The effects of sodium fluoride on the reproductive performance of the male American kestrel (*Falco sparverius*) and the Japanese quail (*Coturnix japonica*)

Laird Shutt

Faculty of Agriculture and Environmental Sciences

Macdonald Campus of McGill University, Montréal

January, 1996

A thesis submitted to the Faculty of Graduate Studies and Research

in partial fulfillment for the degree Doctor of Philosophy

Suggested short title:

Effects of sodium fluoride on reproduction in male kestrels and quail

I would like to dedicate this thesis to my family, including Janet and Stuart, for their encouragement and for teaching me the value of education and also to Jennifer who was an inspiration to me.

Thesis Abstract

In order to evaluate the effects of exposure to fluoride on avian reproduction, male American kestrels (*Falco sparverius*) and Japanese quail (*Coturnix japonica*) were chronically treated with sodium fluoride and paired with untreated females. Forty male American kestrels were randomly assigned to one of the following treatments: 1.65, 3.3, or 6.6 mg NaF or a control solution of 13.2 mg $NaHCO_3$ administered by oral gavage daily for 45 days. In the first year of the study all males were paired with untreated females upon completion of dosing. In the second year dosing of the males continued for 15 days after pairing with undosed females. During the dosing and subsequent pairing periods all males had 1 ml of blood taken weekly for determination of circulating testosterone. All birds were weighed periodically throughout the study. Data were collected on reproductive behaviours as well as the number of fertile eggs produced. Humeri and testes were removed at necropsy to assess fluoride uptake and effects at the tissue level. While the gavage method resulted in bone fluoride burdens increasing in a dose-dependent manner there was no significant effect on the number of fertile eggs produced in either year. Furthermore, no differences were detected in reproductive behaviours or in circulating testosterone levels. Gonadosomatic indices for fluoride-exposed and control kestrels were not significantly different. In order to evaluate the effects of fluoride exposure on semen quality 40 male Japanese quail were given fluoridated drinking water (0, 125, 250, 500 ppm NaF) for 122 days. Semen samples were collected weekly and evaluated for ejaculate volume, motility, live/dead ratio, and sperm concentration. Twenty males were also exposed to 0 or 250 ppm NaF in their drinking water for 64 days. During that time they were briefly paired with a female on 5 separate occasions and the duration and proportion of fertility of eggs resulting from those pairings were recorded. There was no effect on the ability of the male to fertilize eggs or on the duration of fertility as a result of exposure to fluoride. Results from the semen evaluation study support these results. There

was no significant decline in most of the semen parameters measured as a result of chronic exposure to sodium fluoride. The significance of these results to free-living birds is discussed.

RESUMÉ

Afin d'évaluer les effets de l'exposition au fluor sur la reproduction d'oiseaux mâles, des crécerelles d'Amérique (*Falco sparverius*) et des cailles japonaises (*Coturnix japonica*) ont été traitées avec du fluor de sodium pendant une période de temps prolongé puis couplées avec des femelles non traitées. Quarante crécerelles d'Amérique mâles ont été assignées de façon aléatoire à un des traitements suivants: 1,65, 3,3 ou 6,6 mg NaF ou une solution contrôle de 13,2 mg $NaHCO_3$ administrés par gavage oral quotidiennement pendant 45 jours. Durant la première année tous les mâles ont été couplés avec des femelles non traitées dès la complétion du dosage. Durant la deuxième année le dosage des mâles a continué pendant 15 jours après le couplage avec des femelles non dosées. Au cours du dosage de même que durant la période subséquente de couplage, 1 ml de sang a été prélevé à chaque semaine chez tous les mâles afin de déterminer le niveau de testérone en circulation. Tous les oiseaux ont été pesés périodiquement tout au long de l'étude. Des données ont été récoltées sur le comportement lié à la reproduction ainsi que sur la proportion d'oeufs fertiles produits. Un des fémurs et les testicules ont été prélevés à la nécropsie afin de mesurer le niveau de fluor et les effets dans les tissus. Tandis que la méthode du gavage a produit une hausse du fluor dans les os en relation avec une augmentation de la dose, aucun effet significatif n'a été signalé quant au nombre d'oeufs fertiles produits au cours des deux années de l'étude. De plus, aucune difference n'a été détectée sur le comportement de reproduction ou dans les niveaux de testostérone en circulation. En outre les indices gonadosomatiques chez les crécerelles exposées et chez celles servant de contrôle n'ont pas montré de différence significative. Afin d'évaluer les effets de l'exposition au fluor sur la qualité du sperme, de l'eau fluorée (0, 125, 250 ppm NaF) fut donnée à boire à 40 cailles japonnaises mâles pendant 122 jours. A chaque semaine des échantillons de sperme ont été recueillis afin d'évaluer le volume d'éjaculation, la motilité, la proportion de sperme vivant et mort ainsi que la concentration de spermes. Vingt mâles ont également été exposé a 0 et 250

ppm de NaF dans leur eau de consommation pendant 64 jours. Au cours de cette période chaque oiseau a été couplé brièvement avec une femelle, pendant 5 fois séparées; la durée de la fertilité de même que la proportion des oeufs fertiles qui ont résulté de ces couplages ont été notées. L'exposition au fluor n'a causé aucun effet ni sur l'aptitude du mâle à fertiliser les oeufs ni sur la duration de la fertilité. Les résultats de l'étude de spermes vont dans le même sens que ces derniers résultats. L'exposition chronique au fluor de sodium n'a causé aucune réduction significative des mesures de paramètres liés au sperme. La signification des ces résultats pour les oiseaux en liberté est discutée.

TABLE OF CONTENTS

TABLE OF CONTENTS (CONT'D)

TABLE OF CONTENTS (CONT'D)

TABLE OF CONTENTS (CONT'D)

ACKNOWLEDGEMENTS

Any research involving the study of animals in captivity by necessity requires the input of many people. To those I may have neglected to include, I apologize and extend my heartfelt thanks. Ian Ritchie of the Avian Science and Conservation Centre ensured that the birds were properly housed and fed. He also maintained the incubators and most of the equipment required to carry out this project. Most important he provided excellent advice on all aspects of maintaining birds in captivity. Mark Adam fed and cared for the birds as well as helping to keep the quail entrained.

Many people contributed to the collection of data. I would like to thank Jean Cypher, Kelly MacLellan, Andrew Bochenek, Genevieve Savard and Meloni Muir for their help. Meloni also contributed her ideas to other aspects of the study and her friendship was a source of inspiration for me. I would like to acknowledge Donna Leggee for measuring the testosterone levels in the blood samples and Céline Boutin for her translation of the abstract. Brian Collins suffered through my innumerable visits to his office with queries on statistics.

The person who had the most input into this study and to whom I am most grateful is my supervisor and friend Dr. David Bird. He was a constant source of inspiration and support for me through the lean times. To him I am forever thankful.

Financial support was provided by the Wildlife Toxicology Fund of the World Wildlife Fund, NSERC, FCAR and the Avian Science and Conservation Centre.

Finally, I would like to thank Jen who kept me motivated and has had the patience of a saint.

LIST OF TABLES

LIST OF TABLES (CONT'D)

LIST OF TABLES (CONT'D)

LIST OF FIGURES

LIST OF FIGURES (CONT'D)

LIST OF FIGURES (CONT'D)

LIST OF FIGURES (CONT'D)

INTRODUCTION

Fluoride in the environment, from anthropogenic or natural sources, has been demonstrated to accumulate to significant levels in wildlife. While numerous toxicity-based endpoints, including compromised reproductive performance, have been documented in laboratory and domesticated species, comparable information on wildlife is lacking. Ingestion of fluoride in domestic species has been shown to result in reduced egg production in females and compromised semen quality in males. Since wildlife has been found to be sensitive to this element in contaminated areas, the purpose of this research was to investigate the possible impacts of exposure to sodium fluoride on the avian male reproductive system. The common American kestrel (*Falco sparverius*) was selected as a model species since, as a top-level predator, it is likely to be exposed to significant levels of fluoride when occurring in contaminated environments. The male of the species was chosen as the focus of this research since, while the effects of fluoride on egg production in fowl have been well documented, the impacts of similar exposures on the avian male's fertilizing capacity are relatively unknown. Accordingly, the results obtained from this study could be used in a predictive fashion to estimate the likelihood of reproductive failure experienced by pairs breeding in areas contaminated with fluoride.

The ultimate contribution of the male to the breeding unit is the ability to fertilize the female's ova. While various aspects of male reproduction have been assessed in toxicological evaluations, the importance of this measure of reproductive fitness to a free-living species cannot be overstated. Several measures of semen quality have been demonstrated to be good predictors of fertilizing capacity. In order to assess the impacts of fluoride exposure on sperm viability and numbers, male Japanese quail were given fluoridated drinking water and serially collected semen samples were evaluated. Since sperm

concentrations are typically quite low in the American kestrel and the semen is often clouded with urates and cellular debris, the quail was considered a more suitable model species for this aspect of the study.

The purpose of this research was two-fold: to determine which components, if any, of the male reproductive system of an avian species were deleteriously impacted by fluoride exposure and second, to ascertain whether any resulting impacts were significant enough to affect the ability of the breeding pair to produce viable offspring, perhaps the most important endpoint for a free-living species. The endpoints selected were based, to a large extent, on previously published research and are potentially suitable indicators of reproductive dysfunction.

The first chapter of this thesis reviews the literature regarding the natural levels of fluoride in the environment, anthropogenic inputs, and effects on biota with the emphasis being placed on levels and effects documented in wildlife. The majority of published research concerning fluoride toxicity deals with domestic and laboratory animals. Most studies concerning wildlife document levels of fluoride in the skeleton and do not discuss the significance of those exposures. Because of the lack of information on wildlife it was necessary to use domestic and laboratory studies to discuss the various responses resulting from chronic fluoride exposure. The second chapter discusses the two experiments in which male American kestrels were treated with known quantities of sodium fluoride by oral gavage daily and then paired with untreated females. Several aspects of male reproductive performance were evaluated during the subsequent breeding period. In the third chapter male Japanese quail were exposed to sodium fluoride in their drinking water. In the first component of the study treated males were paired with untreated females and the duration of fertility of eggs produced was recorded. Finally, another group of male quail were treated with three levels of

fluoride, also in their drinking water. Semen samples collected were evaluated for motility and sperm concentration as well as several other parameters.

