
Professional Personnel *(Name and Title)*		
Edward B. Brothers, Ph.D. Principal Investigator		$ 14,000.00
Maintenance and Operation		
Temporary wages	0	
Supplies	$ 525.00	
Travel	200.00	
Communications plus secretarial, office, etc.	400.00	1,125.00
Equipment *(Specify)* 3 month use fee for SEM and preparation fees and X-ray microprobe.		1,200.00
(Indirect Costs and Overhead, if applicable: explain)		



Signature of Principal Investigator

Ph. D.

Title:

Received: *(Signature of appropriate administrative official)*

Title:

A PROPOSAL TO THE GREAT LAKES FISHERY COMMISSION

TITLE: Development of otolith marking techniques for the early life history stages of lake trout (*Salvelinus namaycush*).

DATE: September 15, 1984

SUBMITTED BY: Edward B. Brothers, Ph.D.
3 Sunset West
Ithaca, New York 14850

TOTAL AMOUNT REQUESTED: $16,325.00

PROPOSED STARTING DATE: October 1984

DURATION OF PROGRAM: 9 months

PRINCIPAL INVESTIGATOR: Edward B. Brothers

Introduction and General Outline of Proposed Research

The otoliths (in particular the sagitta and lapillus) are the first calcified structures to appear in the unhatched or embryonic developmental stages of salmonids, (Fig. 1) including the lake trout, Salvelinus namaycush (pers. obs., McKern et al., 1974). Once formed, the early otolith growth structures remain intact and unchanged for the entire life of the fish. This observation has been verified for the otoliths of lake trout exceeding 13 years of age and in American eels of nearly 30 years old (pers. obs.). Because the otoliths are the only permanent and persistent structures present in the very earliest life history stages of lake trout, they offer the only possibilities for producing an endogenous and unique mark; i.e. one which does not depend upon the insertion or attachment of some artificial tag (for very small fishes, magnetic microtags are available; however, they have generally not been used for fishes as young as required for the Great Lakes stocking experiments). Other traditional marking procedures such as fin clipping and tatooing would almost certainly prove to be either too difficult, too time consuming, or potentially damaging to the fish, and therefore would be of little practical value. Otolith marking for larval and juvenile fishes has been successfully accomplished by several techniques in a variety of fishes (Brothers, 1978, 1981, and unpublished; Victor, 1982; Tanaka et al., 1981; Campana & Neilsen, 1982; Taubert and Coble, 1977; Mugiya and Muramatsu, 1982; Hettler, 1984; and many others). These experiments were often performed during studies of otolith growth or as part of laboratory work to validate the occurrence of daily growth increments, and not to produce marked fish for release into the wild. Some otolith marking in adults has been used to mark fish for release and subsequent recapture (Lanzing and Hynd, 1966; Jones and Bedford, 1968; Blackler, 1974; Wild and Forman, 1980), however these fish also

carried externally visible tags; the otolith work was still geared to age validation studies.

My experience with otolith microstructure studies on the early life history of many species, including trout, char, and salmon, suggests at least four possible approaches to marking the otoliths of lake trout embryos, alevins and fry. The various methods require different treatments to produce the mark, and more significantly, very different modes of viewing or analysis to reveal the marks. The proposed methods fall into the following categories:

I. Microstructural patterns produced by environmental temperature manipulations. Although light and feeding manipulations are possibilities, my observations on salmonids demonstrate that temperature cues produce the most reliable and striking results (Brothers, 1981: see also Wilson and Larkin, 1980; Campana, 1983; and Volk et al. 1984). The types of marks caused by temperature manipulations are clearly distinguishable with light microscopy. Such analysis requires the least critical, quickest and most economical mark "recovery", particularly in comparison to the chemically-based mark discussed below. The only preparation needed is otolith grinding (or possibly sectioning) to view the microstructure formed during the earlier marking stages. I anticipate that temperature marking of lake trout otoliths will prove to be the most practical and effective of the proposed methods.

