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**AN OTOPATHOLOGICAL ASSESSMENT OF DOGS EXPOSED TO EXCESSIVE DIETARY  
FLUORIDE AND/OR SUSPECTED WATER BORNE TOXINS**

**by**

**Barbara Ann Hannah**

**A Thesis  
Submitted to the  
Faculty of The Graduate College  
in partial fulfillment of the  
requirements for the  
Degree of Master of Science  
Department of Biology and Biomedical Sciences**

**Western Michigan University  
Kalamazoo, Michigan  
April 1987**

AN OTOPATHOLOGICAL ASSESSMENT OF DOGS EXPOSED TO EXCESSIVE DIETARY  
FLUORIDE AND/OR SUSPECTED WATER BORNE TOXINS

Barbara Ann Hannah, M.S.

Western Michigan University, 1987

In 1970, a once very productive Allegan, Michigan dog kennel began experiencing a rash of unexplained perinatal deaths and reproductive anomalies in Shetland pups. Anomalies including cleft palate and lip, kinked tails, twisted feet and convulsions immediately before death plagued the neonates. The adult dogs were reported to have tooth mottling, exostoses and auditory and vestibular problems.

Because the dogs had been on high fluoride diets and because the kennel well water was suspected of harboring toxins, a group of 20 Shetland dogs was used to try to find if there was any correlation between the reproductive problems and the high fluoride food and well water.

The research described in this manuscript examines the temporal bones of eight of these Shetland dogs along with six non-Allegan control dogs in an effort to try to explain the reported auditory and vestibular problems at the kennel. It was determined in the present study that the animals from the high fluoride and well water group showed substantially less otopathology than those from the low fluoride and distilled water group.

## ACKNOWLEDGEMENTS

I would like to dedicate this thesis in loving remembrance of my mother, Elizabeth, whose guidance and support have been and will continue to be a cornerstone for me.

I extend my deepest appreciation to the Upjohn researchers--Tom Marks, Diane Schellenberg, Jo Oostveen and Mary J. Morey who had the incentive and dedication to undertake this massive study. I am also grateful to The Upjohn Company for supplying the control animals and the embedding media.

I also extend my gratitude to Wendy Lubke for her assistance in coverslipping the specimens and to Oris Griffin for her assistance in typing this document.

I thank the members of my thesis committee, Drs. Leonard Beuving and Jaime Benitez, for the input of their technical expertise and suggestions for the final presentation.

With heartfelt gratitude, I extend my deepest appreciation to my major adviser, Dr. Cecil McIntire, who has truly been a mentor exemplar. I will remain grateful to him for his patience, support and words of encouragement which have been most influential and inspirational.

Barbara Ann Hannah

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**Hannah, Barbara Ann, M.S.**

**Western Michigan University, 1987**

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## CHAPTER I

### INTRODUCTION

#### Background of the Problem

During the 1950s and 60s Moribrook kennel in Allegan, Michigan was the epitome of dog kennels. At that time, the kennel produced hundreds of Shetland sheepdogs with 45 American Kennel Club champions. The kennel had successfully operated out of old wood frame buildings with females producing ten to fifteen litters a year and losing very few pups perinatally.

In 1969, a new concrete block building was erected and the kennel was rehoused in the new facility. Beginning in 1970 a rash of perinatal deaths began to sweep through the kennel. In 1974, all pups in fourteen of the newborn litters died. In addition to the preponderance of perinatal deaths, pups were born with severe abnormalities which included spinal defects, cleft palate, and positional anomalies of the limbs. In 1980, each of the eighteen litters whelped between January and June contained some deformed animals. By the end of 1981 there were approximately 100 dead litters and reproduction had almost halted. In the male dogs, the breeder suspected a diminished sperm count which was based on her assessment of an observed drop in libido.

In an attempt to identify the cause(s) of the abnormalities, the breeder boarded out dams during pregnancy and the neonatal period and compared the results of the "away" pregnancies with those at Moribrook.

The dogs were sent to various friends of the breeder in an attempt to compare the Moribrook results with the "away" results. The females were impregnated both at Moribrook and away. It was discovered that 27 of the 30 Moribrook pregnancies resulted in litters with birth defects, perinatal losses or both, while only 4 of the 26 "away" pregnancies resulted in the aforementioned birth defects and/or perinatal deaths.

Not only were dogs affected, but it was observed that Persian queens housed at the Moribrook Cattery stopped cycling for more than one year. A litter of mongrel kittens died between five and six weeks of age and another litter had birth defects.

Not only did birth defects present a problem at the kennel, but beginning in 1979, tooth mottling and bony outgrowths were noted in the dogs. Out of 64 dogs, 39 or 70% of them had palpable bony outgrowths (which were later diagnosed as exostoses). Exostoses was noted on the occipital bones, tibias, scapular spines, ribs and supra-orbital arches. Figures 1, 2 and 3 depict exostoses on the occipital crest and the tibia of two non-study Allegan dogs.

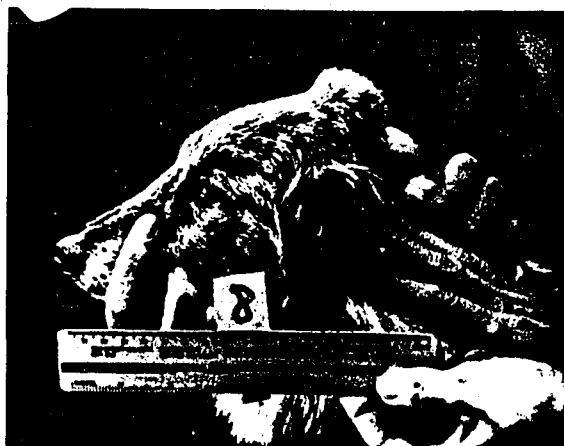


Figure 1. Profile of Large Exostoses on the Occipital Crest of a Nine Year Old Non-Study Allegan Dog.



Figure 2. Frontal View of Large Exostoses on the Occipital Crest of a Nine Year Old Non-Study Allegan Dog.



Figure 3. Tibial Exostoses of a Six Year Old Non-Study Allegan Dog.

Some of the dogs also presented with enlarged zygomatic arches.

Concurrently, two other Michigan dog kennels, both within five miles of Moribrook, reported similar bone and tooth pathologies. One of the kennels, Wil-O-Lane, a producer of Collies and Cocker Spaniels, reported that between May 1981 and April 1983 all pups in three of the nine Cocker Spaniel litters and all pups in 4 of 47 Collie litters were born deformed. The other kennel, Allegan Veterinary Clinic reported no birth defects but did report tooth mottling and exostoses. In addition to these aberrations the owner observed and reported an empirical assessment of auditory loss and dizziness in 75% of the dogs at Moribrook.

A team of researchers from The Upjohn Company, Michigan State University, the United States Department of Agriculture and the Michigan Department of Health came together to investigate the problems at Allegan. Due to the tooth mottling and exostoses noted, fluorosis was suspected. Urinalysis of 18 of the Moribrook dogs showed that their urine levels ranged from 30 to 190 ppm of fluoride while those of control animals ranged from 4 to 12 ppm. Fluoride assays of the dog food used at Moribrook showed fluoride levels between 460 and 500 ppm as compared with other commercial brands which contained 50 to 100 ppm. The high fluoride levels were attributed to rock phosphate which had been added by the manufacturer as a calcium and phosphate source.

#### Organization of the Study

The research team quickly came to suspect that a possible correlation might exist between the high fluoride levels in the food and/or the Moribrook well water, and the reproductive anomalies. It was

postulated that two parameters to which the dogs were constantly exposed—food and water—could easily be measured for possible contaminants. This led to the development of a two-way factorial study to examine the effects that fluoride and/or Moribrook well water had on reproduction. The two-way factorial study was designed to try to answer the following questions: (a) does high fluoride in dog food affect female reproduction, (b) does Moribrook Kennel water affect female reproduction, and (c) is there any interaction between fluoride content and Moribrook well water?

The present thesis project represents one segment of the total study and is designed to assess a considerable variety of hard tissues that are found within the temporal bone, as well as the auditory and vestibular organs.

## CHAPTER II

### REVIEW OF RELATED LITERATURE

#### Water--A Possible Link to Toxicity

Water, the universal solvent, in its natural state contains a great variety of solutes, serves as a vehicle for a multitude of particulate types, and is the home of a menagerie of living organisms. When water is used for domestic and industrial purposes even more materials are added.

Pathogenic bacteria and viruses from humans and other mammals are commonly transferred to water supplies by fecal contamination. The most common water borne diseases that affect humans are typhoid fever, dysentery and cholera. Low level radioactivity is also present naturally in water, and industrial use of radioactive materials has significantly increased the probability that these substances will be excessively high in some water supplies. Products such as detergents, artificial fertilizers and pesticides have also become pollutants in water supply systems.