Data collection, analysis and manuscript preparation were conducted independently by the senior author. Manuscripts from chapters II and III will be submitted for publication with D.M. Bird as co-author.

REGULATION ON THESIS PRESENTATION

The following is included in accordance with the regulations of the Faculty of Graduate Studies and Research of McGill University:
"Candidates have the option of including, as part of the thesis, the text of a paper(s) submitted or to be submitted for publication, or the clearly-duplicated text of a published paper(s). These texts must be bound as an integral part of the thesis.

If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Presentation". The thesis must include: A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list. Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored

papers. Under no circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis".

Chapter 1.

The effects of fluoride on wildlife: a review of the literature

INTRODUCTION

Fluorine is a member of the halogen family and is the most electronegative of the elements, making it extremely reactive [1]. As such, it is rarely found in the free state in nature but occurs in the ionic form (F^-) or bound in a non-ionic matrix of either biological or mineral origin. It is the inorganic complexes of fluorine which have the most toxicological significance [2]. Fluorine, which is soluble and potentially mobile, is ubiquitous in the environment [3], comprising 0.06-0.09% of the earth's crust. In addition to weathering of fluorine- containing ores, natural releases of fluoride include geothermal and volcanic activity as well as the cycling of this compound between the biosphere, the hydrosphere and the atmosphere. In nature, fluoride occurs most commonly as fluorite (CaF_2) and fluorapatite ($CaF_2 \cdot 3Ca_3(PO_4)_2$) [4]. Because this element occurs so widely it often makes the delineation of the contribution of anthropogenic versus natural fluoride to incidences of toxicity somewhat problematic. However, the preponderance of environmental fluoride which is bioavailable and results in toxicosis is of anthropogenic origin [5]. Since elemental fluorine is rarely detected in nature, previous conventions will be observed, and the term 'fluoride' will be used when referring to fluorine-containing compounds.

SOURCES AND LEVELS OF FLUORIDE IN THE ENVIRONMENT

As a result of natural and/or human processes fluoride may accumulate to significant levels in environmental media. The occurrence of elevated levels of fluoride compounds in soil, water and plants is widespread globally but variable.

In Canada the principal sources of industrial fluorides are, in descending order of importance, the aluminum industry, production of phosphate fertilizers, and the iron and steel industry [5]. The superphosphate fertilizer industry contributes almost half of the total fluorides released to the environment. Total current

production of fluorides from these sources is difficult to estimate. Canadian data, compiled in the 1970's, indicate a release of approximately 11,700 metric tonnes of airborne fluorides per year [5]. Data collected in the late 1980's indicate that these industries continue to be the major contributors of anthropogenic fluorides to the environment [6,7]. By 1993 estimated releases of airborne fluorides had dropped to 5,400 metric tonnes with the total release of fluorides into the Canadian environment being estimated at 23,000 metric tonnes, 58% of which was in surface waters [8].

Airborne fluorides (which constitute 23% of the total fluorides released) are emitted as gases or airborne particles which are carried downwind and deposited on plants, soils or surface waters in a dry form or washed out in precipitation. Ambient levels of fluoride (gaseous and particulate) in Canadian air are low to undetectable (<0.05 $\mu g/m^3$), although concentrations in the vicinity of industrial centres may reach 0.1 to 0.85 $\mu g/m^3$ [8].

Generally speaking, levels of fluoride in surface waters in Canada are naturally low, with a mean level of 0.05 mg F/L [9]. Water supplies fluoridated for the prevention of dental caries in humans generally contain 0.73 to 1.25 mg F/L [8], while maximal concentrations in surface waters near industrial facilities have exceeded 30 mg F/L [8]. In addition, large amounts of fluoride may be added to surface waters as a component of industrial effluents [10]. Fluorine is a natural component of most soil types. In Canada, concentrations of fluoride in soils are quite variable, ranging from 20 to 1000 µg/g [11] with the mean levels for surface soils being 160 µg/g [12].

TOXICOKINETICS OF FLUORIDE

The most significant route of exposure to fluoride is via consumption of contaminated food or water. Fluoride absorption occurs as a passive non-ionic diffusion process taking place largely in the intestine and stomach [13] and is dependent on the solubility of the fluoride compound as well as numerous other factors, including duration of ingestion and species of animal involved [14]. It has been estimated that 50- 95% of a single dose of fluoride diffuses across the intestinal wall depending on how it was administered (absorption is almost complete if administered as a liquid) [15]. Zipkin and Likins [16] noted that fluoride from covalently bound (and physiologically inert) compounds, such as KPF_6, is much more rapidly absorbed than electrovalently bound fluoride exemplified by NaF and typically found in the ionic form. Several authors have demonstrated that the uptake of fluoride is significantly reduced when it is co-administered with food [17,18] or as a salt complexed with calcium, aluminum or magnesium [19,20]. Fluoride is rapidly converted to hydrogen fluoride in the stomach, which easily diffuses across cell membranes [13]. Upon absorption, fluoride is rapidly distributed throughout the body, crossing cell membranes including the erythrocyte [21]. Radio-labelled fluoride partitioned largely (79%) into plasma within 2 minutes when injected into dogs [22]. Migration from the blood follows a triphasic pattern, mixing initially with body water, then the skeleton and finally excreted in the urine [23].

Although plasma fluoride levels were once thought to be homeostatically regulated [24], it has since been demonstrated that levels fluctuate in response to changing fluoride intake [25]. Fluoride is also transported across the placenta and will accumulate in the fetus [3,4,26,27]. The principal route of elimination of fluoride is via the kidney. Wallace-Durbin [28] estimated that up to 29% of a single orally or intravenously administered dose of radio-labelled fluoride was excreted in the urine of laboratory rats after 9 hours. Approximately 98% of the

body's fluoride burden is sequestered in calciferous tissue [4]. Fluoride levels in bone tend to increase with age, given a relatively constant rate of intake [2,29]. In fact, skeletal fluoride levels have been demonstrated to increase in humans where intake rates from food and water are very low [21].

ACUTE AND CHRONIC EXPOSURE TO FLUORIDE

The literature describing the effects of fluoride on man and animals is extensive (for reviews see [2,3,5,21,30,31]) and somewhat diverse due, in part, to the interest in the prophylactic properties of fluoride on dental caries. The majority of the literature discussing fluoride effects on domestic animals (largely cattle) deals with chronic exposure resulting from consuming mineral supplements containing elevated levels of fluoride or contaminated forage plants [14,32,33]. In addition, fluoride has been used as an active ingredient in the manufacture of rodenticides (sodium fluoroacetate, fluoroacetamide) and insecticides (sodium fluoride, sodium hexafluorosilicate) [34]. Non-target species may suffer from acute exposure as a result of consumption of these or other fluoride compounds [35]. However, the number of cases of acute poisoning compared to the number of animals, both wild and domestic, which have been chronically exposed to fluoride is extremely small.

Exposure to fluoride may occur on an acute or chronic basis. However, a distinction cannot always be made between subacute and chronic situations [14]. Acute exposure is relatively rare and usually results from consumption of fluoridated pesticides or insecticides [33]. Wildlife and cattle are commonly exposed to fluoride over a period of months or possibly years. It is under these conditions that fluorotic lesions will develop.

Fluoride toxicity was first noted about 1,000 years ago when sheep were found to be sick and crippled during years of volcanic activity [30]. However, the linkage

between chronic exposure to fluoride and modification of calciferous tissues, the primary lesion associated with fluoride exposure, was not made until 1912 when Bartolucci noted a disease resembling osteomalacia in cattle near a superphosphate plant in Italy. The relationship between excessive fluoride intake and dental disfigurement (a sensitive indicator of long-term exposure to fluoride) was first reported by McCollum and co-workers working with laboratory rats in 1925 [36]. By the early 1930's the first reports of fluorosis in man appeared in the scientific literature. Roholm's landmark treatise on fluoride toxicology published in 1937 [30] clearly established the relationship between exposure to fluoridated forage and resulting bone disorders. During the following 10 years, legislation was put in place in the U.S. to limit the fluoride content of animal feeds [14].

LEVELS OF FLUORIDE IN WILDLIFE

Historically, the diagnosis of fluorosis has been largely restricted to humans and domestic animals, primarily cattle, which are exposed to fluoride-contaminated water and/or food or have been administered fluoridated mineral supplements. However, wildlife species, like domestic animals, are also exposed to fluoride from the air, water and their diet [37]. The number of published reports discussing fluoride burdens in free-living animals are relatively few and usually involve sampling animals around sources of known contamination.

The majority of cases of fluorosis involving wildlife may be attributed to environmental pollution [38]. While the largest proportion of industrial fluoride released into the environment is added to surface waters, airborne particulates and gases which are deposited or washed out onto vegetation are probably the most significant threat to wildlife. Fluorides may be inhaled, ingested directly from vertebrate or invertebrate prey, plants, soil and water or indirectly through preening. The single most significant route of exposure for wildlife is the consumption of forage plants containing elevated levels of fluoride resulting from

their proximity to industrial facilities [4]. In addition to anthropogenic sources, natural processes may also increase the levels of fluoride in various media. Mineral deposits, e.g. fluorite, fluorapatite, may leach fluorides into ground water and soil. Certain plant species can actively accumulate fluorides from the soil [39].