II. Chemical markers. Included in this category are methods which utilize chemicals to either alter "normal" otolith growth patterns, or which become directly incorporated in the otolith structure.

A. Tetracycline. This compound is a well known (Weber and

Ridgway, 1962, 1967; Choate, 1964; Kobayashi et al., 1964) marker for calcified structures (including otoliths) in fish. For larger fish it is generally injected, however. There has also been success by adding the compound to food, or by immersion, especially for young marine fishes (Lanzing and Hynd, 1966; Hettler, 1984: P.Schmidt, pers. comm.). Immersion is the only practical batch procedure for life stages too young to feed or too small to inject. There is very little published work on immersion marking of young freshwater fish; however this is a distinct possibility which should be examined further. I have successfully marked the otoliths of swim-up fry rainbow trout by mixing powdered tetracycline-hydrochloride with a standard feed in a closed system. In this case marking may have been achieved by feeding, immersion, or both. Viewing of a tetracycline-marked otolith, particularly if the specimen is from an adult fish, requires grinding of the otolith and examination of a fluorescent band with U.V. illumination in a compound light microscope. A vertical illumination arrangement gives superior results. Tetracycline marks are known to be somewhat light-labile, however good marks are known to persist for at least several years in internal structures such as bones and otoliths (Blackler, 1974).

B. Acetazolamide marking. Successful marking of the otoliths of young fishes has been obtained with the carbonic anhydrase inhibitor, acetazolamide (Mugiya, 1977; Mugiya & Muramatsu, 1982; and R. Radtke and W. Haake, pers. comm.). The mark

produced appears as a growth disruption or interruption, an abrupt cessation of aragonite crystallite deposition, delineated by a thin protein layer. The mark can be seen with the light microscope, however confirmation and optimal viewing requires the use of the scanning electron microscope (SEM). The only normally occurring growth interruption in the otoliths of very young salmonids is sometimes produced at hatching. Preparation for SEM viewing involves grinding, polishing, etching with an acidic agent, and coating (Brothers et al., 1976; Brothers & McFarland, 1981; Haake et al., 1981). Embedding of the otolith will also be required for small specimens. To my knowledge, acetazolamide has only been administered by injection, however it is water soluble and immersion experiments should be attempted for the youngest lake trout stages.

C. Trace element marking. Sagittae are almost entirely composed of calcium carbonate (as aragonite) and a fibrous protein called otolin (ref. cited in Brothers, 1984; Carlstrom, 1963; Degens et al., 1969; Pannella, 1980). The remaining fraction (usually less than 1% by weight) is made up of water and a variety of naturally occurring trace elements which are incorporated or substituted into the otolith structure and apparently remain intact for the life of the fish. The trace element content of the otoliths of wild fish has been used as natural markers of stock origin or even age (Gauldie et al., 1980; Bennett et al., 1982; Papadopoulou et al., 1978, 1980; D. Martin, pers. comm.). Analysis has been by atomic absorption

spectrometry and the X-ray microprobe. In addition, Sr/Ca ratios have been examined as possible indicators of the temperature of carbonate formation in invertebrate skeletons and otoliths (R. Radtke, pers. comm.). Recently several workers have attempted to utilize laboratory introduced trace elements to mark the otoliths and other tissues of fishes. Marking has been by addition to the diet and by immersion (D. Martin, pers. comm.; R.J. Muncy et al., in prep., and H. Poston, pers. comm.). Although the work was not performed on salmonid fishes, the concept and general procedures should be applicable to the lake trout situation. Possible elements of interest include copper, zinc, fluoride, strontium and various rare earth elements. Analysis of single or perhaps multiple exposures and resulting deposition layers would involve use of the X-ray microprobe in conjunction with a SEM. Marks should appear as discrete bands superimposed over the SEM image when the probe is used in the elemental "map" mode. Preparation for this analysis includes grinding, polishing, etching, coating and then X-ray spectral analysis with counting periods of approximately 10 minutes or more per specimen. It will be a relatively expensive and time consuming procedure.