In attempting to determine the route and identification of environmental toxins, certain parameters may be difficult or even impossible to measure. Air pollution composition and surface contact contaminants are two variables that are virtually impossible to assess completely. However, food and water are possible routes of contamination that can be measured with relative ease. For a variety of reasons, the Moribrook well water was believed to be a possible source

of contamination that warranted examination (Marks, personal communication).

### The Relationship Between Fluoride, Bone and Enamel

Bone, as a rigid, structural support system, has fascinated philosophers, scientists and authors since the beginning of recorded time. Due to its solidity it can serve as a more permanent memorial after the flesh has decayed.

After discovering the deposition of red coloring in the bones of sheep fed madder root, Lemnius in 1564 concluded that bone was a living and changing entity that reflected the environment and lifestyle of the organism (Lutwak, 1975).

Eighteenth century investigators were able to show that new bone was continuously being deposited while old bone was being resorbed. Within the past 50 years, with the advent of radioactive isotopes, it has been confirmed that bone is a living and dynamic tissue that is remodeled throughout life and is under the control of both the internal and external environment (Lutwak, 1975).

Bone is affected by a variety of chemicals including fluoride. Fluorine, the most reactive chemical element, rarely occurs in the free state but chemically combines to form fluorides. Fluoride is found within the earth's crust and invariably occurs in the food chains which involve vegetation, animals and man. In nature, fluorine occurs most commonly as fluorite and fluoroapatite (Shupe, Olson, & Sharma, 1972). Fluorite is a common halide mineral occurring in lead and silver ores, sedimentary rocks and hot springs. Fluoroapatite, a common phosphate mineral occurs in both igneous and metamorphic rocks.

The possible consequences of fluoride were first noted in 1916, when as a result of high fluoride levels, tooth mottling was observed (Shaumbaugh & Petrovic, 1968). Lukomsky (1941) advocated the use of 7.5 mg of sodium fluoride per day for the treatment of osteomyelitis and periodontal disease. He believed that fluoride stimulated calcification and gave rise to a more resistant bone structure (Kristoffersen, Bang, & Meyer, 1970).

It is now known that fluoride is required for normal dental and skeletal growth with 1 to 4 mg being the daily requirement (Farley, Wergedal & Baylink, 1983). The requirement of fluoride in children has been well established and it appears that older adults can also reap the benefits (Rich & Ensick, 1961). Shaumbaugh (1971) reports that in Aurora, Illinois, where the fluoride water content was 1.2 ppm, there were less than half as many cavities in the teeth of school children as in Grand Rapids, Michigan where there was a low fluoride water content. When fluoride levels in Grand Rapids were increased tooth decay was reduced. Today fluoride is being added to the drinking water of many communities across the United States.

### The Chemistry of Fluoride

Ingestion of sodium fluoride is usually done via drinking water, with the compound being readily absorbed in the intestinal tract and excreted by the kidneys (Okazaki, Ophaug & Singer, 1985). Very little fluoride is retained by soft tissues and most is taken up by the teeth and bones (Purves, 1962). In assays determining fluoride content the following ratios were found in five tissue types: (1) tooth enamel-



—fluoride ranging from 571 to 2,290 ppm, (2) bone—fluoride ranging from 200 to 1300 ppm, (3) soft tissue—fluoride at less than 1 ppm, (4) saliva—fluoride at .1 ppm, and (5) blood fluoride—at .01 ppm (Shaumbaugh & Petrovic, 1968).

In teeth and bones, fluoride becomes incorporated by replacing the hydroxy ion of the hydroxyapatite crystals with fluoride. This results in the formation of fluoroapatite (Biller, Yosipovitch, & Gedalia, 1977). Fluoroapatite is considerably less soluble and reactive than pure hydroxyapatite. The result is harder teeth and bones (Posner, Eanes, Harper & Zipkin, 1963; Purves, 1962).

Larsen and Thorsen (1984) found that in vitro experiments using liquid saturated with hydroxyapatite crystals there was supersaturation with fluoroapatite when fluoride was added, indicating the replacement of the hydroxy ion with fluoride. When the activity of the fluoride had diminished, the rate of dissolution of the hydroxyapatite brought the system back to its original saturation.

Within the body, fluoroapatite accumulates at a rate that is proportional to the amount ingested (Purves, 1962). Fluoride selectively accumulates in bone and teeth with a cumulative effect that normally continues throughout life. The cumulative effect is directly related to the metabolism of the tissue so that rapidly forming tissues (teeth and bone) have a greater uptake of fluoride (Shupe et al., 1972).

The increased density and decreased solubility of bone following excess fluoride uptake renders it less susceptible to osteoclastic resorption (Biller et al., 1977; Faccini, 1967; Lutwak, 1975). How-

ever, if blood calcium levels decrease significantly due to decreased osteoclast activity, there is a compensatory increase in osteoclast activity and number in an effort to maintain calcium homeostasis (Lutwak, 1975; Ream & Pendergrass, 1982). This correlates well with Healy's observation of increased osteoclast numbers in the temporal bones of rats exposed to excessive fluoride (Healy, 1983). Even with an increase in osteoclastic activity and number, high fluoride bone still remains less susceptible to resorption, and thus results in a decrease in blood calcium levels (Faccini, 1967). Ream and Pendergrass have found that in rats given 150 ppm fluoride for 10 weeks, numerous Howship's lacunae appeared on the surface of trabecular bone indicating extensive resorption and remodelling. Mild secondary hyperparathyroidism stimulated by the decrease in calcium levels is another result of excess fluoride ingestion (Baylink, Duane, Farley & Farley, 1983; Faccini, 1967).

Fluoride is readily absorbed by the intestine and the presence of other ions is a determining factor in its absorbability. Fluoride in the presence of large amounts of calcium, magnesium and aluminum forms less soluble complexes and its absorbability is reduced (Shupe et al., 1972). Conversely, in acute magnesium deficiency, elevated levels of fluoride can retard the mobilization of magnesium. The deficiency of magnesium produces a reduction in skeletal mineral accretion which had been increased by the administration of fluoride (Ophaug & Singer, 1976). In experiments with rats it has been shown that increases in dietary calcium caused decreases in intestinal fluoride absorption (Harrison, Hitchman, Hasany, Hitchman & Tam, 1983).

### The Effect of Fluoride on Carbohydrates, Lipids and Vitamins

Glycosaminoglycans, principally chondroitin-4-sulfate and dermatan sulfate, have been reported to be increased in intervals of prolonged exposure to fluoride (Prince & Navia, 1983).

Wolinsky, Simkin and Guggenheim (1972) report a decreased content in the lipid associated with bone in animals treated with high fluoride.

Hauck, Steenbock and Parsons (1933) have demonstrated that vitamin D reduces the toxicity of fluoride fed to rats on low calcium diets. Muhler (1958) showed that vitamin C increased the storage of fluoride in guinea pigs.

### Topical Application of Fluoride and the Effect of Fluoride on Osteoid

Snow and Anderson (1985) report increases in the cortical area, the percentage of osteoid seams and circumference of osteoid seams in beagles having a high fluoride intake. They also report an increase in bone formation and resorption.

In topical applications of fluoride to defects in the parietal bones of rats, Biller et al. (1977) report an increase mean bone weight in the fluoride treated rats. Branemark (1967) found that topically applied fluoride exacerbated already existing gingival inflammation.

### Hazards of Excessive Fluoride Intake

If the daily intake of fluoride is in the range of 15 to 40 mg, bone becomes exceedingly resistant to resorptive stimuli and can

become abnormal (Lutwak, 1975). In cases where 225 mg of sodium fluoride have been administered to patients, symptoms such as nausea, vomiting and abdominal pain were reported. The lethal dose of fluoride is 5000 mg (Rich & Ensick, 1961).

Instances of high fluoride intake have been reported in India, China and Japan where fluoride content ranges from 8 to 16 ppm (Shaumbaugh & Petrovic, 1968). Ingestion of large quantities of fluoride results in endemic fluorosis, a condition which leads to pitting and discolored spots on the teeth, and exostoses and ankylosis of the joints. Mild cases of endemic fluorosis have been reported in Great Britain and the U. S. but without skeletal changes (Rich & Ensick, 1961). In severe cases of endemic fluorosis, there have been reports of progressive calcification of ligaments and interosseous membranes as well as neurological damage in the extremities resulting from exostoses pressing on the spinal cord (Shaumbaugh & Petrovic, 1968).

Eckerlin, Maylin and Krook (1986) have reported skeletal fluorosis, mastitis, decreased milk production, stunted calves and a high rate of calf mortality in dairy cattle on high fluoride.