The vast majority of data on the uptake of fluorides by free-living animals concerns cattle grazing on contaminated forage plants. Relatively little information has been collected on fluoride levels in wildlife in Canada or elsewhere. Numerous studies have examined fluoride levels in the bones of deer species (since, like cattle, they are likely to be exposed to fluoride through the consumption of contaminated forage plants) obtained near industrial facilities in the USA, while only one such investigation was conducted in Canada [40]. Appreciably less information is available on fluoride levels in free-living birds. Tables 1 and 2 provide levels of fluoride measured in the bones of mammals and birds, respectively. The data presented are largely from North America and Europe and indicate that, like domestic animals, wildllife species are capable of accumulating aappreciable burdens of fluoride in their skeletons.

While it has been hypothesized that skeletal fluoride burdens may increase in concentration with increasing trophic status [10], this has not been demonstrated by the data collected on wildlife to date [39,41]. The results from these studies indicate that fluoride levels in wild animals collected from both contaminated and uncontaminated environments are similar to those in domestic animals, with skeletal burdens in excess of 1,500 ppm (dw) being indicative of fluorosis [14]. Also, older birds have higher levels of bone fluoride than younger birds [39], following the trend observed in mammals [2].

Several studies have attempted to measure the uptake of fluorides by free-living birds in contaminated [42-44] and uncontaminated environments [42,45]. Bone

measurements indicate that wild birds efficiently accumulate fluoride relative to the degree of contamination of their environment. Henny and Burke [44] measured fluoride in the femurae of black-crowned night herons (*Nycticorax nycticorax*) collected near a phosphate processing complex. Bone levels increased significantly with age, however there were no significant differences between the sexes. In a survey to measure fluoride burdens in species collected in uncontaminated environments, Stewart *et al.* [46] concluded that differences in bone levels could, in part, be explained by differences in diet as well as age. van Toledo [47] measured fluoride levels in eggshells from several avian species collected in the vicinity of an aluminum smelter and found that food type played an important role in fluoride uptake. In order to assess if predatory species accumulate higher levels than those occupying lower levels in the food chain, Seel and Thomson [41] and Seel *et al.* [39] measured fluoride in the bones of several species of hawks and owls in Britain. In *Accipiter nisus* and *Falco tinnunculus* the males were significantly higher than females and the two diurnal raptors had higher bone fluoride levels than the two owl species. Raptors which consumed primarily birds were found to have higher bone fluoride levels than those species whose diets were composed largely of mammals. The authors suggested that diet composition and the ability of the consumer to digest bone, the principal site of fluoride storage, were major determinants of skeletal fluoride burdens.

FLUORIDE TOXICITY IN ANIMALS

Animals normally ingest small quantities of fluorides on a regular basis with no negative impacts on their health [48]. In fact, fluoride ingested in small quantities may have beneficial properties [2]. However, chronic intake of elevated levels of fluoride-containing material may result in the expression of a number of pathological conditions [14]. The vast majority of the data extant on the effects

of fluoride exposure on health involves domestic animals. However, the symptoms of injury in wildlife resulting from exposure are consistent with those observed in laboratory and domestic species [49]. While dental and osteofluorosis are the two most commonly described lesions associated with fluoride exposure, (and potentially the most significant to wildlife health), secondary symptoms such as appetite suppression and a concomitant loss of body condition [4] may also seriously affect the health of an organism in the wild and, ultimately, its survivability.

EFFECTS ON HARD TISSUES

The most significant lesions which occur as a result of chronic exposure to fluoride appear in the teeth and bone and are commonly referred to as chronic fluorosis or fluoride toxicosis [33]. It is this suite of lesions which are most commonly documented in wildlife and domestic animals [38]. Mottling and deformation of the calciferous tissues could result in significant impairment to the health of exposed wildlife through loss of mobility and impaired feeding efficiency. Numerous authors have demonstrated restricted movement in the leg joints [14,50] and excessive pitting in the incisors and erosion of the molars [4,21,37] as a result of chronic exposure to environmental fluoride. The effects of fluoride exposure on wild mammals and birds are summarized in Tables 3 and 4, respectively.

Potentially the most debilitating lesion arising as a result of chronic exposure to fluoride is gross pathological changes in bone. These changes involve primarily metabolically active bone and will occur throughout the life of the animal [33]. The bones lose their smooth, shiny appearance and become chalky white and porous with a roughened periosteal surface and increased density [14,48]. In addition, exostoses of the long bones may result from periosteal hyperostosis [48]. These calcified protrusions are most commonly located on the medial

surfaces of the long bones of the legs as well as the ribs and mandibles and are indicative of chronic fluoride exposure. Alterations to the structure of the bone results when the fluoride ion [F] replaces the hydroxyl group [OH] in the hydroxyapatite crystal [$Ca_{10}(PO_4)_6(OH)_2$] forming fluorapatite [$Ca_{10}(PO_4)_6(F)_2$] [51]. Other conditions associated with fluoride toxicosis include osteosclerosis, osteoporosis and osteomalacia [33]. Enlargement of the metabolically active joints results from the larger size of the fluoride-containing crystal. It is the ensuing loss of mobility which characterizes the crippling effects of chronic osteofluorosis.

The most obvious primary lesions associated with long-term exposure to elevated levels of fluoride appear in the permanent dentition [52]. As is the case for bone, the rate of uptake of fluoride is a function of the metabolic state of the calcified tissue; developing teeth will accumulate fluoride most rapidly [4]. The nature and severity of fluoride-induced dental lesions correlates well with the rate of ingestion of soluble fluorides [38]. Severely affected incisors are often chalky, mottled and may exhibit signs of hypoplasia and hypocalcemia [4]. The molars exhibit symptoms of erosion and, in extreme cases, may be worn to the level of the gums [30]. Frequently this results in excessive erosion and attrition of the enamel which may greatly impact an animal's ability to masticate and break up its food. Because dental lesions correlate well with osteofluorosis and the amount of fluoride ingested, they are frequently used in the clinical diagnosis of fluoride intoxication [14].

The first discussion of the impacts of fluoride ingestion on wildlife was made by Robinette *et al* [53] while investigating the causes of tooth wear in mule deer. More recently, research on the uptake and impacts of exposure to environmental fluorides has primarily involved ungulates [24,54-59]. Several studies have used the skeletal fluoride burdens of small mammals including rodents [60-64] and foxes [65] as indicators of ecosystem contamination by fluoride. The majority of

investigations involving wildlife which document bone fluoride levels do not assess the implications of this exposure to the health of the animal, although Newman [66] suggests that wildlife may be at least as sensitive to the debilitating effects of exposure to fluoride as cattle.

EFFECTS ON SOFT TISSUES

Unlike calciferous tissues which can sequester up to 98% of an administered dose of fluoride, early reports indicated that fluoride did not accumulate significantly in or have an effect upon soft tissues (see [2,21,31]). However, it has been demonstrated (by using radio-labelled F^{18}) that fluoride crosses cell membranes with ease [31]. Cattle chronically exposed to fluoride will exhibit slight increases in concentration of fluoride in soft tissues. In addition, chickens fed a high fluoride supplement produced egg yolks elevated in fluoride [21]. More recently, animals acutely exposed under controlled laboratory conditions have exhibited changes in the structure and function of numerous tissues as a result of fluoride administration. Investigators have studied the impacts of fluoride on the liver [67-69], kidney [68,70,71], thyroids [67,72-74], parathyroids [75,76], adrenals [77] and the neurosecretory system [78,79]. Many of the responses documented in soft tissues may be related to the ability of fluoride to inhibit enzyme systems, including protein synthesis and glycolysis (see below). In addition, research has also been conducted on the effects of fluoride on various systems, including the digestive tract and reproduction (for reviews see [2,80]).

While many studies have demonstrated fluoride to have an impact on the structure and function of various soft tissues under experimental conditions, there is little evidence of histological changes in the soft tissues of chronically exposed domestic animals, specifically cattle [2] and there remains a paucity of data on the effects of chronic fluoride exposure on the soft tissues of free-living wildlife.

Genotoxicity of fluoride

The question of whether fluoride has the capacity to cause genetic damage has not been resolved satisfactorily. In order to assess the genotoxic impacts resulting from acute or chronic exposure to fluoride, investigators have used several different models, including sperm morphology tests [81-83], sister chromatid exchange rates in cultured Chinese hamster cells [84,85], rates of DNA strand breakage [86,87] and chromosomal aberrations in cultured cell lines [88]. While positive results have been obtained in several of the above studies, the effects were induced using near lethal concentrations of NaF (extrapolated to whole animal levels). As a result there remains no established concensus regarding the genotoxic properties of fluoride on cells *in vivo* [89].

Effects on enzymes

Fluoride is known to inhibit or promote the activity of numerous enzymes and enzyme systems [90,91]. Fluoride is highly electronegative and therefore binds trace elements critical to the normal functioning of many enzymes [35]. The mechanism by which this occurs appears to involve the fluoride ion forming hydrogen bonds with specific components of the enzyme complex, resulting in disruption of the active site [92]. The altered configuration of the active site prevents binding with the normal substrate.

Numerous authors have documented significant increases in adenylate cyclase activity *in vitro* and *in vivo* [93-95]. The enhancement of this activity results in an increase in the formation of cAMP, a 'second messenger' for many hormones (including steroid hormone synthesis) and a regulator of a wide range of cellular processes [96]. While these responses have been demonstrated repeatedly *in vitro*, whole animal tests have failed to produce similar results.

The addition of fluoride to intact cells and cell membrane fragments has been shown to alter carbohydrate metabolism. In fact, it is believed that the ability of fluoride to halt aerobic and anaerobic carbohydrate metabolism may, in part, explain its toxicity *in vivo* [35]. A single large dose of fluoride resulted in an elevation of serum glucose associated with stimulation of glucose-6-phosphatase activity in the liver and kidney of exposed rats [97]. McGown and Suttie [98] reported that fluoride-induced hyperglycaemia in the rat was mediated by epinephrine produced by the adrenal medulla. Catabolic processes such as glycogenolysis and glycolysis were enhanced and inhibited, respectively, by addition of NaF to hepatocytes [95]. However, while fluoride does stimulate the production of adenosine 3',5'-cyclic monophosphate (cAMP), no such effect was observed for several glycolytic enzymes and phosphatases [99]. Fluoride administration is also known to reduce anaerobic glycolysis through the inhibition of enolase [98].