The objective of this proposed research project is to develop methods and techniques of marking otoliths in the early life stages of lake trout, for fish to be used in stocking experiments in the Great Lakes.

Research Plan

I propose to evaluate the feasibility of marking lake trout embryos, alevins and fry by the four approaches discussed above. The experimental work would be carried out at the Tunison Laboratory of Fish Nutrition (U.S. Fish and Wildlife Service, Cortland, New York). The Tunison Laboratory has excellent facilities for experimental work and environmental control of young salmonids in a "hatchery-type" atmosphere. The laboratory director, Dr. Gary Rumsey , has expressed interest in the proposed research and a willingness to cooperate by making necessary fish and facilities available for the project. In addition, one of the research faculty, Dr. Hugh Poston, has had specific experience with rare earth element marking of striped bass (R.J. Muncy et al.,in prep.) and will cooperate in the related portions of the proposed lake trout study. The Tunison Laboratory maintains a brood stock of "Marquette strain", Lake Superior lake trout and will have ample supplies of eggs and fish at all stages of development. Otoliths of Tunison Laboratory yearlings have been compared with specimens obtained from the Jordan River National Fish Hatchery (which utilizes stock of the same origin as the Tunison fish). Otoliths from both lots of fish were found to be comparable in the appearance of the early otolith microstructure, in the presence of discrete daily growth increments (validated by known ages), and in the relatively low incidence of abnormal or "crystalline" otoliths (approximately 30%). Otolith features of these fish were also found to be quite similar to those of adult lake trout stocked in Cayuga Lake (N.Y.) as yearlings and captured up to 13 years later (Fig. 2). I conclude that the Tunison Laboratory lake trout are entirely suitable as model experimental subjects since they are equivalent to the stocks of lake trout that will be utilized in the projected Great Lakes stocking programs. Furthermore, the Tunison Laboratory has superb facilities including

environmental chambers and temperature controlled aquaria and the capacity to hold and maintain large numbers of experimental lots of fish under constant supervision. The staff is willing to cooperate and the Laboratory is located about 15 minutes from my home and base of operations. Microscope equipment to be utilized in the study is located at Cornell University. I have complete and free access to an excellent compound microscope with photographic, video and fluorescence capabilities. SEM and X-ray microprobe facilities are also available at Cornell for a user fee.

The research will be carried out along four avenues of investigation, following the four proposed marking techniques. Greatest emphasis will be placed on "temperature marking" of otolith microstructure, since this has demonstrated success in other salmonids and appears to offer the most practical means for marking <u>and</u> mark recognition for a large stocking operation.

I. The time of sagittal primordium and core formation will be determined by daily examination of developing lake trout eggs. Embryos will be dissected out of the egg membranes, fixed and dehydrated in absolute ethanol, cleared and examined with polarized light microscopy. This technique has been developed in my laboratory and successfully applied to other salmonids (pers. obs.). Once incremental growth is detected around the multiple cores, experimentation will proceed. A group of control fish, held in "hatchery conditions" and not manipulated will be maintained and periodically sampled to document "normal" otolith developments. The experimental stock will be divided into lots of approximately 30 fish. In some cases multiple experiments can be performed on the same fish, since otolith responses will be permanently recorded in the microstructure and fish may be examined after a series of manipulations. Most of the fish will

be maintained under typical hatchery conditions (relatively constant temperatures, ca. 8° C; spring fed, open system, water supply; natural and/or artificial lighting approximating a natural diel cycle). These fish will be periodically exposed to controlled temperature conditions which will consist of one to several days of diel temperature cycles with an amplitude of about 5° C. Other groups will be exposed to periods (to 10 days or more) of slowly rising or falling temperatures. Such manipulations have produced characteristic marks in the otoliths of rainbow trout embryos, alevins and fry (Figs. 3 and 4).