According to Shupe et al. (1972) the main source of fluoride to animals is from mineral ores. In certain industrial operations, these fluorides can escape from the heating ores and be emitted into the atmosphere where they ultimately settle on the vegetation around the industry. Animals grazing in the area are then exposed to the fluoride. Animals can usually ingest small amounts of fluoride without adverse effects.

The onset of chronic fluoride toxicity in animals is gradual and insidious, and its symptoms mimic osteoarthritis. Dental lesions are quite obvious, appearing as chalk-like mottled areas. Hypocalcification is also noted. Livestock exposed to high levels of fluoride have been noted to mobilize themselves on their knees intermittently due to an inability to stand. The first palpable bony lesions in livestock usually appear on the metatarsals with subsequent lesions occurring on the mandible, ribs and metacarpals. During the recovery period from chronic fluoride toxicity, animals ingesting higher quantities for a short time are more prone to eliminate the fluoride than are animals with the same level of fluoride intake over a longer duration. Urinary fluoride levels remain high even after an animal is removed from fluoride.

#### Fluoride and its Possible Transfer to the Fetus

The incidence of selected congenital malformations in areas with fluoride supplementation of public water supplies was comparable with the incidence in areas where the water supply is deficient in fluoride. Comparison of the incidences of several common birth defects (including Down's syndrome, cleft palate and cleft lip) in fluoridated and non-fluoridated areas revealed no substantial differences in the two areas (Erickson, Oakley, Flynt, & Hay, 1976).

Feltman and Kosel (1955) found that in pregnant women who had diets supplemented with calcium fluoride tablets or sodium fluoride tablets there was an increase in the concentrations of fluoride in the periphery of the placenta. One possible explanation proposed was that

since the calcium content is greatest in the periphery of the placenta there is a possible chemical attraction. A second proposal was that there exists a protective mechanism to keep the fluoride from the center of the placenta where there is the highest maternal-fetal transfer.

### Fluoride in the Treatment of Disease

Fluoride has been determined to be therapeutic in the treatment of certain diseases. According to Shaumbaugh and Petrovic (1968) sodium fluoride was found to be the best treatment for prevention of osteoporosis. Fluoride in osteoporosis functions by increasing the rate of bone formation (Farley et al., 1983). Shaumbaugh (1971) contends that there is an optimum daily dosage of fluoride for osteoporosis below which there is a lessened effect or no effect and a higher dosage which becomes less and less effective and could lead to eventual death. He surmised that in the treatment of osteoporosis 40 to 60 mg per day is optimum.

Rich and Ensick (1961) report that in Paget's disease, osteitis deformans, there was considerable relief from bone pain in 14 of 16 patients on 60 mg per day of fluoride. They also report an improvement in calcium balance in four of five cases.

Fluoride also decreases abnormal calcification of the aorta—a common condition in the elderly (Shaumbaugh, 1971).

### The Relationship Between Fluoride and the Temporal Bone

According to Shaumbaugh and Petrovic (1968), sodium fluoride is therapeutic in the disease, otosclerosis which is an osteoporosis of

the labyrinth capsule. In the otosclerotic temporal bone, foci of new, highly vascular bone replace the original endochondral and almost avascular bone of the labyrinth capsule. Shaumbaugh and Petrovic (1968) believe there is a greater incidence of stapes fixation in low fluoride areas as compared with high. Daniel (1969) found that stapedia otosclerosis was four times higher in a low fluoride area (.6 ppm) than it was in a high fluoride area (1.9 ppm). In the treatment of otosclerosis, Stuntzmann (cited in Gunby, 1979) says that sodium fluoride operates by restraining bone resorption. As a consequence of this, the pH is restored to normal, enzymes and catabolites released into the perilymph are reduced and hearing impairment ceases. He believes that fluoride's action on bone resorption is of secondary importance (cited in Gunby, 1979).

In the Indian village of Kammaguda in Nalgonda district, Andhra Pradesh, the fluoride in the drinking water measures 11.8 ppm (Rao & Siddiqui, 1962) which has led to numerous cases of endemic fluorosis. Radiograms of resident skulls showed sclerosis of the cranial vault, petrous portion of the temporal bone and the internal auditory meatus.

In a study involving twelve temporal bones of males from proven cases of fluorosis, dehiscence was found in the facial nerve canal of all bones. The three ossicles, joints, ligaments and tendons were normal (Thapar, Singh & Singh, 1977). There was no conductive deafness noted in the advanced cases of fluorosis, only mild sensorineural deafness due to presbycusis.

Fluoride and/or Moribrook Well Water and the Effect on the Temporal  
Bones of Rats and Dogs

A group of researchers from The Upjohn Company, Michigan State University, the USDA and the Michigan Department of Health came together for the purpose of investigating reproductive anomalies at the Moribrook Kennel in Allegan, Michigan. The study utilized a two-way factorial approach to try to ascertain the possible relationship between high fluoride dog food and/or Moribrook Kennel well water on the reproductive anomalies.

Because the gestation period for rats is much shorter than for dogs, a six month study was conducted with Sprague-Dawley rats to try to assess the effects of fluoride and suspected water borne toxins. The rat study was similar in design to the Allegan dog study with the same three treatment groups and one control group. Each of the rat groups contained nine females and 18 males. The rats were housed in steel cages at Moribrook. There was no duplication of the reproductive problems in the rats that had persisted in the dogs. The teeth of the high fluoride rats were larger and whiter than the low fluoride rats and they had to be cut to allow the animals to eat.

Healy (1983) found no changes in the lengths and widths of mid-modilar cochleas of the Sprague-Dawley rats exposed to excessive fluoride and suspected water borne toxins.

A pilot study was conducted by Hannah (1986) with the temporal bones of six dogs (five of them exposed to high fluoride). Four of the high fluoride animals had mottled teeth and detached retinas. They were sacrificed and the temporal bone examined in an effort to determine the correlation, if any, that might exist between detached



retinas and vestibular abnormalities. One of the high fluoride animals was a Shetland neonate born with hydrocephalacy, cleft palate and abnormally short limbs and tail. The neonate was from a Shetland kennel that was not experiencing the reproductive anomalies nor was the kennel administering high fluoride dog food to the animals. The sixth animal was a high fluoride resident dog of the Moribrook Kennel that was reported to have an auditory loss and exostoses. With the exception in dog six, of pyknotic neurons of both geniculate and Scarpa's ganglia as well as a collapsed tectorial membrane, all animals presented a normal temporal bone histological picture.

#### The Present Study

The major aspect of the present thesis project is concerned with the histopathological analysis of temporal bones from eight dogs at the Moribrook Kennel and six outside control dogs. The eight dogs represent the three treatment groups and the one internal control (housed at Moribrook) group. Because some of the animals were reported to have auditory and vestibular problems, the temporal bone is ideal not only for assessment of the effect fluoride has on various hard tissues, but also for isolating a possible cause for the observed auditory and vestibular problems. In addition, the temporal bone was conveniently available for analysis after other tissues had been removed by other researchers (McIntire, personal communication).

The temporal bone is composed of both lamellar and spicular bone and in addition contains the most compact bone in the body—that of the otic capsule. The calcium carbonate stones, otoliths or otoconia,

of the sacculle and utricle are present nowhere else in the body. Diarthrodial (synovial) joints, muscle, epithelium and sensorineural tissues are also present. These aspects of the temporal bone justify examining it in an effort to try to ascertain the causes of the reported developmental, skeletal, sensorineural and reproductive problems at Moribrook Kennel.

## CHAPTER III

### MATERIALS AND METHODS

#### Animals and Treatment

Twenty purebred Sheltie dogs (16 females and 4 males) were brought to Allegan as study dogs. The animals were purchased in New York, Illinois, Indiana and Michigan. Criteria for selection of the dogs included age (the animals had to be between 2 and 8 years old), no history of reproductive problems, and no brucellosis. The males had to be proven studs and the females had to have whelped at least one healthy litter. Four groups were delineated and the dogs were randomly assigned (five animals per group). The three treatment groups chosen were: (1) group A—high fluoride and Moribrook well water, (2) group B—high fluoride and distilled water, and (3) group C—low fluoride and Moribrook well water. Group D—low fluoride and distilled water served as the control group for both variables. Each of the groups contained four females and one male.

As the study dogs were acquired they were boarded at Ravenwood Kennel in Kalamazoo for prestudy evaluations. These evaluations included body weight, serum chemistries, urinalysis, sperm evaluation, urine fluoride assay, hair analysis, blood count, brucellosis test and palpation for possible bony exostoses examination. Pittsburgh paint sample strips were utilized for analysis of tooth color. Prestudy treatment of the animals included deworming and shots to prevent

distemper, hepatitis, leptospirosis, parainfluenza and parvovirus. The dogs were in good condition prior to the study with two exceptions. Sprite and Charlie Boy were treated for heartworm with arsenic-containing caparsolate.