In addition to modifying intra and extracellular carbohydrate levels, fluoride exposure has also been demonstrated to affect lipid and protein metabolism. Fluoride is known to be a cytotoxin [100], causing growth inhibition [101]. It is believed that the mechanism of toxicity may involve inhibition of protein synthesis [102]. Lipase in the thyroid may be inhibited by fluoride with a resulting increase in triglycerides [74]. Plasma cholesterol levels were significantly elevated in Guinea pigs exposed to HF [103] but reduced in the lungs of rabbits receiving subcutaneous injections of NaF [104]. Long term exposure of rats to fluoride resulted in a reduction in the rate of protein synthesis [105] but not in hepatic microsomal protein content of rats exposed to fluoride in their water [106]. Mice exposed to fluoride for 4 weeks exhibited significantly reduced protein synthesis in several organs, possibly as a result of reduced RNA transcription [70]. Imai and coworkers [102] noted inhibition of synthesis of a number of proteins when sodium fluoride was added to cultured human carcinoma cells. Fluoride is also known to inhibit the Krebs cycle *in vitro* [103], which reduces the energy

produced by the exposed cells.

The effects of the various impacts of fluoride on enzyme systems in the whole animal are not well documented. Furthermore, enzymatic effects documented on intact cells or cell membranes were elicited at what would be, in some cases, toxic levels *in vivo*. Many of the effects observed *in vitro* are not reproducible *in vivo*. While these effects could disrupt the energetic balance of an organism, similar information for free-living animals is not available.

Effects on reproduction

Concerns over the possible impacts of fluoride exposure on reproduction in animals originated with the diagnosis of fluorosis in cattle early in the 20th century. Since then, numerous studies have investigated various aspects of reproductive impairment at several levels of organization ranging from the cells to whole animals. Traditionally, research has focused primarily on the female of the species with the emphasis on production of healthy offspring [107-111] or eggs in the case of domestic fowl [112-114]. More recently, fluoride exposure has been demonstrated to alter various components of the male reproductive system as well. While laboratory studies suggest a correlation between fluoride ingestion and reproductive dysfunction, long-term surveillance of chronically exposed cattle do not support these results [14,33,38] but suggest a possible secondary role in reproductive impairment involving poor body condition, lameness, and reduced feed utilization. The apparent discrepancy between laboratory and field results may be related, in part, to the suitability of using biochemical/histological endpoints to predict reductions in the production of young. The effects of fluoride ingestion on the reproductive performance of free-living wildlife have never been adequately assessed. Based on the results obtained in cattle and in laboratory studies, the potential risk experienced by exposed wildlife should be evaluated. A summary of reproductive impacts

resulting from fluoride exposure for mammals and birds is presented in Tables 5 and 6, respectively.

Numerous investigations have been carried out to assess the reproductive success of laboratory animals exposed to fluoride in their diet. In one of the earliest publications Schultz and Lamb [115] documented increasing mortality in litters from rats fed fluoride in their ration. In a series of well designed studies Phillips *et al.* [107] demonstrated that several levels of dietary fluoride had no impact on reproductive function in rats. Observed cessation of oestrus could be attributed to inanition and production of gonadotropins from the anterior pituitary of exposed animals was apparently not affected. However, Messer *et al.* [116,117] reported that mice maintained on a low fluoride diet produced fewer litters than groups exposed to a higher level of dietary fluoride. While the authors suggested that fluoride may be essential for normal reproduction, subsequent research indicated that the observed decrease in pup production may have been attributable to anemia in the dams and pups caused by mineral deficiencies in the diet [109].

The rodent model has been used extensively to examine the effects of fluoride exposure on histological, physiological and biochemical components of the male reproductive system (Table 5). Male mice were administered 10 or 20 mg/kg NaF in their food for 30 days. Testicular succinate dehydrogenase was significantly reduced, suggesting a reduction in testicular oxidative metabolism while cholesterol and serum tesosterone levels were unchanged [118]. Reductions in sperm count and motility have been observed in rats orally exposed to fluoride with a concommitant drop in epididymal ATPase activity [119]. Alterations in sialic acid and acid phosphatase activity were documented along with increased numbers of deformed sperm in rats where NaF was injected directly into the vas deferens [120]. In addition there was significant damage to the integrity of the caudal epididymis, vas deferens and testis. Changes to the testicular

ultrastructure have also been confirmed in studies where fluoride was administered to mice [121,122], voles [111], and rabbits [123]. Circulating androgen levels in fluoride-exposed animals were generally unaffected by the treatment [118,119]. While these effects are significant, none of the studies discussed above attempted to relate altered function with the ability to fertilize ova and produce viable offspring.

Many studies have investigated the impacts of fluoride ingestion on reproductive success of livestock eating contaminated forage. Udall and Keller [124] discussed reductions in birth rate and agalactia in cows living in the vicinity of an aluminum plant, which they attributed to consumption of fluoride-contaminated fodder. However, in earlier research Phillips *et al* [125] found that experimental fluorosis produced in cattle did not result in reproductive dysfunction. Cattle given several levels of fluoridated water in South Africa over a four year period were found to produce fewer calves as the proportion of fluoride in the water increased [126]. In a chronic exposure study Shupe *et al* [108] administered several levels of fluoride and mineral supplements to cows in their diet over a period of 7.5 years. While the animals receiving the highest doses (49 and 93 ppm) exhibited symptoms of dental and osteofluorosis, no indications of reductions in reproductive efficiency were noted.

The effects of fluoride exposure on laying hens have been well documented since fluoride-contaminated rock phosphate is routinely used as a source of supplementary dietary calcium and phosphate. Initially, domestic fowl were found to exhibit symptoms of osteofluorosis when their ration was supplemented with rock phosphate [127]. Guenter and Hahn [114] noted reductions in weight gain and egg production in hens fed diets supplemented with 1,000 and 1,300 ppm sodium fluoride. Merkley and Sexton [128] found that 100 ppm NaF added to the diet of male and female chickens had no deleterious effects on reproductive performance. Guenter [129] found that 200 ppm NaF added to the

diet had a positive effect on egg production and that levels up to 800 ppm had no impact on fertility or hatchability of eggs. However, Mehdi *et al* [130] fed 98 day old male and female chickens up to 600 ppm NaF to the diet and detected a delay in the initiation of spermatogenesis in the high dose group. While growth rates were not significantly affected by any of the fluoride treatments, egg production dropped with increasing dietary fluoride content. Overall, they found poultry to be more tolerant of high fluoride intake than mammals. Al-Azawi *et al.* [131] added three levels of NaF to the drinking water of four groups of roosters over a period of 24 weeks. The addition of the two higher doses (800 and 1200 ppm) resulted in various degenerative changes in the testicular ultrastructure in birds exposed for 24 weeks. The diets of a high eggshell strength (HES) and low eggshell strength (LES) strain of chicken were supplemented with up to 1200 ppm NaF [113]. Reductions in weight gain and egg production were noted in the HES group. Because of the economic considerations associated with egg production, fluoride toxicity has been documented primarily in hens when rock phosphate is used as a calcium supplement. As a result, data on comparable effects in males are few, although toxicity has been documented in several cases.

The reproductive success of wild mammal populations exposed to fluoride is relatively undocumented. None of the studies which have assessed fluoride exposure in ungulates or rodents considered any of the parameters used to measure reproductive output. Several researchers used wild species in captivity to assess the impact of fluoride on reproductive function. Krasowska [111] fed krill (*Euphasia superba*) which are naturally high in fluoride to bank voles (*Clethrionomys glareolus*). Fewer litters were produced by the females receiving the high fluoride diet (96 ppm). In addition, the seminiferous tubules of the males on the high fluoride diet showed extensive degenerative changes, including a diminution of the germinal epithelium and the presence of vacuoles between the germinal cells. Silver foxes (*Vulpes vulpes*) were unknowingly fed a diet

containing up to 137 ppm fluoride [132]. Elevated kit mortality was probably a result of agalactia in the vixens caused by ingestion of high levels of fluoride. While males were not examined, there was no reported reduction in the number of kits produced, indicating no significant loss in fertilizing capacity.

Whereas the effects of dietary fluoride on reproductive performance of domestic fowl have been well documented, considerably less is known regarding impacts on wild birds. While no studies were found which assessed reproductive impacts on free-living birds, several authors have used wild birds in a captive environment to measure fluoride effects on reproduction. Pattee *et al* [133] exposed captive-raised screech owls (*Otus asio*) to several levels of fluoride in their diet and noted a 40% reduction in hatching success in those pairs exposed to 200 ppm NaF. Hoffman *et al* [134] analysing the same data, reported that eggs from the 40 and 200 ppm groups were significantly smaller than those produced by the control owls. Chicks produced from pairs exposed to 200 ppm NaF weighed 10% less than controls and had shorter lengths of wing and leg bones.

Carriere *et al.* [135] exposed breeding pairs of American kestrels to cockerels which had been fed three levels of fluoridated water for 10 days. Pairs fed the chicks with high bone fluoride levels laid fewer eggs with a lower percent fertility than the lower fluoride groups. As in the case of the screech owl study, the contribution of each sex to the observed reduced reproduction was not determined.

The result of these studies indicate that like laboratory species, free living animals may be negatively impacted by exposure to elevated levels of fluoride. Free-living herbivores, domestic or wild, have been found to accumulate fluoride from the environment in sufficient quantity to result, in some cases, in toxicity. Considerably less information is available concerning the likelihood and

significance of exposure of free-living carnivores. In a study conducted in Britain, foxes known to be eating mice with elevated levels of skeletal fluoride were found to have relatively low levels of bone fluoride themselves, presumably since they did not digest the skeletons of their prey [65].