Temperature manipulations will be performed in closed systems (aquaria) with good aeration, filtration, circulation and adequate volume to prevent any significant changes in water quality (dissolved oxygen, pH and perhaps nitrogen or ammonia levels will be tested to verify this). Manipulations will be interspersed between periods in which the experimental groups will be maintained in the open "hatchery" conditions. Lots will be isolated in vertical or cylindrical rearing containers (an established procedure at the Tunison Lab). Feeding after swim-up will utilize the standard prepared Tunison diets on a regular schedule.

Experiments will test the ability to produce visible and characteristic marks in the sagittae of embryos, alevins and fry. A few fish will be sacrificed within a week or two after a manipulation to get an immediate impression of the response and to make an assessment of needed adjustments in the temperature conditions. Most fish will be maintained to fingerling size (less than 10 cm, TL) before terminating the experiments and examining otoliths. Expected

otolith microstructure responses include:

1. well defined daily growth increments formed during periods of elevated temperatures and strong diel temperature cycles
2. poorly defined daily increments in a broad and relatively translucent band when fish were exposed to gradually rising, warm, temperatures
3. poorly defined daily increments in a narrow and relatively opaque band when fish were exposed to constantly falling, cool temperatures.

Similar patterns have been produced by laboratory manipulations of other salmonids, particularly rainbow trout (Figs. 3 and 4).

II. Groups of lake trout (at different ages) will be exposed to tetracycline-hydrochloride in a closed system for periods of one to several days. Immersion solutions containing dimethyl sulfoxide (DMSO) may also be tested following consultation with R.C. Johnson (Natl. Mar. Fish. Serv., Rufus, Oregon). For feeding fish, diets will be prepared containing a measured quantity of tetracycline. Dosages and feeding periods will be adjusted to approximate a total exposure of at least 20 mg/kg of body weight, an average effective dosage for injections. Average food consumption rates will be monitored to determine actual dosages. Some lots will experience multiple exposures (separated by at least 1 to 2 weeks) to determine the feasibility of producing multiple marks. Additional fish will receive tetracycline injections to produce marks for comparison with the immersion and feeding experiments. Otoliths will be prepared for fluorescence light microscopy and examined for tetracycline deposition.

III. Acetazolamide experiments will follow a protocol similar to those for tetracycline. Otoliths will be examined with both light microscopy and SEM.

IV. There are numerous possible elements to test as markers in otoliths. Due to the anticipated lower efficiency and practicality of elemental analysis of otoliths as a large scale effort, this portion of the study will be limited to testing only a few possible markers. The mode of exposure to be examined will be immersion (and possibly feeding), with a few injected specimens and controls for comparison. Candidates for testing include copper, iron, zinc, cadmium, strontium, fluoride, and one rare earth metal such as Terbium or Europium. D. Martin (pers. comm.) has successfully done some immersion marking of otoliths for the larvae and juveniles of three freshwater (or euryhaline) species. He also found that marking of unhatched embryos may be severely limited due to greatly reduced chorion penetration by some of these elements. Otolith specimens will be prepared for the SEM and analyzed with the X-ray microprobe. Dr. Hugh Poston of the Tunison Laboratory of Fish Nutrition will be a collaborator on this portion of the project.

Results from all experiments will be summarized and photo-documented where possible. A final report will be prepared which will assess the feasibility of the different marking techniques, describe all necessary materials and techniques, and make recommendations for practical applications in stocking experiments.

Schedule of Work

Activity	Time Period
1. Preparation and set up of equipment, materials, supplies and experimental manipulation and holding facilities	Oct. - Nov. 1984
2. Fertilization of lake trout eggs; establishment of experimental groups and monitoring of early development	Oct. - Nov. 1984
3. Experimental treatments of embryos, alevins and fry; preliminary monitoring and analysis of responses	Nov. 1984 - March 1985
4. Analysis of otoliths	Jan. - April 1985
5. Preparation of final report	April - June 1985

Previous Work and Qualifications of the Principal Investigator

As principal investigator, I have over ten years of experience working with otolith microstructure research. A large portion of these investigations has dealt specifically with the early life history stages of fishes. I am a recognized leading authority in the field and have been invited to chair and produce a major review paper for the otolith session of next year's International Symposium on Age and Growth of Fish.