Prestudy plasma fluoride levels (ppm) ranged from 0.074 to 0.178 in the twenty dogs as compared with 0.260 to 1.060 at the end of one year in the high fluoride animals. The range in the low fluoride animals was 0.034 to 0.050. Plasma fluoride levels were examined quarterly as was urine, hair and blood. The animals were transferred to Moribrook and the study was commenced on March 26, 1982. The dogs were housed near the resident dogs but were not allowed access to the soil and plants of the exercise area.

Purina dog chow was obtained commercially and ground to a meal prior to being mixed with powdered rock phosphate which was added to increase the fluoride content. The resulting high fluoride dog food contained 460 ppm and the low fluoride food contained 10 ppm. The food was stored at Wil-O-Lane Kennel while the distilled water being used was stored in tanks at Moribrook Kennel.

The present thesis study utilizes eight of the twenty dogs of the original Allegan dog study and six non-Allegan control dogs. The temporal bones of all animals were histologically prepared through a celloidin method to minimize shrinkage and maximize the possibility for best results.

The eight Allegan study dogs selected for the histopathological analysis were from the following groups: two had been given high fluoride and Moribrook well water; three, high fluoride and distilled

water; one had been given low fluoride and well water; and two, low fluoride and distilled water. Background data on the Allegan animals is presented in Table 1.

Table 1  
Allegan Study Animals

Animal Name	Sex	Age at Death	Date of Death	Treatment
Sprite	F	6 years	7-12-84	high fluoride; well water
Eve	F	7 years	11-14-84	high fluoride; well water
Plain Jane	F	8 years	11-14-84	high fluoride; distilled water
Jellybean	F	9 years	1-6-84	high fluoride; distilled water
Bear	M	8 years	11-14-84	high fluoride; distilled water
Becky	F	7 years	6-5-85	low fluoride; well water
Sassy	F	6 years	5-25-83	low fluoride; distilled water
Tika	F	5 years	6-5-85	low fluoride; distilled water

The non-Allegan control dogs for this study included five beagles from the Upjohn Beagle colony and one German Shepherd also donated by the Upjohn Company. Because of the ease of availability and convenience in acquiring them, these two species of dogs were utilized as opposed to the Shetland species which were impossible to obtain at the

time. Background data on the non-Allegan control animals is presented in Table 2.

Table 2  
Non-Allegan Control Animals

Animal Name	Sex	Species	Age at Death	Date of Euthanasia
Academy	M	Beagle	11 mos.	4-30-85
Jennifer	F	Beagle	10 mos.	3-26-85
Lady	F	Beagle	10 mos.	3-19-85
Madame President	F	Beagle	10 mos.	3-26-85
O Henry	M	Beagle	10 mos.	3-12-85
Quincy	M	German Shepherd	5 yrs.	4-17-85

Although all of the non-Allegan control dogs were euthanized with chlorolose, various causes were responsible for the deaths of the eight Allegan animals. Table 3 includes these causes along with all recorded clinical and postmortem observations relating to the Allegan study dogs.

Table 3  
Clinical Observations and Method of Demise

Animal Name	Cause of Death	Observations During Study	Postmortem Observations
Sprite	Pyometra and infection	Numerous staggering episodes	Subcutaneous hemorrhage; exo-

Table 3--Continued

Animal Name	Cause of Death	Observations During Study	Postmortem Observations
			stoses in parietal bone
Eve	T-61 (anesthetic)	Good Health	Unremarkable
Plain Jane	T-61 (anesthetic)	Good Health	Unremarkable
Jellybean	Endotoxic shock	Alopecia; weight loss; dermatitis	Unreported
Bear	T-61 (anesthetic)	Low testosterone response levels; eosinophilic myositis	Lymphocytic infiltration into pancreas and kidney
Becky	T-61 (anesthetic)	Good Health	Multicystic ovaries
Sassy	Struck by truck	Good Health	Injuries related to accident
Tika	T-61 (anesthetic)	Good Health	Dark and spotted lungs; discolored thyroid gland

The bullae of the six non-Allegan control dogs, Tika and Becky were opened and 10% neutral buffered formalin was injected into the bullae within 20 to 30 minutes after death (Appendix A). One non-Allegan control dog, Jennifer was an exception. This animal's body was refrigerated for eight hours before the temporal bones were removed and placed in formalin.

The remaining six animals were acquired and placed into fixative by another researcher. The exact times between death and placement in

the formalin are uncertain and probably variable. However, all available information indicates that this time period did not exceed 16 hours.

Hair, connective tissue, and excess bone were removed from the temporal bones and the bullae were examined for serous, mucoid, hemorrhagic, and/or purulent middle ear exudate. With the exception of Eve and Jennifer, all middle ears were clear. Jennifer presented with a clear right bullae but hemorrhage was noted on the left. Tympanic membrane examination revealed that all were normal except for a massive perforation on the right side of Eve. There was also a considerable amount of granular exudate attached to the tympanic membrane of this animal.

#### Histological Preparation of the Tissues

##### Fixation and Decalcification

The bones of all but three of the animals were placed en bloc into 10% neutral buffered formalin. The bones of Eve, Bear and Plain Jane were sectioned along the midsagittal plane before being placed in formalin. The amount of time in formalin varied from two days to two weeks. Bones were then placed in Heidenhain-Susa fixative for two days. A second change of Heidenhain-Susa was made and the bones remained there overnight (Appendix A).

Following Heidenhain-Susa fixation, decalcification of the bones was accomplished using a 5% trichloroacetic acid solution. Decalcification time varied from two to four weeks depending upon the size of the temporal bone. The acid was changed every 48 hours and a chemical



endpoint test using 5% ammonium oxalate and 5% ammonium hydroxide was used to detect the presence of calcium in the solution. Two milliliters of the solution from the decalcifying temporal bone was mixed with one milliliter of ammonium oxalate and one milliliter of ammonium hydroxide. Formation of a white precipitate indicated the presence of calcium and therefore incomplete decalcification. If no precipitate formed the bones were then placed into a 5% solution of sodium sulfate for neutralization. The neutralization procedure lasted 24 hours and the bones were then rinsed in distilled water.

#### Dehydration

Dehydration of the bones was accomplished using increasing concentrations of ethyl alcohol followed by anhydrous ethyl ether (Appendix B). Iodine was used to remove most of the residual mercury remaining from the Heidenhain-Susa fixative.

#### Embedding

Following dehydration, the bones were embedded in increasing concentrations of celloidin, a highly purified form of cellulose nitrate (Appendix C). Celloidin serves as an embedding media that limits tissue shrinkage as the block hardens.

After three weeks in the most concentrated celloidin solution (15%), the temporal bone blocks were sufficiently hard to mount on wooden blocks. This was accomplished by using extra 15% celloidin as an adhesive. The tissue blocks were next placed in chloroform overnight for additional hardening to facilitate sectioning. The bones

were then stored in 80% ethanol until the time of sectioning.

### Sectioning

Each bone was sectioned at 20 micrometers using a Spencer sliding microtome. The blocks were placed in the microtome chuck and sectioned from the most superior aspect to the most inferior. Beginning with a section just superior to the cristae of the superior semicircular canal and ending with a section just inferior to the cristae of the posterior semicircular canal, every section was saved and every fifth section was stored separately for staining. During sectioning, eighty percent ethanol was continuously dripped onto the block to prevent drying. The sections were then stored in 80% ethanol permanently or until staining.

### Staining

Staining was done using a routine hematoxylin and eosin method for celloidin. Ten to twelve sections were placed into a wire cassette and submerged into the appropriate liquid for the appropriate time (Appendix D). The cassettes were gently agitated to assure thorough penetration of the liquids.

### Mounting

After submergence in the one-to-one solution of xylene and terpineol at the end of the staining procedure the tissue sections were coverslipped. The sections were oriented with the anterior aspect of the cochlea apex pointing toward the top of the slide. A synthetic Permount resin by Fischer was used for coverslipping. Sizes of cover-

slips varied with the size of the tissue section but were either 22 x 40 mm or 22 x 50 mm. The coverslipped sections were placed under weights for two weeks to help keep the tissues flat and to reduce the occurrence of air bubbles.

#### Otopathological Analysis

The slides were randomized by an uninvolved observer, who was unfamiliar with the research design in terms of animal groupings. The double blind randomization was necessary because the animals were acquired at different times, and throughout the processing of the bones, the researcher was aware of the identity of each specimen. The uninvolved observer assigned random coded identifiers to the temporal bone slide sets and the code was not disclosed until the end of the histopathological analysis. The code identifier consisted of a letter followed by a number. Animals were assigned two separate code identifiers—one for the right side and one for the left side—thus a total of 28 coded identifiers were used.