Summary

It has been well documented that exposure to dietary fluoride results in dental and skeletal lesions in domestic cattle which, in turn, may affect mastication, growth and ultimately body condition. In addition, several studies have noted reduced production of eggs or young in chronically exposed animals. While free-living animals may be exposed to significant burdens of fluoride from a variety of sources, mostly of anthropogenic origin, considerably less is known regarding routes of uptake and potential effects on reproductive performance. Several studies have confirmed that birds of prey will accumulate fluoride in the skeleton and exposed females will produce fewer fertile eggs.

REFERENCES CITED

1. **Neumüller, O.-A.** 1981. *Römpps Chimie Lexikon.* Franck'sch Verlaghandlung. Stuttgart, Germany.

2. **National Academy of Sciences.** 1974. The Effects of Fluorides in Animals. National Research Council. Washington,D.C.

3. **Smith, F.A. and H.C. Hodge.** 1979. Airborne Fluorides and Man: Part 1. *C. R. C. Crit. Rev. Environ. Control* 8:293-371.

4. **Shupe, J.L., A.E. Olson and R.P. Sharma.** 1972. Fluoride toxicity in domestic and wild animals. *Clin. Toxicol.* 5:195-213.

5. **Rose, D. and J.R. Marier.** 1977. Environmental Fluoride 1977. NRCC 16081. Publications, NRCC. Ottawa.

6. **Fuge, R.** 1988. Sources of halogens in the environment, influences on human and animal health. *Environ. Geochem. Health* 10:51-61.

7. **Fuge, R. and M.J. Andrews.** 1988. Fluorine in the UK environment. *Environ. Geochem. Health* 10:96-104.

8. **Environment Canada and Health Canada.** 1993. Canadian Environmental Protection Act. Inorganic Fluorides (Priority substances list assessment report). Canada Communications Group. Ottawa, ON.

9. **Geological Survey of Canada.** 1991. National geochemical reconnaissance survey database for fluoride (1973-1991). Geological Survey of Canada. Ottawa.

10. **Groth E.** 1975. Fluoride pollution along the food chain. *Environment* 17:29-38.

11. **Davison, A.W.** 1983. Uptake, transport and accummulation of soil and airborne fluorides by vegetation. In Shupe, J.L. eds., *Fluorides: Effects on Vegetation, Animals and Humans.* Paragon Press Inc. Salt Lake City, pp. 61-82.

12. **Schuppli, P.A.** 1985. Total fluorine in CSSC reference soil samples. *Can. J. Soil. Sci.* 65:605-607.

13. **Ekstrand, J. and C.-J. Spak.** 1990. Fluoride pharmacokinetics: its implications in the fluoride treatment of osteoporosis. *J. Bone Miner. Res.* 5 Supplement 1:S53-S61.

14. **Shupe, J.L. and A.E. Olson** 1983. Clinical and pathological aspects of fluoride toxicosis in animals. In Shupe, J.L. eds., *Fluorides: Effects on Vegetation, Animals and Humans.* Paragon Press Inc. Salt Lake City, pp. 319-338.

15. **Cerklewski, F.L. and J.W. Ridlington.** 1985. Influence of zinc and iron on dietary fluoride utilization in the rat. *J. Nutr.* 115:1162-1167.

16. **Zipkin, I. and R.C. Likins.** 1957. Absorption of various fluorine compounds from the gastrointestinal tract of the rat. *Am. J. Physiol.* 191:549-550.

17. **Jowsey, J. and B.L. Riggs.** 1978. Effect of concurrent calcium ingestion on intestinal absorption of fluoride. *Metabolism* 27:971-974.

18. **Trautner, K. and J. Einwag.** 1989. Influence of milk and food on fluoride bioavailability from NaF and Na_2FPO_3 in man. *J. Dent. Res.* 68:72-77.

19. **Harrison, J.E., A.J.W. Hitchman, S.A. Hasany, A. Hitchman and C.S. Tam.** 1984. The effect of diet calcium on fluoride toxicity in growing rats. *Can. J. Physiol. Pharmacol.* 62:259-265.

20. **Rao, G.S.** 1984. Dietary intake and bioavailability of fluoride. *Ann. Rev. Nutr.* 4:115-136.

21. **Underwood, E.J.** 1977. *Fluorine.* Academic Press. New York.

22. **Carlson, C.H., W.D. Armstrong and L. Singer.** 1960. Distribution, migration and binding of whole blood fluoride evaluated with radiofluoride. *Am. J. Physiol.* 199:187-189.

23. **Perkinson, J.D.J., I.B. Whitney, R.A. Monroe, W.E. Lotz and C.L. Comar.** 1955. Metabolism of fluorine 18 in domestic animals. *Am. J. Physiol.* 182:383-389.

24. **Singer, L. and W.D. Armstrong.** 1960. Regulation of human plasma fluoride concentration. *J. Appl. Physiol.* 15:508-510.

25. **Whitford, G.M. and J.L. Williams.** 1986. Fluoride absorption: Independence from plasma fluoride levels. *Proc. Soc. Exp. Biol. Med.* 181:550-554.

26. **World Health Organization.** 1984. Fluorine and Fluorides. 36. World Health Organization. Geneva.

27. **Hudson, J.T., G.K. Stookey and J.C. Muhler.** 1967. The placental transfer

of fluoride in the guinea pig. *Archs. Oral. Biol.* 12:237-246.

28. **Wallace-Durbin, P.** 1954. The metabolism of fluorine in the rat using F18 as a tracer. *J. Dent. Res.* 33:789-800.

29. **Machoy, Z. and A. Machoy-Mokrzynska.** 1990. Mechanisms of fluoride elimination and detoxification in living organisms. *Fluoride* 23:151-153.

30. **Roholm, K.** 1937. *Fluorine Intoxication: A Clinical-hygenic Study with a Review of the Literature and Some Experimental Investigations.* H.K. Lewis and Co. Ltd. London.

31. **Smith, F.A.** 1993. Metabolism of Inorganic Fluoride. In Smith, F.A. eds., *Pharmacology of Fluorides. Part 1.* Springer-Verlag,

32. **Shupe, J.L.** 1970. Fluorine toxicosis and industry. *Amer. Indust. Hygiene* 31:240-247.

33. **Shupe, J.L.** 1980. Clinicopathologic features of fluoride toxicosis in cattle. *J. Anim. Sci.* 51:746-758.

34. **British Crop Protection Council.** 1991. *The Pesticide Manual.* British Crop Protection Council. Farnham, Surrey.

35. **McIvor, M.E.** 1990. Acute fluoride toxicity. Pathophysiology and management. *Drug Saf.* 5:79-85.

36. **McCollum, E.V., N. Simmonds, J.E. Becker and R.W. Bunting.** 1925. The effect of additions of fluorine to the diet of the rat on the quality of the teeth. *J. Biol. Chem.* 63:553-562.

37. **Walton, K.C.** 1988. Environmental fluoride and fluorosis in mammals. *Mammal Rev.* 18:77-90.

38. **Shupe, J.L., H.B. Peterson and A.E. Olson** 1979. Fluoride toxicosis in wild ungulates of the western United States. In Nielsen, S.W. eds., *Animals as Indicators of Environmental Pollutants.* National Academy of Science, Washington,D.C. pp. 253-266.

39. **Seel, D.C., A.G. Thompson and R.E. Bryant** 1987. Bone fluoride in four species of predatory bird in the British Isles. In Coughtrey, P.J., M.H. Martin and M.H. Unsworth, eds., *Pollutant Transport and Fate in Ecosystems. Special Publication Number 6 of the British Ecological Society.* Blackwell Scientific Publications, Oxford,

40. **Karstad, L.** 1967. Fluorosis in deer (*Odocoileus virginianus*). *Bull. Wildl. Dis. Assoc.* 3:42-46.

41. **Seel, D.C. and A.G. Thompson.** 1984. Bone fluoride in predatory birds in the British Isles. *Environ. Pollut. (Ser. A)* 36:367-374.

42. **Seel, D.C.** 1982. Fluoride in the magpie. 73. Institute of Terrestrial Ecology. Gwynedd, Great Britain.

43. **Andreasen, J.K. and R.K. Stroud.** 1987. Industrial halide wastes cause acute mortality of snow geese in Oklahoma. *Environ. Tox. and Chem.* 6:291-293.

44. **Henny, C.J. and P.M. Burke.** 1990. Fluoride accumulation and bone strength in wild Black-crowned Night-Herons. *Arch. Environ. Contam. Toxicol.* 19:132-137.

45. **Turner, J.C., S.R.B. Solly, J.C.M. Mol-Krijnen and V. Shanks.** 1978. Organochlorine, fluorine, and heavy metal levels in some birds from New Zealand estuaries. *N. Z. J. Sci.* 21:99-102.

46. **Stewart, D.J., T.R. Manley, D.A. White, D.L. Harrison and E.A. Stringer.** 1974. Natural fluorine levels in the Bluff area, New Zealand. *N. Z. J. Sci.* 17:105-113.

47. **van Toledo, B.** 1978. Fluoride content in eggs of wild birds (*Parus major* L. and *Strix aluco* L.) and the common house-hen (*Gallus domesticus*). Fluoride 11:198-207.

48. **Suttie, J.W.** 1977. Effects of fluoride on livestock. *J. Occup. Med.* 19(1):40-48.

49. **Newman, J.R.** 1979. Effects of industrial air pollution on wildlife. *Biol. Conserv.* 15:181-190.

50. **Shupe, J.L., R.H. Bruner, J.L. Seymour and C.L. Alden.** 1992. The pathology of chronic bovine fluorosis: a review. *Toxicol. Pathol.* 20:274-288.

51. **Grynpas, M.D.** 1990. Fluoride effects on bone crystals. *J. Bone Miner. Res.* 5, Suppl. 1:S169-S175.

52. **Smith, G.E.** 1985. A surfeit of fluoride? *Sci. Prog. Oxf.* 69:429-442.

53. **Robinette, W.L., D.A. Jones, G. Rogers and J.S. Gashwiler.** 1957. Notes on tooth development and wear for Rocky Mountain Mule Deer. *J. Wildl.*

Manage. 21:134-152.