With regards to the particular research proposed here, I have a great deal of experience working with the otoliths of salmonid fishes, including those of lake trout. The majority of these studies have been aimed at determining the relative importance of exogenous and endogenous environmental factors in determining the appearance and composition of discrete patterns in otolith microstructure. This work has included a substantial amount of laboratory manipulations to produce specific patterns and to mimic naturally occurring ones. I am familiar with all of the basic techniques and procedures included in the research plan, and have personally pioneeded the development of several of them. Otolith marking of embryonic, alevin and fry stages of trout (primarily rainbow trout) has already been accomplished in my laboratory and I forsee no major complications in applying similar methods to lake trout for the purposes of identifying stocking groups or treatments. For several years now I have utilized "natural marks", usually produced by anomolous or discrete temperature fluctuations in the wild, or in hatchery environments, to track and identify trout origins. I have also had experience with tetracycline marking of fish otoliths (in mackeral, tuna, trout and eels) and am highly qualified in the use of the SEM. Finally, the X-ray microprobe instrumentation is familiar to

me and I have taken a course covering its application to biological investigations.

My C.V. and attached reprints should serve to confirm my qualifications to do the proposed research.

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Figures

Figure 1 - Early growth of rainbow trout otoliths.

A. Separate sagittal primordia and cores in an embryonic (pre-hatch) trout.

B. Beginning of fusion and incremental growth in an early alevin.

C. Fusion into a single sagittal otolith in a late alevin.

Figure 2 - Early growth of lake trout otoliths as seen in ground sections from adult fish. Pre-hatching and pre-swim-up marks are evident. Scale bar equals 20 um.

A. Primordia & cores from a 13 year old lake trout from Cayuga Lake, N.Y. This was a hatchery reared fish.

B. Similar view from a 10 year old fish.

Figure 3 - Characteristic marks produced in rainbow trout otoliths by temperature manipulations.

A. Marks produced in the embryonic and alevin stages. Point 1 and zone 2 were induced by eliminating diel temperature cycles.(Photomicrograph; magnification about 1500x)

B. Abnormally wide increments in a juvenile fish produced by skipping the nocturnal temperature drop for 1 day, returning to a normal diel cycle and then skipping another night's drop before resuming the diel cycle. (Photomicrograph; magnification about 2000x)

C. SEM micrograph of the same specimen. (Magnification about 3300x)

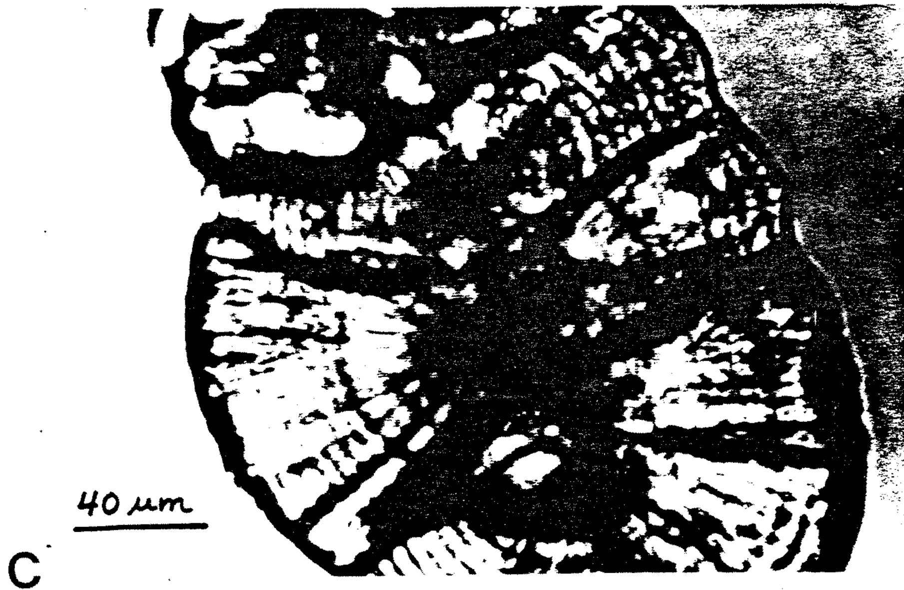
Figure 4 - Unique marks produced in rainbow trout otoliths by longer term temperature manipulations.