The temporal bone morphology was analyzed by the investigator and the research adviser using the classical histopathological paradigm. Slides were analyzed with a double headed One-Ten Phase Star white light American Optical microscope. The structures assessed and the reasons for the assessment are presented in Table 4.

Table 4

## Temporal Bone Histopathological Assessment Procedure

Structure	Reason for Assessment
Bone marrow	To compare the ratio of red marrow to yellow marrow
Muscles tensor tympani stapedius	For cell population density and morphology
Superior Semicircular Canal endolymphatic area  perilymphatic area hair cells and support cells	For the presence of exudate and hydrops For the presence of exudate For cell population density, morphology and organizational integrity
Cranial nerves CN VII (facial) CN VIII (vestibulocochlear)	For cell population density and cell and fiber morphology
Ossicles malleus incus stapes	For fraying and/or calcification in the joints, integrity of the chain, morphology of the bones, and condition of the mucoperiosteum
Middle Ear Cavity	For exudate, hemorrhagic products, and inflammatory development
Middle Ear Lining	For thickening or alteration of the mucus membrane
Lateral Semicircular Canal endolymphatic area  perilymphatic area hair cells and support cells	For the presence of exudate and hydrops For the presence of exudate For cell population density, morphology, and organizational integrity
Utricle endolymphatic area  perilymphatic area	For the presence of exudate and hydrops For the presence of exudate

Table 4--Continued

Structure	Reason for Assessment
hair and support cells	For cell population density, morphology, and organizational integrity
otolithic membrane	For morphology and organizational integrity
otoconia	For morphology
<b>Sacculle</b>	
endolymphatic area	For the presence of exudate and hydrops
perilymphatic area	For the presence of exudate
hair and support cells	For cell population density, morphology, and organizational integrity
otolithic membrane	For morphology and organizational integrity
otoconia	For morphology
<b>Cochlea</b>	
scalas	For the presence of exudate, hemorrhagic products, and inflammatory development
organ of Corti	
hair and support cells	For morphology, population density and organizational integrity
tectorial membrane	For morphology
vestibular membrane	For thickening and hydrops
stria vascularis	For morphology and thickness
spiral ligament	For morphology
<b>Posterior Semicircular Canal</b>	
endolymphatic area	For the presence of exudate and hydrops
perilymphatic area	For the presence of exudate
hair and support cells	For cell population density, morphology and organizational integrity
<b>Round Window Membrane</b>	For thickness and structural alterations
<b>Round Window Niche</b>	For exudate, hemorrhagic products, and alterations in the mucus membrane

Table 4—Continued

Structure	Reason for Assessment
Tympanic Membrane	For thickness and structural alterations

#### Grouping the Histopathological Observations

Histopathological assessment consisted of both investigators (principal investigator and adviser) jointly discussing and assessing each structure in Table 4. The results of the ongoing discussion were tape recorded and later transcribed onto data sheets for each animal.

The data from the sheets were compiled into chart form to facilitate comparison and synthesis of the categories. The data chart includes the structures assessed in Table 4 as column headings. Each structure was assigned a value or a descriptive term representing the degree or kind of pathology observed. The data chart and otopathological assessments for four of the temporal bone specimens is presented in Appendix E.

The investigators next assessed the chart data and converted that material into temporal bone summaries which are presented in Appendix F. The overall otopathology for each temporal bone was determined by both investigators separately and classified as to whether there was no profound otopathology, profound middle ear pathology only, profound inner ear pathology only, or profound pathology in both middle and inner ears. There were no substantial disagreements between the evaluations given by the two investigators. Minor disagreements were discussed and a consensus was always reached. Sometimes a review of

the histopathology was helpful in clarifying differences of observation.

After determining the magnitude of otopathology for each temporal bone, the code was broken and quantitative assessments for each animal were placed within the appropriate group. The four Allegan study dog groups were: (1) high fluoride and well water, (2) high fluoride and distilled water, (3) low fluoride and well water, and (4) low fluoride and distilled water. There was also a fifth group consisting of the six non-Allegan control dogs. The quantitative assessment consisted of the following ratings: (a) zero, for no profound middle or inner ear pathology bilaterally; (b) one, for profound unilateral middle or inner ear pathology; (c) two, for profound pathology in two of the possible four locations; (d) three, for profound pathology in three of the possible four locations; and (e) four, for profound pathology in all four possible locations.

## CHAPTER IV

### RESULTS

#### Reproduction During the Study Period

During the two year study period, from 1982 to 1984, group C was the most prolific of the four groups with one animal producing three litters and a total of 20 pups. Concurrently, the resident Shelties (non-study dogs) above five years of age produced fewer offspring than did their younger counterparts. The opposite held true for the study animals.

From the two high fluoride groups no serious congenital pathologies were noted in the offspring, with two exceptions. A deep lumbar spinal cleft and inability to use the hind legs was noted in one pup and another pup presented with an esophageal malformation. Other anomalies present in all four groups included polydactyly, positional abnormalities of the limbs such as twisted hind and fore feet, and kinked tails.

Prior to coming to Moribrook all females had produced at least one normal litter. The resident dogs continued to produce offspring with palatal defects, kinked tails and pups that screamed and convulsed immediately before death. Two of the high fluoride animals, Sprite and Bear developed palpable bony exostoses after the first year. Exostoses were frequently found on the occipital crest and limbs. Figures 1, 2 and 3 (chapter I) depict this exostoses in two non-study



animals. There was no notable exostoses in the low fluoride animals.

### Histopathology of the Temporal Bones

From the detailed histopathological assessments presented in the data chart (Appendix E) and summaries (Appendix F), four categories were used to assess the overall magnitude of otopathology for each temporal bone. The four categories were: (1) no profound otopathology, (2) profound middle ear pathology only, (3) profound inner ear pathology only, and (4) profound pathology in both middle and inner ears. The results for each temporal bone are presented in Table 5.

Table 5  
Temporal Bone Pathologies

No Profound Pathology	Middle Ear Pathology Only	Inner Ear Pathology Only	Both Middle and Inner Ear Pathology
M2 (r. Lady)	D9 (1. Madame President)	C7 (1. Bear)	T13 (r. O Henry)
L7 (r. Sprite)	E1 (1. Academy)	S3 (1. Becky)	Z5 (r. Tika)
F3 (1. Lady)	K6 (1. O Henry)	R2 (1. Jelly-bean)	Y3 (r. Bear)
F5 (r. Madame President)			Z17 (1. Jennifer)
W4 (1. Eve)			A7 (r. Plain Jane)
P6 (r. Becky)			P5 (r. Jelly-bean)
O7 (r. Academy)			Q8 (r. Quincy)
G2 (1. Sprite)			H9 (1. Plain Jane)

Table 5—Continued

No Profound Pathology	Middle Ear Pathology Only	Inner Ear Pathology Only	Both Middle and Inner Ear Pathology
R3 (r. Eve)			B4 (l. Tika)
L4 (l. Quincy)			C1 (r. Sassy)
			X11 (r. Jennifer)
			R5 (l. Sassy)

Otopathology scores were assigned to each dog using the following ratings: (a) zero, for no profound middle or inner ear pathology bilaterally; (b) one, for profound unilateral middle or inner ear pathology; (c) two, for profound pathology in two of the possible four locations; (d) three, for profound pathology in three of the possible four locations; and (e) four, for profound pathology in all four possible locations. Table 6 shows these scores for the five groups of dogs.

Table 6  
Otopathological Scores

Non-Allegan Controls		High Fluoride Distilled Water	High Fluoride Well Water	Low Fluoride Well Water	Low Fluoride Distilled Water
Lady	0	P. J. 4	Sprite 0	Becky 1	Tika 4
Quincy	2	Bear 3	Eve 0		Sassy 4
Academy	1	J. B. 3			
O Henry	3				

Table 6--Continued  
Otopathological Scores

Non-Allegan Controls	High Fluoride Distilled Water	High Fluoride Well Water	Low Fluoride Well Water	Low Fluoride Distilled Water
Jenny 4				
M. Pres. 1				

The only two groups with alpha values at .1 using the Kruskal-Wallis one-way analysis of variance were the high fluoride and well water group and the low fluoride and distilled water group.

## CHAPTER V

### DISCUSSION

#### Histopathological Observations

##### Hemorrhage Into the Middle and Inner Ear

A number of animals presented with blood in the middle ear. This was most probably due to hemorrhage at the time of sacrifice (McIntire, personal communication).