54. **Kay, C.E.** 1975. Fluoride distribution in different segments of the femur, metacarpus and mandible of Mule Deer. *Fluoride* 8:92-98.

55. **Kay, C.E., P.C. Tourangeau and C.C. Gordon.** 1975. Fluoride levels in indigenous animals and plants collected from uncontaminated ecosystems. *Fluoride* 8:125-133.

56. **Kay, E., P.C. Tourangeau and C.C. Gordon.** 1976. Populational trends of fluoride parameters in wild ungulates from the western United States. *Fluoride* 9:73-90.

57. **Newman, J.R. and M. Yu.** 1976. Fluorosis in Black-tailed deer. *J. Wildl. Dis.* 12:39-41.

58. **Shupe, J.L., A.E. Olson, H.B. Peterson and J.B. Low.** 1984. Fluoride toxicosis in wild ungulates. *J. A. V. M. A.* 185:1295-1300.

59. **Suttie, J.S., R. Dickie, A.B. Clay, P. Neilson, W.E. Mahan, D.P. Baumann and R.J. Hamilton.** 1987. Effects of fluoride emissions from a modern primary aluminum smelter on a local population of White-tailed deer (*Odocoileus virginianus*). *J. Wildl. Dis.* 23:135-143.

60. **Wright, D.A., A.W. Davidson and M.S. Johnson.** 1978. Fluoride accumulation by Long-tailed field mice (*Apodemus sylvaticus* L.) and Field voles (*Microtus agrestis* L.) from polluted environments. *Environ. Pollut.* 17:303-310.

61. **Andrews, S.M., J.A. Cooke and M.S. Johnson.** 1989. Distribution of trace element pollutants in a contaminanted ecosystem established on metalliferous fluorospar tailings - 3. Fluoride. *Environ. Pollut.* 60:165-179.

62. **Walton, K.C.** 1986. Fluoride in moles, shrews and earthworms near an aluminium reduction plant. *Environ. Pollut. (Ser. A)* 42:361-371.

63. **Walton, K.C.** 1985. Fluoride in bones of small rodents near an aluminium reduction plant. *Water Air and Soil Pollut.* 26:65-70.

64. **Kay, E.** 1974. An inquiry into the distribution of fluoride in the environment of Garrison, Montana. *Fluoride* 7:7-31.

65. **Walton, K.C.** 1984. Fluoride in fox bone near an aluminum reduction plant in Anglesey, Wales, and elsewhere in the United Kingdom. *Environ. Pollut. (Ser. B)* 7:273-280.

66. **Newman, J.R.** 1984. Fluoride standards and predicting wildlife effects. *Fluoride* 17:41-47.

67. **Ogilvie, A.L.** 1953. Histologic findings in the kidney, liver, pancreas, adrenal, and thyroid glands of the rat following sodium fluoride administration. *J. Dent. Res.* 32:386-397.

68. **Singh, M.** 1984. Biochemical and cytochemical alterations in liver and kidney following experimental fluorosis. *Fluoride* 17:81-93.

69. **Geeraerts, F., G. Gijs, E. Finne and R. Crokaert.** 1986. Kinetics of fluoride penetration in liver and brain. *Fluoride* 19:108-113.

70. **Zhavoronkov, A.A. and L.S. Strochkova.** 1981. Fluorosis: geographical pathology and some experimental findings. *Fluoride* 14:182-191.

71. **Kessabi, M., A. Hamliri, J.P. Braun and A.G. Rico.** 1985. Experimental acute sodium fluoride poisoning in sheep: renal, hepatic, and metabolic effects. *Fund. Appl. Toxicol.* 5:1025-1033.

72. **Bobek, S., S. Kahl and Z. Ewy.** 1976. Effect of long-term fluoride administration on blood thyroid hormone levels in rats. *Endocrinol. Exp.* 10:289-295.

73. **Tsuchida, M., I. Okayasu, Y. Kohyama, H. Kurihara, H. Tanaka, F. Yanagisawa, C. Date, M. Hayashi, K. Mui and M. Asada.** 1986. Effects of long term, low dose ingestion of fluoride on the thyroid gland in rats. *Fluoride Research 1985. Stud. Environ. Sci.* 27:307-312.

74. **Shashi.** 1988. Biochemical effects of fluoride on thyroid gland during experimental fluorosis. *Fluoride* 21:127-129.

75. **Faccini, J.M. and A.D. Care.** 1965. Effect of sodium fluoride on the ultrastructure of the parathyroid glands of the sheep. *Nature* 207:1399-1401.

76. **Teotia, S.P.S., M. Teotia, R.K. Singh, N.P.S. Teotia, D.R. Taves, S. Heels and V.P. D'Mello.** 1978. Plasma fluoride, 25-hydroxycholecalciferol, immunoreactive parathyroid hormone and calcitonin in patients with endemic skeletal fluorosis. *Fluoride* 11:115-119.

77. **De, S. and A.K. Sarkar.** 1988. Effect of sodium fluoride on adrenal and hypothalamus in Weaver Bird. *Sci. Cult.* 54:344-345.

78. **Mietkiewski, K., M. Walczak and R. Trojanowicz.** 1966. The effect of

sodium fluoride on the neurosecretory system of the guinea-pig. *Pol. Endocrinol.* 17:72-80.

79. **Singh, K.B. and C.J. Dominic.** 1975. Sodium fluoride-induced changes in the hypothalamic neurosecretory system of the spotted owlet, *Athene brama* Temminck. *Endocrinol. Exp.* 9:149-155.

80. **Monsour, P.A. and B.J. Kruger.** 1985. Effect of fluoride on soft tissues in vertebrates. *Fluoride* 18:53-61.

81. **Li, y., A.J. Dunipace and G.K. Stookey.** 1987. Effects of fluoride on the mouse sperm morphology test. *J. Dent. Res.* 66:1509-1511.

82. **Pati, P.C. and S.P. Bhunya.** 1987. Genotoxic effect of an environmental pollutant, sodium fluoride, in mammalian *in vivo* test system. *Caryologia* 40(1-2):79-87.

83. **Dunipace, A.J., W. Zhang, T.W. Noblitt, y. Li and G.K. Stookey.** 1989. Genotoxic evaluation of chronic fluoride exposure: micronucleus and sperm morphology studies. *J. Dent. Res.* 68:1525-1528.

84. **Li, y., N.A. Heerema, A.J. Dunipace and G.K. Stookey.** 1987. Genotoxic effects of fluoride evaluated by sister-chromatid exchange. *Mutat. Res.* 192:191-201.

85. **Li, y., W. Zhang, T.W. Noblitt, A.J. Dunipace and G.K. Stookey.** 1989. Genotoxic evaluation of chronic fluoride exposure: Sister-chromatid exchange study. *Mutat. Res.* 227:159-165.

86. **Edwards, M.J. and Parry, J.M.** 1986. Sodium fluoride mediated DNA damage and DNA replication in mammalian cells. *Mutagenesis* 1. p.77

87. **Skare, J.A., K.R. Schrotel and J.P. Nixon.** 1986. Lack of DNA-strand breaks in rat testicular cells after *in vivo* treatment with sodium fluoride. *Mutat. Res.* 170:85-92.

88. **Aardema, M.J., D.P. Gibson and R.A. LeBoeuf.** 1989. Sodium fluoride-induced chromosome aberrations in different stages of the cell cycle: a proposed mechanism. *Mutat. Res.* 223:191-203.

89. **Li, y., A.J. Dunipace and G.K. Stookey.** 1988. Genotoxic effects of fluoride: A controversial issue. *Mutat. Res.* 195:127-136.

90. **Berry, R.J.** 1969. Effects of fluoride on cells and tissues in culture. *Fluoride*

Q. Rep. 2:157-167.

91. **Strochkova, L.S. and A.A. Zhavoronkov.** 1983. Fluoride as an activator of enzymatic systems. *Fluoride* 16:181-186.

92. **Edwards, S.L., T.L. Poulos and J. Kraut.** 1984. The crystal structure of fluoride-inhibited Cytochrome c peroxidase. *J. Biol. Chem.* 259:12984-12988.

93. **Weiss, B.** 1969. Similarities and differences in the norepinephrine and sodium fluoride-sensitive adenyl cyclase system. *J. Pharmacol. Exper. Ther.* 166:330-338.

94. **Singh, M. and A.K. Susheela.** 1982. Adenyl cyclase activity and cyclic AMP levels following F ingestion in rabbits and human subjects. *Fluoride* 15:202-207.

95. **Shahed, A.R. and D.W. Allmann.** 1984. Stimulation of adenylate cyclase activity by Na2PO3F and NaF in intact rat hepatocytes and plasma membrane fractions. *Fluoride* 17:210-217.

96. **Stryer, L.** 1981. *Biochemistry.* W.H. Freeman and Co. New York.

97. **Suketa, Y., Y. Asao, Y. Kanamoto, T. Sakashita and S. Okada.** 1985. Changes in adrenal function as a possible mechanism for elevation of serum glucose by a single large dose of fluoride. *Toxicol. Appl. Pharmacol.* 80:199-205.

98. **McGown, E.L. and J.W. Suttie.** 1977. Mechanism of fluoride-induced hyperglycemia in the rat. *Toxicol. Appl. Pharmacol.* 40:83-90.

99. **Sato, T., M. Niwa, Y. Nishada and T. Tsuji.** 1986. The effects of fluoride on the activities of several enzymes and on cyclic AMP levels in human lymphocytes *in vitro. Fluoride Research 1985. Stud. Environ. Sci.* 27:257-261.

100. **Imai, T., M. Niwa and M. Ueda.** 1983. The effects of fluoride on cell growth of two human cell lines and on DNA and protein synthesis in HeLa cells. *Acta Pharmacol. Toxicol.* 52:8-11.

101. **Berry, R.J. and W. Trillwood.** 1963. Sodium fluoride and cell growth. *Brit. Med. J.* 2:1064.