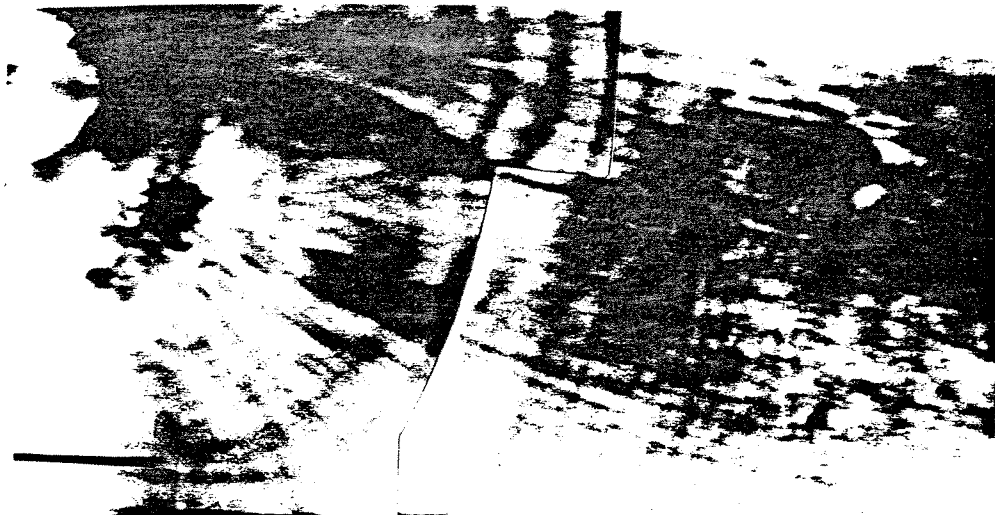
A. Ten day period without diel temperature cycle.