One of the low fluoride and distilled water animals had extensive hemorrhage into the scala tympani. This was believed to be a result of the impact of the truck accident in which she was killed. This is compatible with Schuknecht's (1974) observation that in five of nine cats that experienced severe head blows, there was hemorrhage into the perilymphatic spaces of the inner ear. Blood was reported in the cochlear duct in one of these animals. The remaining three had no blood in the cochlea. Schuknecht (1974) also reported that within three weeks of the head blows, the leukocytes present in the hemorrhagic area disappeared.

Another animal, a high fluoride and distilled water animal, presented with moderate to heavy hemorrhage into the scala tympani. It is unsure as to why this was present.

##### Fused and Displaced Otoconia

Mammalian otoconia are composed of calcium carbonate crystals and

an organic matrix, which is believed to be sulfated glycosaminoglycans and glycoproteins (Lim, 1983). Developing otoconia tend to be spindle, dumbbell, trigonal or quadrilobed shaped. As otoconia mature, they take on a barrel shape with pointed tips (Lim, 1983).

Certain agents including ototoxic drugs, infection, trauma and aging have been reported to have degenerative effects on otoconia. Ototoxic drugs such as ethacrynic acid, streptomycin and neomycin are reported to cause abnormal otoconia (Harada & Sugimoto, 1977). It has been proposed that abnormal giant otoconia were formed in some mammals by the fusion of smaller crystals as a result of ototoxicity (Johnson, Rouse, Wright, Henry & Hawkins, 1982).

Profound otolithic fusion was noted bilaterally in both the utricle and saccule of one of the low fluoride and distilled water animals and unilaterally in the utricle of another animal from this same group. Substantial otolithic fusion was also present bilaterally in both the utricle and saccule in the animal which had been on the low fluoride and well water regimen. A slight hint of otolithic fusion was also suggested in one of the animals from the high fluoride and distilled water group. The above pattern seems to suggest a predilection of the low fluoride animals to a degree of otolithic fusion, whether it be profound or minimal.

Figure 3 depicts normal saccular otoliths from a non-Allegan control animal and Figure 4 shows saccular otolithic fusion from a low fluoride and distilled water animal.



Figure 3. Normal Saccular Otoliths From a Non-Allegan Control Dog.  
(magnification 150x)



Figure 4. Fused Saccular Otoliths From a Low Fluoride and Distilled  
Water Dog (magnification 375x)

One of the low fluoride and distilled water animals presented unilaterally with small basophilic deposits lying adjacent to the base of the cristae in the posterior semicircular canal. Schuknecht (1974) reported that in cupulolithiasis, histological studies have revealed basophilic deposits on the cupula of the posterior canal. These deposits show no evidence of a fibrillar structure and bear no relationship to the state of preservation of the temporal bone, the time interval between death and fixation nor the condition of the otolithic membrane. Schuknecht (1974) also found the deposits to be present as often in normal bones as in pathological ones. Although it is not suggested that cupulolithiasis was present in any one of the 28 temporal bones of the present study, the above observation by Schuknecht (1974) is included as the only reference that addresses similar observations.

#### Thickening of the Middle Ear Lining, Tympanic Membrane and the Round Window Membrane

The middle ear is subject to a variety of diseases including secondary involvement from generalized systemic infection and conditions of the external ear canal and auditory tube (Paparella & Shumrick, 1980). Because the middle ear constitutes an extension of the upper respiratory tract, it is highly susceptible to bacterial invasion via the auditory tube (Schuknecht, 1974). Mild infections of the middle ear are characterized by thickening of the mucus membrane from edema and invasion with acute inflammatory cells.

Tympanic membrane thickening was present unilaterally in both of the low fluoride and distilled water animals and unilaterally in one

of the high fluoride and distilled water animals. Three of the non-Allegan control animals presented with tympanic membrane thickening. None of the Allegan study animals on the well water regimen showed any tympanic membrane thickening.

Thickening of the middle ear lining (mucus membrane) was found in 58% of the non-Allegan control animals and in 56% of the Allegan animals. Of the Allegan animals with mucus membrane thickening, three of them were from the high fluoride and distilled water group, three were from the low fluoride and distilled water group and one was from the high fluoride and well water group.

Round window membrane thickening was present in 5 of the 12 non-Allegan control specimens. The only Allegan study group with round window membrane thickening was the high fluoride and distilled water group. All three animals of this group presented unilaterally with round window membrane thickening.

The overall data for middle ear involvement shows that the animals on the Moribrook well water had a much lower incidence of thickening of the membranes of the middle ear.

#### Exudate in the Inner Ear

Serofibrinous exudate with pink staining strands of precipitate and similar to that which has been described by Paparella in the early stages of labyrinthitis was seen in the inner ears of a number of specimens from the present study. Paparella and Capps (1973) describe the earliest stage of labyrinthitis as a time when there is no detectable disruption of sensory epithelium and end organ function is preserved. This condition is believed to be reversible and is a condi-



tion that was seen to some extent in the majority of the animals in the present study.

More rarely the animals of the present study displayed a limited fibroblastic inner ear condition which is compatible with the earliest stages of Paparella and Capp's (1973) fibrous labyrinthitis. The fibrous stage of labyrinthitis is characterized not only by fibroblastic proliferation in the inner ear, but also by the formation of granulation tissue. This last condition apparently separates the present study animals from classical labyrinthitis animals in that no granulation tissue was seen in any of the inner ears of the present study. While otopathology of the present study bears some resemblance to classical labyrinthitis, the resemblance is limited, and probably the etiology and prognosis are not the same.

Extensive exudate was noted throughout the cochlea and/or labyrinth in 63% of the Allegan animals. All of the remaining Allegan animals, except two presented with slight exudate in the inner ear. The two exceptions were both from the high fluoride and distilled water group, and these animals presented unilaterally with absolutely no exudate in the cochlea. One of these animals had scant exudate in the labyrinth. Moderate exudate was present in the inner ears of 33% of the non-Allegan control dogs.

The amount of inner ear exudate did not vary greatly between the four Allegan study groups but the low fluoride and distilled water group did predominate in the amount of exudate present. In both of the low fluoride and distilled water animals the labyrinth including the cochlea, presented with substantial serofibrinous exudate.

### Ossicular Pathology

Forty-three percent of the Allegan animals presented with some type of ossicular pathology. There was some degree of ossicular pathology in both of the low fluoride and distilled water animals. One of these animals presented bilaterally with fraying and ossification of the incudomalleal joint as well the incus being attached to the lateral wall. The other animal of this group presented bilaterally with calcification and fraying within the incudomalleal joint.

The low fluoride and well water animal presented with fraying and calcification of the incudomalleal joint, while one of the high fluoride and distilled water animals showed increased adhesion between the lateral wall and the ossicles along with thickening of the periosteum of the incus. Only one of the non-Allegan control animals presented with any ossicular pathology, and that was unilateral attachment of the incus to the lateral wall.

The animals on the low fluoride diets seemed to have a predilection towards ossicular pathology. These same animals that presented with the ossicular pathology were the ones that had otolithic fusion. There could be a possible link between the causative agent that interacted with the inorganic matrices of both the otoconia and the ossicles.

### Other Observed Pathologies

In one of the non-Allegan control dogs, a phenomenon was present which was not found in any of the other animals. Bilaterally, this animal presented with slate blue spherical structures within the geniculate ganglia. These structures, outlined by glial cells were

approximately the size of two ganglia. The composition of the deposits and the reason for their presence is unknown.

#### Comment

Based on the clinical observations of fluorosis (tooth mottling and exostoses) observed at the dog kennels and the possibility of infectious agents being transmitted to the animals via the well water, it was postulated that the high fluoride and well water animals would present with numerous anomalies and the low fluoride and distilled water animals would be completely healthy. The reverse was found to be true. In neither of the two high fluoride and well water animals was there any profound middle nor inner ear pathology. However both the Allegan control animals (low fluoride and distilled water) presented profound bilateral middle and inner ear pathology.

It was postulated in 1984, that although fluoride was responsible for the observed bone and tooth anomalies, there was no correlation between fluoride and the reproduction anomalies observed at the kennels. The present thesis study was undertaken to assess the possibility that fluoride might be related to the auditory and vestibular problems empirically observed by the Moribrook Kennel owner. One interpretation that is suggested by data from the present study is that the high fluoride and well water could have exerted a protective function against the agent which was causing, at minimum, the auditory and vestibular problems. The two low fluoride and distilled water animals were unprotected against the causative agent(s), thus they tended to present with more pathologies. It must also be considered that the truck accident killing one of the low fluoride and distilled

water animals may have produced some of the observed otopathology. In the high fluoride and distilled water animals it can be postulated that these animals had the protection of one variable, high fluoride, which could account for them not presenting with as much pathology as the low fluoride and distilled water animals. A similar postulation may be made for the low fluoride and well water animal. Is it possible that the well water alone afforded some protection against the otopathology and that since this animal was not on high fluoride she did not receive the maximum protection? The non-Allegan control animals presented with a degree of otopathology which is accepted as baseline average for non-Allegan dogs.