102. **Imai, T., K. Gojo, T. Sato, R. Nishikawa, M. Niwa and T. Tsuji.** 1986. Induction of protein in HeLa cells by sodium fluoride. *Fluoride Research 1985. Stud. Environ. Sci.* 27:263-266.

103. **Dousset, J.C., C. Rioufol, C. Philibert and P. Bourbon.** 1987. Effects of

inhaled HF on cholesterol, carbohydrate and tricarboxylic acid metabolism in Guinea pigs. *Fluoride* 20:137-141.

104. **Shashi, A., J.P. Singh and S.P. Thapar.** 1989. Effect of fluoride in excess on lipid constituents of respiratory organs in albino rabbits. *Fluoride* 22:33-39.

105. **Hongslo, J.K., C.F. Hongslo, H. Oystein and R.I. Holland.** 1980. Reduced fluoride sensitivity of liver cells from rats chronically exposed to fluoride. *Acta Pharmacol. Toxicol.* 47:355-358.

106. **Hongslo, J.K., J. Holme, C.F. Hongslo, K.A. Eliassen, E. Dybing, R.I. Holland and J. Ekstrand.** 1983. No effects of prolonged fluoride exposure on cytochrome P-450 and associated monooxygenases or on the level of polyamines in the rat. *Acta Pharmacol. Toxicol.* 53:250-253.

107. **Phillips, P.H., A.R. Lamb, E.B. Hart and G. Bohstedt.** 1933. Studies on fluorine in the nutrition of the rat II. It's influence upon reproduction. *Am. J. Physiol.* 106:356-364.

108. **Shupe, J.L., M.L. Miner, D.A. Greenwood, L.E. Harris and G.E. Stoddard.** 1963. The effect of fluorine on dairy cattle II. Clinical and pathologic effects. *Am. J. Vet. Res.* 24:964-984.

109. **Tao, S. and J.W. Suttie.** 1976. Evidence for a lack of an effect of dietary fluoride level on reproduction in mice. *J. Nutr.* 106:115-1122.

110. **Eckerlin, R.H., G.A. Maylin, L. Krook and D.T. Carmichael.** 1988. Ameliorative effects of reduced food-borne fluoride on reproduction in silver foxes. *Cornell Vet.* 78:385-391.

111. **Krasowska, A.** 1994. Influence of low-chitin krill meal on reproduction of *Cletherionomys glareolus* (Schreber, 1780). *Comp. Biochem. Physiol.* 94C:313-320.

112. **Merkley, J.W.** 1981. The effect of sodium fluoride on egg production, egg quality, and bone strength of caged layers. *Poult. Sci.* 60:771-776.

113. **van Toledo, B. and G.F. Combs Jr.** 1984. Fluorosis in the laying hen. *Poult. Sci.* 63:1543-1552.

114. **Guenter, W. and P.H.B. Hahn.** 1986. Fluorine toxicity and laying hen performance. *Poult. Sci.* 65:769-778.

115. **Schulz, J.A. and A.R. Lamb.** 1925. The effect of fluorine as sodium

fluoride on the growth and reproduction of albino rats. *Science* 61:93-94.

116. **Messer, H.H., W.D. Armstrong and L. Singer.** 1972. Fertility impairment in mice on a low fluoride intake. *Science* 177:893-894.

117. **Messer, H.H., W.D. Armstrong and L. Singer.** 1973. Influence of fluoride intake on reproduction in mice. *J. Nutr.* 103:1319-1326.

118. **Chinoy, N.J. and E. Sequeira.** 1989. Fluoride induced biochemical changes in reproductive organs of male mice. *Fluoride* 22:78-85.

119. **Chinoy, N.J., P.K. Pradeep and E. Sequeira.** 1992. Effect of fluoride ingestion on the physiology of reproductive organs of male rat. *J. Environ. Biol.* 13:55-61.

120. **Chinoy, N.J., M.V. Rao, M.V. Narayana and E. Neelakanta.** 1991. Microdose vasal injection of sodium fluoride in the rat. *Reprod. Toxicol.* 5:505-512.

121. **Kour, K. and J. Singh.** 1980. Histological finding of mice testes following fluoride ingestion. *Fluoride* 13:160-167.

122. **Chinoy, N.J. and E. Sequeira.** 1989. Effects of fluoride on the histoarchitecture of reproductive organs of the male mouse. *Reprod. Toxicol.* 3:261-267.

123. **Chinoy, N.J., E. Sequeira and M.V. Narayana.** 1991. Effects of vitamin C and calcium on the reversibility of fluoride-induced alterations in spermatozoa of rabbits. *Fluoride* 24:29-39.

124. **Udall, D.H. and K.P. Keller.** 1952. A report on fluorosis in cattle in the Columbia River Valley. *Cornell Vet.* 42:159-184.

125. **Phillips, P.H., E.B. Hart and G. Bohstedt.** 1934. Chronic toxicosis in dairy cows due to the ingestion of fluorine. *Research Bulletin (Univ. Wisconsin Ag. Exp. Stn.)* 123:1-29.

126. **Van Rensburg, S.W.J. and W.H. De Vos.** 1966. The influence of excess fluoride intake in the drinking water on reproductive efficiency in bovines. *Onderstepoort J. Vet. Res.* 33:185-194.

127. **Jacob, K.D. and D.S. Reynolds.** 1928. The fluorine content of phosphate rock. *Assoc. Off. Anal. Chem.* 11:237-250.

128. **Merkley, J.W. and T.J. Sexton.** 1982. Reproductive performance of white Leghorns provided fluoride. *Poult. Sci.* 61:52-56.

129. **Guenter, W.** 1979. Fluorine toxicity and laying hen performance. *Poult. Sci.* 58:1063.

130. **Mehdi, A.W.R., K.A. Al-Soudi, N.A.J. Al-Jiboori and M.K. Al-Hiti.** 1983. Effect of high fluoride intake on chicken performance, ovulation, spermatogenesis and bone fluoride content. *Fluoride* 16:37-43.

131. **Al-Azawi, S.H., K.A. Al-Soudi and A.W.R. Mehdi.** 1986. Effect of fluoride intake on the structural-functional aspects of the reproductive system of the male domestic fowl. *J. Agric. Water Reso. Res.* 5:285-300.

132. **Eckerlin, R.H., L. Krook, G.A. Maylin and D. Carmichael.** 1986. Toxic effects of food-borne fluoride in silver foxes. *Cornell Vet.* 76:395-402.

133. **Pattee, O.H., S.N. Wiemeyer and D.M. Swineford.** 1988. Effects of dietary fluoride on reproduction in Eastern Screech-Owls. *Arch. Environ. Contam. Toxicol.* 17:213-218.

134. **Hoffman, D.J., O.H. Pattee and S.N. Wiemeyer.** 1985. Effects of fluoride on screech owl reproduction: Teratological evaluation, growth, and blood chemistry in hatchlings. *Toxicol. Lett.* 26:19-24.

135. **Carriere, D., D.M. Bird and J.W. Stamm.** 1987. Influence of a diet of fluoride-fed cockerels on reproductive performance of captive American kestrels. *Environ. Pollut.* 46:151-159.

136. **Newman, J.R. and J.J. Murphy.** 1979. Effects of industrial fluoride on Black-tailed deer (preliminary report). *Fluoride* 12:129-135.

137. **Kay, C.E., P.C. Tourangeau and C.C. Gordon.** 1975. Industrial fluorosis in Mule deer and Whitetail deer from Western Montana. *Fluoride* 8:182-191.

138. **Shupe, J.L. and R.P. Sharma**. 1976. Fluoride distribution in a natural ecosystem and related effects on wild animals. Conference Proceeding. Trace Substances In Environmental Health: Proceedings of the Tenth University of Missouri Annual Conference. pp.137-144.

139. **Murray, F.** 1981. Fluoride cycles in an estuarine ecosystem. *Sci. Tot. Environ.* 17:223-241.

140. **Walton, K.C.** 1987. Tooth damage in field voles, wood mice and moles in

areas polluted by fluoride from an aluminum reduction plant. *Sci. Tot. Environ.* 65:257-260.

141. **Cooke, J.A., S.M. Andrews and M.S. Johnson.** 1990. Lead, zinc, cadmium and fluoride in small mammals from contaminated grassland established on fluorspar tailings. *Water Air Soil Pollut.* 51:43-54.

142. **Tourangeau, P.C., C.C. Gordon and C.E. Carlson.** 1977. Fluoride emissions of coal-fired power plants and their impact on plant and animal species. *Fluoride* 10:47-62.

143. **Culik, B.** 1987. Fluoride turnover in Adélie penguins (*Pygoscelis adeliae*) and other bird species. *Polar Biol.* 7:179-187.

144. **Suttie, J.W., R.J. Hamilton, A.C. Clay, M.L. Tobin and W.G. Moore.** 1985. Effects of fluoride ingestion on White-tailed deer (*Odocoileus virginianus*). *J. Wildlife Dis.* 21:283-288.

145. **Newman, J.R. and D. Markey.** 1976. Effects of elevated levels of fluoride on deer mice (*Peromyscus maniculatus*). *Fluoride* 9:47-53.

146. **Shupe, J.L., A.E. Larsen and A.E. Olson.** 1987. Effects of diets containing sodium fluoride on mink. *J. Wildl. Dis.* 23:606-613.

147. **Newman, J.R.** 1977. Sensitivity of the house martin, (*Delichon urbica*), to fluoride emissions. *Fluoride* 10:73-76.

148. **Fleming, W.J., C.E. Grue, C.A. Schuler and C.M. Bunck.** 1987. Effects of oral doses of fluoride on nestling European starlings. *Arch. Environ. Contam. Toxicol.* 16:483-489.

149. **Bird, D.M. and C. Massari.** 1983. Effects of dietary sodium fluoride on bone fluoride levels and reproductive performance of captive American Kestrels. *Environ. Pollut. (Ser. A)* 31:67-76.

150. **Tash, J.S. and A.R. Means.** 1982. Regulation of protein phosphorylation and motility of sperm by cyclic adenosine monophosphate and calcium. *Biol. Reprod.* 26:745-763.