B. Alternating long temperature "days" (about 50 hours) with short "days" (about 18 hours).

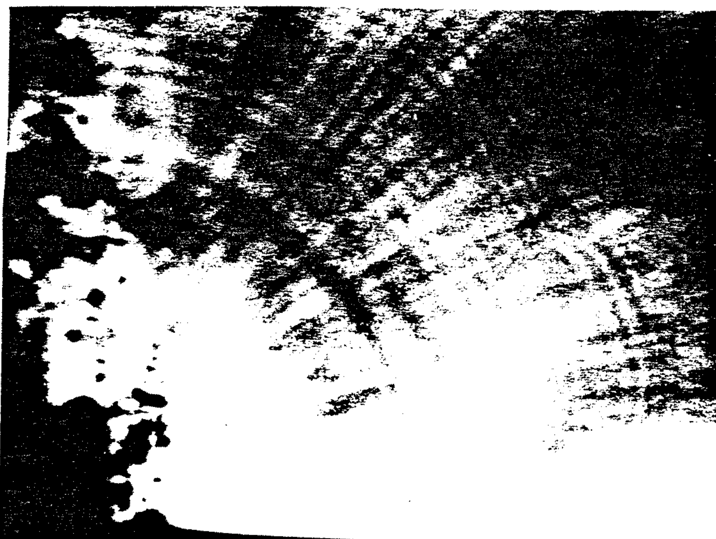
10µm
A

10µm
B


40 µm
C



A



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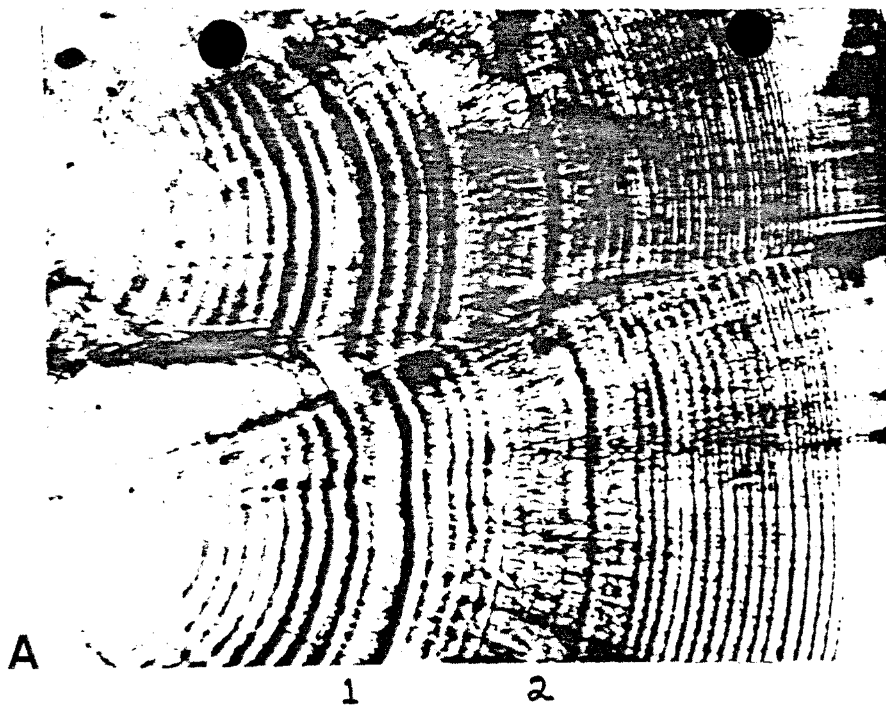
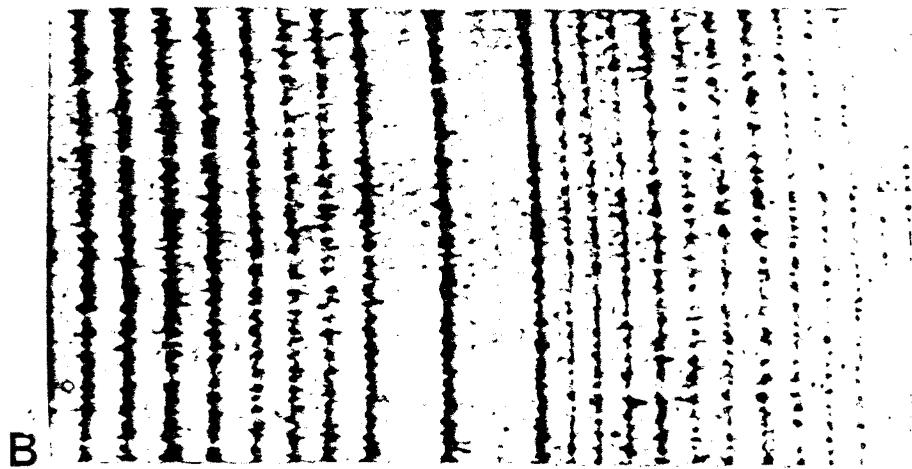