## CHAPTER VI

### CONCLUSION

The results of the present thesis can be easily misconstrued if there are attempts to extrapolate beyond the context of the study. Specifically, this research project describes correlations between treatment groups and otopathology. The question of whether there are meaningful correlations between the observed otopathology and more generalized or systemic pathology, has not been addressed. Also the question of whether there are meaningful correlations between the observed otopathology and the reported reproductive problems at Moribrook has not been addressed.

However it can be said with some degree of confidence that the types of observed otopathology were generally not auditory and/or vestibular specific. There was not a single instance where the specific sensory epithelium of these systems was damaged; rather the nature of the observed otopathology closely resembled moderate to profound acute inflammatory reactions, chronic inflammatory reactions or one of a variety of repair stages. These changes may or may not be related to the reproductive aberrations.

The problems at the Moribrook Dog Kennel are both baffling and ongoing with no solutions in sight. The evidence does seem to point to some environmental agent since the dogs have a much greater reproductive success rate away from the kennel.

Although it had already been discovered that the high fluoride dog

food and Moribrook well water were probably not responsible for the reproductive anomalies, the present thesis project was designed to mainly address the reported auditory and vestibular problems in the study dogs.

Presented here are the pathological and normal findings from the temporal bones of 14 animals that represent the three treatment groups, one Allegan control group, and one non-Allegan control group. The findings support a cautious suggestion that high fluoride in combination with Moribrook well water may serve to minimize the auditory and vestibular pathological effects which could be the result of the same toxic agent(s) that is/are causing the reproductive anomalies.

## **APPENDICES**

## **Appendix A**

### **Fixatives**



## Appendix A

## Fixatives

10% Neutral Buffered Formalin

37-40% formalin	10 ml
Distilled water	90 ml
Sodium phosphate, monobasic	.4 g
Sodium phosphate, dibasic	.65 g

Heidenhain-SusaStock Solution

Mercuric Chloride	2.25 g
Sodium Chloride	0.25g
Distilled Water	40 ml
Trichloroacetic acid	1 g
Glacial acetic acid	2 ml

The working solution is prepared at the time of use by mixing four parts of stock solution with one part of full strength formaldehyde solution.

## **Appendix B**

### **Serial Dehydration**

## Appendix B

## Serial Dehydration

70% ethanol with iodine*	24 hours
80% ethanol with iodine	24 hours
95% ethanol with iodine	24 hours
95% ethanol with iodine	24 hours
absolute ethanol	24 hours
absolute ethanol	24 hours
absolute ethanol and ethyl ether (1:1)	24 hours
absolute ethanol and ethyl ether (1:1)	24 hours

The tissues were then embedded in celloidin.

\*Iodine was used for the purpose of removing residual mercury remaining from fixation. Mercury deposits in the tissue appear as histological artifact and tend to damage the knife during sectioning.

## **Appendix C**

### **Serial Embedding**

## Appendix C

## Serial Embedding

5% celloidin	1 week
5g cellulose nitrate	
100 ml of a 1:1 mixture of absolute ethanol and ether	
10% celloidin	2 weeks
10g cellulose nitrate	
100 ml of a 1:1 mixture of absolute ethanol and ether	
15% celloidin	3 weeks
15g cellulose nitrate	
100 ml of a 1:1 mixture of absolute ethanol and ether	

## **Appendix D**

### **Hematoxylin and Eosin Y Staining Procedure**

## Appendix D

## Hematoxylin and Eosin Y Staining Procedure

Tap Water	Rinse
Tap Water	Rinse
Lugol's iodine	10 minutes
5% Sodium thiosulfate	5 minutes
Tap Water	Rinse
Tap Water	Rinse
Harris Hematoxylin	13 minutes
Tap Water	Rinse
Tap Water	Rinse
70% Acid Alcohol	10 seconds
Tap Water (running)	30 seconds
5% ammonia water	2 minutes
5% ammonia water	2 minutes
5% ammonia water	2 minutes
Tap Water	Rinse
Tap Water	Rinse
Eosin Y	25 seconds
80% ethanol	1 minute
80% ethanol	1 minute
80% ethanol	1 minute
95% ethanol	30 seconds
Chloroform and Absolute Ethanol (1:1)	1 minute

The tissues were then transferred to a petri dish containing a 1:1 solution of xylene and terpineol and coverslipped an average of one week later.

## **Appendix E**

### **Otopathological Assessment of Four Temporal Bone Specimens**



## Appendix E

## Otopathological Assessment of the Middle Ears of Four Specimens

<u>Structure</u>	<u>Z5</u>	<u>L7</u>	<u>Y3</u>	<u>P6</u>
Bone marrow ratio red:yellow	15:85	50:50	1:99	50:50
Ossicles				
Malleus	N	N	N	N
Incus	N	N	N	N
Incudomalleal Joint	Fr & O	N	N	Fr & C
Stapes	N	N	E-1	H-1
Incudostapedial Joint	N	N	N	Fr
Tympanic Membrane	N	N	N	N
Round Window Membrane	N	N	ST	N
Middle Ear Cavity	N	N	H-1 & E-1	H-1
Middle Ear Lining	N	N	MT	N
Round Window Niche	N	N	N	N
Perilymphatic Space of the Round Window Area	F-4	N	N	E-2

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Z5 is from the low fluoride and distilled water group.

L7 is from the high fluoride and well water group.

Y3 is from the high fluoride and distilled water group.

P6 is from the low fluoride and well water group.

C=Calcification  
E=Exudate  
F=Fibroblasts  
H=Hemorrhage

MT=Moderately Thickened  
N=Normal  
O=Ossification  
ST=Slightly thickened  
T=Thickened

Degree of Pathology  
0=no pathology  
1=less than 25%  
2= 25 to 50%  
3= 50 to 75%  
4= greater than 75%

## Appendix E

## Otopathological Assessment of the Inner Ears of Four Specimens

<u>Structure</u>	<u>Z5</u>	<u>L7*</u>	<u>Y3</u>	<u>P6</u>
Superior Canal				
hair cells	Missed	D	N	N
support cells	due to	N	N	N
exudate (endo- lymphatic area)	angle of cut	2	0	2
exudate (peri- lymphatic area)		0	0	0
Lateral Canal				
hair cells	N	N	N	N
support cells	N	N	N	N
exudate (endo- lymphatic area)	2-G	1	1	0
exudate (peri- lymphatic area)	2-F	0	0	0
Utricle				
hair cells	N	D	D	N
support cells	N	D	D	N
exudate (endo- lymphatic area)	1	2	3	1
exudate (peri- lymphatic area)	2	0	2	2
otoconia	Fu	N	N	N
otolithic membrane	D	D	N	N

D = Disarrayed  
 F = Fibrous  
 Fu = Fused  
 G = Granular  
 N = Normal  
 \* = Postmortem Artifact

Degree of Exudate Present  
 0 = no exudate  
 1 = Less than 25%  
 2 = 25 to 50%  
 3 = 50 to 75%  
 4 = greater than 75%

Structure	<u>Z5</u>	<u>L7*</u>	<u>Y3</u>	<u>P6</u>
Sacculle				
hair cells	D	D	N	N
support cells	N	D	N	N
exudate (endo-lymphatic area)	4	3	2	1
exudate (peri-lymphatic area)	2	4	0	2
otoconia	Fu	N	N	N
otolithic membrane	N	D	N	N
Cochlea				
exudate (endo-lymphatic area)	d/2	a/1	b/3	c/4
exudate (peri-lymphatic area)	c/2	a/1	b/2	c/2
blood	0	0	2	0
vestibular membrane	T	N	N	T & Di
tectorial membrane	N	S	S	N
organ of Corti	N	D	D	N
Posterior Canal				
hair cells	N	N	N	N
support cells	N	N	N	N
exudate (endo-lymphatic area)	3	2	3	0
exudate (peri-lymphatic area)	0	1	0	0

## Amount of Scala Affected

a = less than 25%  
b = 25 to 50%  
c = 50 to 75%  
d = over 75%

D = Disarrayed

Di = Disrupted

Fu = Fused

\* = postmortem degeneration

N = Normal

S = Swollen

T = Thickened

## Degree of Exudate and/or Blood

0 = none  
1 = less than 25%  
2 = 25 to 50%  
3 = 50 to 75%  
4 = over 75%

## **Appendix F**

### **Temporal Bone Summaries**

## Appendix F

## Temporal Bone Summaries

Temporal Bones With no Profound Otopathology

## M2 (r. Lady)

There was a slight serofibrinous exudate in all scalas of the cochlea. Slate blue spherical bodies were found within the geniculate ganglia.

## L7 (r. Sprite)

An excessive amount of postmortem artifact was present in this specimen. A moderate serofibrinous exudate was present in the endolymphatic areas of the cochlea, utricle and saccule.

## F3 (l. Lady)

There was slight serofibrinous exudate noted in the all scala of the cochlea. Slate blue spherical bodies were found within the geniculate ganglia.

## F5 (r. Madame President)

Fibroblasts were present along the footplate of the stapes along with a serofibrinous exudate in the middle ear. There was moderate thickening of the middle ear lining. Air bubble damage made most aspects of the inner ear impossible to analyze.

## W4 (l. Eve)

Scarpa's ganglia were pyknotic in this specimen. There was a high level of exudate in both the endolymphatic and perilymphatic areas of the cochlea and a low amount of exudate in the vestibular system.

## P6 (r. Becky)

There was fraying of the articular cartilage in both the incudo-

malleal and incudostapedial joints. Moderate serofibrinous exudate was present in all parts of the cochlea. The vestibular membrane was thickened in some turns and absent in others.

O7 (r. Academy)

There was a slight thickening of the tympanic membrane. Moderate exudate was present in both the endolymphatic and perilymphatic areas of the cochlea. There was a slight accumulation of localized fibroblasts in the perilymphatic area of the round window area.

G2 (l. Sprite)

Postmortem degeneration was noted in this specimen. A slight thickening of the middle ear lining was present along with a slight exudate adjacent to the stapes. Moderate exudate was present in the endolymphatic area of the cochlea and labyrinth and a moderate to heavy exudate in the perilymphatic area of the cochlea. A slight exudate was present in the perilymphatic area of the labyrinth. There was minimal fibroblastic buildup in the perilymphatic area of the round window area.

R3 (r. Eve)

The middle ear lining was slightly thickened with slight exudate in the middle ear cavity. There was increased basophilia on the head of the malleus. A slight exudate occupied a small portion of the endolymphatic area of the cochlea and a greater portion of the endolymphatic areas of the utricle and saccule. The perilymphatic area of the cochlea had some exudate. Fibroblasts were found in the perilymphatic duct of the round window area.

L4 (l. Quincy)

Both the endolymphatic and the perilymphatic areas of the cochlea

contained moderate exudate as did these areas of the saccule. There was slight exudate in the perilymphatic area of the round window area.

#### Temporal Bones With Profound Middle Ear Pathology

D9 (1. Madame President)

The middle ear lining of this specimen was severely thickened. There was slight exudate throughout the inner ear. The perilymphatic area of the round window area contained moderate exudate and slight fibroblastic invasion.

E1 (1. Academy)

There was ankylosis between the incus and the middle ear wall. The middle ear lining was slightly thickened. There was a slight exudate in the perilymphatic areas of the cochlea.

K6 (1. O Henry)

The round window membrane was slightly thickened as was the middle ear lining. There was slight fibroblastic invasion into the area of the stapes and the perilymphatic space of the round window area. Slight hemorrhage and exudate was present in the round window niche.

#### Temporal Bones With Profound Inner Ear Pathology

C7 (1. Bear)

There was moderate exudate in all scala of the cochlea. Blood occupying 35% of the scala tympani was noted.

S3 (1. Becky)

There was moderate serofibrinous exudate in both the endolymphatic and perilymphatic areas of the inner ear with a high level present in

the saccule. There was profound otolithic fusion in both the utricle and saccule.

R2 (l. Jellybean)

Moderate exudate was present in the lateral aspect of the middle ear cavity along with moderate exudate around the stapes. The vestibular membrane was four to five times thicker than normal and had a heavy exudate resting upon it.

Temporal Bones With Profound Middle and Inner Ear Pathology

T13 (r. O Henry)

The lining of both the tympanic and round window membranes were thickened in this specimen. There was slight hemorrhage and moderate fibroblastic invasion in the perilymphatic area of the round window area.

Z5 (r. Tika)

There was an abnormally high red to yellow bone marrow ratio (15:85). The incus was displaced and there was fraying and ossification within the incudomalleal joint. The cartilage adjacent to the annular ligament was frayed. There was extensive serofibrinous exudate throughout the entire cochlea and saccule. Serofibrinous exudate was moderate in the lateral canal and high in the posterior canal. There was profound otolithic fusion in both the utricle and saccule.

Y3 (r. Bear)

There was local hemorrhage and exudate in the middle ear cavity. The middle ear lining and the round window membrane were thickened. There was a moderate to high exudate throughout the endolymphatic area



of the inner ear and a slight amount of exudate in the perilymphatic area of the inner ear. The basal turn of the last one-half of the scala tympani contained hemorrhagic products.

Z17 (l. Jennifer)

Extensive hemorrhagic products and fibroblasts were present in the middle ear. Both the middle ear lining and the round window membrane were thickened. There was moderate fibroblastic invasion into the perilymphatic duct area. Both the endolymphatic and perilymphatic areas of the inner ear contained exudate ranging from moderate to heavy.

A7 (r. Plain Jane)

Extensive middle ear involvement was present and thickening of the tympanic membrane, round window membrane, and middle ear lining. There was substantial exudate accumulation in the area around the stapes and in the middle ear cavity. There was variable and inconsistent hydrops in both the medial and lateral turns of the cochlea. The vestibular membrane was thickened.

P5 (r. Jellybean)

Both fluid and hemorrhagic products were present in the middle ear cavity. There was increased adhesion between the malleus and incus along with thickening of the periosteum of the incus. Both the round window membrane and the middle ear lining were thickened. Fibroblasts were present in the perilymphatic space of the round window area and thought to be continuous with the round window membrane. There was a slight hint of otolithic fusion in the saccule. There was moderate exudate in the perilymphatic area of the cochlea and saccule and slight exudate in the endolymphatic area of the vestibular system.

## Q8 (r. Quincy)

There was extensive hypocellularity in the temporal bone marrow. The red to yellow bone marrow ratio was 1:99. Hemorrhagic products resembling cholesterol needles were present in the middle ear cavity and round window niche. There was thickening of the round window membrane. There was excessive space between adjacent endoneurial structures of the spiral ganglia. A slight exudate was present in the endolymphatic area of the cochlea and utricle. The endolymphatic area of the saccule had substantial exudate. There was moderate exudate in the perilymphatic area of the cochlea and slight exudate in the perilymphatic area of the utricle.

## H9 (l. Plain Jane)

There was moderate serofibrinous exudate in the middle ear along with mild fibroblastic buildup. The middle ear lining was slightly thickened with exudate attached to it. Both the endolymphatic and perilymphatic areas of the cochlea and vestibular system had moderate exudate. Extensive fibroblastic buildup continuous with the round window membrane was present in the perilymphatic area.

## B4 (l. Tika)

There was fraying of and possible calcification within the incudomalleal joint. The middle ear lining and the tympanic membrane were slightly thickened. There was moderate fibroblastic buildup in the perilymphatic space of the round window area and slight fibroblastic buildup throughout the scala tympani. Moderate serofibrinous exudate was present in the endolymphatic area of the cochlea as well as the utricle and saccule. The perilymphatic area of the cochlea had sub-

stantial exudate throughout. The vestibular membrane was thickened and had exudate accumulated upon it.

C1 (r. Sassy)

The bone marrow ratio of red to yellow was 20:80. The middle ear lining was sporadically thickened on the lateral side and there was slight calcification and fraying of the incudomalleal joint. Significant hemorrhagic products were present in the middle ear cavity as well as between the crura and the round window niche. Exudate was substantial in all scala of the cochlea and slight in the endolymphatic area of the vestibular area. The vestibular membrane was thickened. There was significant fibroblastic invasion in the round window perilymphatic area that extended medially beyond midmodiolar.

X11 (r. Jennifer)

The middle ear lining, tympanic membrane, the footplate and the annular ligament were thickened. The endolymphatic area of the cochlea and saccule had slight exudate while the perilymphatic area of the cochlea and saccule had substantial exudate. There was exudate and moderate fibroblastic invasion into the perilymphatic area adjacent to the round window membrane.

R5 (l. Sassy)

The bone marrow ratio of red to yellow was 1:99. There was a slight hint of calcification on the lateral side of the incudomalleal joint. Both the tympanic membrane and the middle ear lining were thickened. Hemorrhagic products were very substantial in the middle ear cavity around the footplate and in the round window niche. The round window niche also had excessive fibroblastic invasion and exudate. The scala tympani was 60% filled with blood and contained

leukocytes symmetrically arranged. The vestibular membrane was thickened. The endolymphatic area of the cochlea and the labyrinth contained moderate exudate and the perilymphatic areas of the cochlea and labyrinth had heavy exudate. There was possible otolithic fusion in the utricle.

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