A
1
2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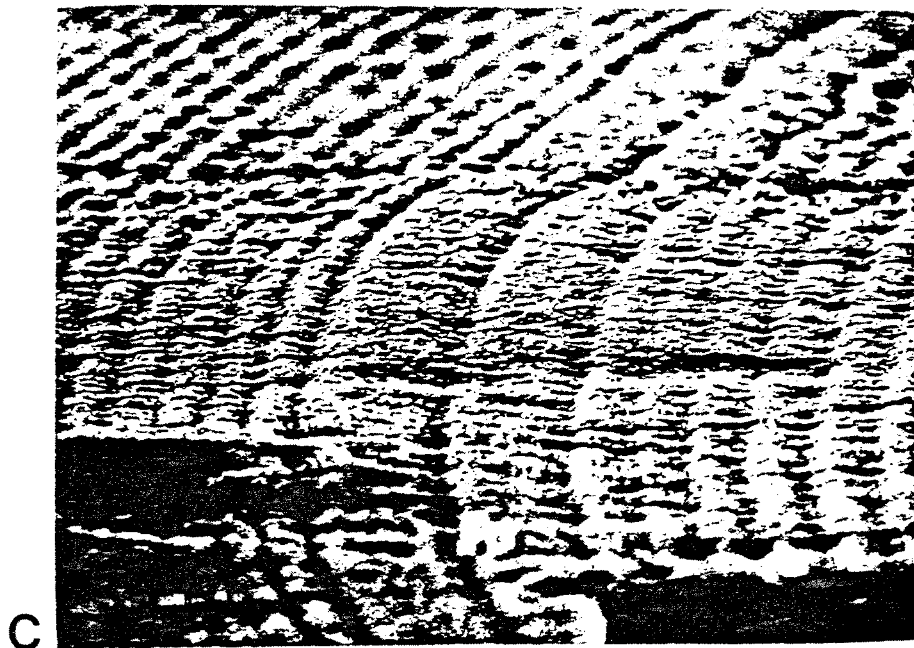
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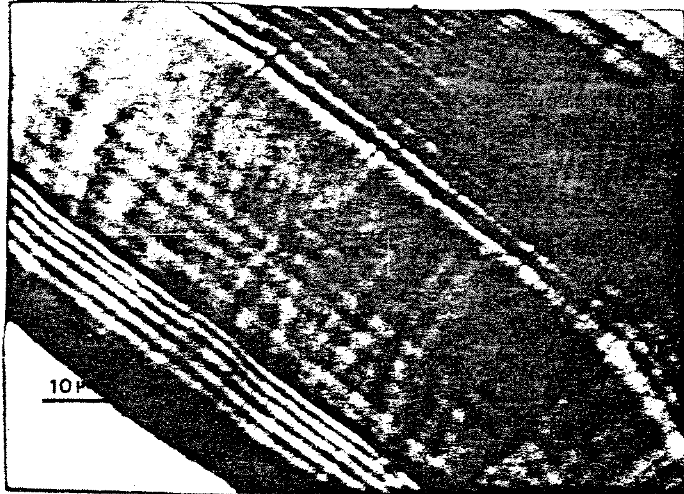

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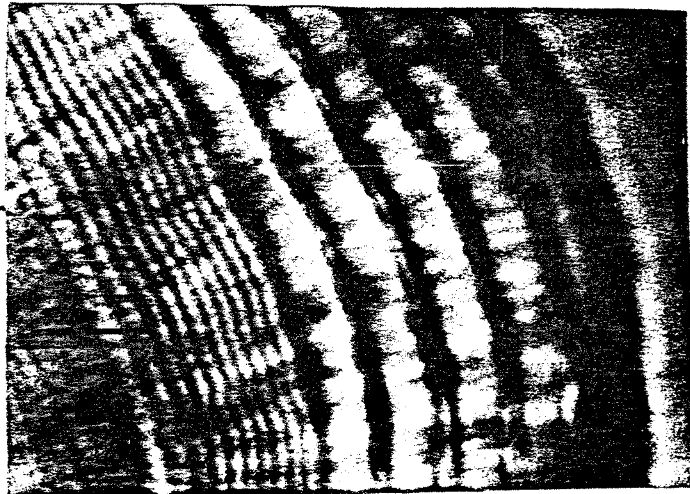

B