151. **Schoff, P.K. and H.A. Lardy.** 1987. Effects of fluoride and caffeine on the metabolism and motility of ejaculated bovine spermatozoa. *Biol. Reprod.* 37:1037-1046.

152. **Pillai, K.S., A.T. Mathai and P.B. Deshmukh.** 1988. Effect of subacute

dosage of fluoride on male mice. *Toxicol. Lett.* 44:21-29.

153. **Mandrik, F.I. and Yakubovskaya, Y.L.** 1986. Gonado- and embryotoxicity of fluorine. *Fluoride* 19: 134.

154. **Krasowska, A. and T. Wlotowski.** 1992. The effect of high fluoride intake on tissue trace elements and histology of testicular tubules in the rat. *Comp. Biochem. Physiol.* 103C:31-34.

155. **Shellenberg, D., T.A. Marks, C.M. Metzler, J.A. Oostveen and M.J. Morey.** 1990. Lack of effect of fluoride on reproductive performance and development in shetland sheepdogs. *Vet. Hum. Toxicol.* 32:309-314.

156. **Hahn, P.H.B. and W. Guenter.** 1986. Effect of dietary fluoride and aluminum on laying hen performance and fluoride concentration in blood, soft tissue, bone and egg. *Poult. Sci.* 65:1343-1349.

157. **Kuhl, H.J. and Sullivan, T.W.** 1976. Effect of sodium fluoride and high fluorine fertilizer phosphates on performance of laying chickens and eggshell quality. *Poult. Sci.* 55:2055

Table 1. Levels of fluoride in the tissues of wild mammals.

Species	Mean level in tissue in µg F/g dry weight (d.w.)	Location	Comments	Reference
UNGULATES:				
Moose, ***Alces alces*** 5.5 yrs old	248 in bone	Montana	Uncontaminated site	[55]
Black-tailed deer, ***Odocoileus hemionus columbianus***	control site: 157, 465, contaminated site: 2820, 6809 in rib	Washington	Adjacent to an aluminum plant	[57]
Black-tailed deer	638-3411 in adults from a contaminated site	Washington	In the vicinity of an aluminum plant	[136]
Roe deer, ***Capreolus capreolus***	466 in 5 yr. old males and females	Germany	Uncontaminated ecosystem	[24]
White-tailed deer, ***Odocoileus virginianus***	1,132 a.w.* in adults at contaminated site, 260 a.w. at clean site	South Carolina	Near an aluminum smelter	[59]
White-tailed deer	3475-5625 in femurae of adults from contaminated site	Montana	Near a phosphate ore concentrator and an aluminum plant	[137]

* a.w.= ash weight

Table 1. Levels of fluoride in the tissues of wild mammals (cont'd).

Species	Mean level in tissue in µg F/g dry weight (d.w.)	Location	Comments	Reference
White-tailed deer	389-7125 in mandibles in adults from contaminated site	Ontario	Unnamed industrial facility	[40]
Mule deer, ***Odocoileus hemionus***	610-6950 in young and adults from contaminated site	Montana	Near a phosphate ore concentrator	[137]
Mule deer, 2 yr. old	1500 in premolars, wt. basis ?	Utah	Near a steel plant	[53]
Mule deer	12-176 in mandibles of 0.5-6 yr. old males	Montana	Uncontaminated site	[56]
Mule deer	1398 in metatarsals from fawns and adults	Western USA	Uncontaminated site	[138]
Dall sheep, ***Ovus dalli dalli***	432-905 in mandibles of 0.5-8 yr. olds	Alaska	Uncontaminated site	[56]
Bighorn sheep, ***Ovus canadensis***	241-784 in mandibles, increased with age at clean sites	Montana	Uncontaminated site	[56]

* a.w.= ash weight

Table 1. Levels of fluoride in the tissues of wild mammals (cont'd).

Species	Mean level in tissue in µg F/g dry weight (d.w.)	Location	Comments	Reference
Elk, ***Cervus canadensis***	16-65 in .5-3 yr. old males	Montana	Uncontaminated site	[56]
RODENTS: Beaver, ***Castor canadensis***	149 in bone	Montana	Uncontaminated site	[55]
House mouse, ***Mus musculus***	between 3000 and 4000 a.w.* in bone from a contaminated site	New South Wales	Near several industrial fluoride sources	[139]
Meadow vole, ***Microtus pennsylvanicus***	139 in bone	Montana	Uncontaminated site	[55]
Meadow vole	137 in bone from control site, 750 from contaminated site	Montana	Phosphate defluorination plant	[64]
Field vole, ***Microtus agrestis***	7148 in bone at contaminated site	Wales	Aluminum reduction plant	[140]
Field vole	588 (range 68-3700) in bone from a contaminated site	Wales	Aluminum reduction plant	[63]

* a.w.= ash weight

Table 1. Levels of fluoride in the tissues of wild mammals (cont'd).

Species	Mean level in tissue in µg F/g dry weight (d.w.)	Location	Comments	Reference
Field vole	551 in femurae from a contaminated grassland	Derbyshire, UK	Fluorspar tailings dam	[141]
Field vole	2195 in femurae from contaminated site	Peak District, UK	Fluorspar tailings dam	[60]
Field vole	1106 in femurae from a contaminated site	UK	Fluorspar tailings dam	[61]
Wood mouse, ***Apodemus sylvaticus***	8430 in bone at contaminated site	Wales	Aluminum reduction plant	[140]
Wood mouse	1437 (range 98-8500) in bone	Wales	Aluminum reduction plant	[63]
Wood mouse	344 in femur from contaminated grassland	Derbyshire, UK	Fluorspar tailings dam	[141]
Wood mouse	4387 in femurae from contaminated site	Peak District, UK	Fluorspar tailings dam	[60]

* a.w.= ash weight

Table 1. Levels of fluoride in the tissues of wild mammals (cont'd).

Species	Mean level in tissue in µg F/g dry weight (d.w.)	Location	Comments	Reference
Deer mouse, ***Peromyscus maniculatus***	144 in bone	Montana	Uncontaminated site	[55]
Deer mouse	501 in femurae from contaminated site	Montana	Near coal-fired electrical generating station	[142]
Deer mouse	144 in femur from control site, 1995 from contaminated area	Montana	Phosphate defluorination plant	[64]
INSECTIVORES:				
Vagrant shrew, ***Sorex vagrans***	475 in bone	Montana	Uncontaminated site	[55]
Masked shrew, ***Sorex cinereus***	589 in bone	Montana	Uncontaminated site	[55]
Shrew (species unknown)	10,500 in bone from contaminated site	Montana	Phosphate defluorination plant	[64]
Common shrew, ***Sorex araneus***	1069 in femurae from contaminated grasslands	Derbyshire, UK	Fluorspar tailings dam	[141]
Common shrew	1827 in femurae from a contaminated site	Peak District, UK	Fluorspar tailings dam	[60]

* a.w.= ash weight

Table 1. Levels of fluoride in the tissues of wild mammals (cont'd).

Species	Mean level in tissue in µg F/g dry weight (d.w.)	Location	Comments	Reference
Common shrew	1553 in femurae from a contaminated site	UK	Fluorspar tailings dam	[61]
Common shrew	9590 in bones collected at a contaminated site	Anglesey, Wales	Aluminum reduction plant	[62]
Mole, ***Talpa europaea***	7740 in bones from a contaminated site	Anglesey, Wales	Aluminum reduction plant	[62]
CARNIVORES: Lynx, ***Lynx canadensis***	176 in bone	Montana	Uncontaminated site	[55]
Coyote, ***Canis latrans***	257-321 in bone	Montana	Uncontaminated site	[55]
Mink, ***Mustela vison***	500 in bone	Montana	Uncontaminated site	[55]
Fox, ***Vulpes vulpes***	1650 in bone from contaminated site, 551 from uncontaminated areas	Wales	Aluminum reduction plant	[65]

* a.w.= ash weight

Table 2. Levels of fluoride in the tissues of wild birds.

Species	Mean level in tissue in µg F/g dry weight (d.w.)	Location	Comments	Reference
Black-crowned night heron, ***Nycticorax nycticorax***	5409-6042 a.w.* in femurae of adults	Pocatello, Idaho	Phosphate processing complex	[44]
Grey heron, ***Ardea cineria***	980.9 in femurae	Britain	Collected on roadsides	[41]
White-faced heron, ***Ardea novaehollandiae***	1006-3264 a.w.* in bone	New Zealand	Natural background levels	[46]
Kestrel, ***Falco tinnunculus***	1568 in femurae	Britain	Collected on roadsides	[41]
Kestrel	994-1482 in femurae	Britain	Country-wide collection	[39]
Merlin, ***Falco columbarius***	2693 in femurae	Britain	Collected on roadsides	[41]
Sparrow hawk, ***Accipiter nisus***	2051 in femurae	Britain	Collected on roadsides	[41]
Sparrow hawk	1594-2055 in femurae	Britain	Country-wide collection	[39]

* a.w.= ash weight

Table 2. Levels of fluoride in the tissues of wild birds (cont'd).

Species	Mean level in tissue in µg F/g dry weight (d.w.)	Location	Comments	Reference
Harrier hawk, ***Circus approximans***	379-4775 a.w.* in bone	New Zealand	Natural background levels	[46]
Barn owl, ***Tyto alba***	112.7 in femurae	Britain	Collected on roadsides	[41]
Barn owl	137 in femurae	Britain	Country-wide collection	[39]
Tawny owl, ***Strix aluco***	531.6 in femurae	Britain	Collected on roadsides	[41]
Tawny owl	606 in females, 640.2 in male femurae	Britain	Country-wide collection	[39]
Tawny owl	240 a.w.* in eggshells	Germany	Near an aluminum plant	[47]
Blue grouse, ***Dendragapus obscurs***	321 in femur	Montana	Uncontaminated site	[55]
Ruffed grouse, ***Bonasa umbellus***	128 in femur	Montana	Uncontaminated site	[55]
Sage grouse, ***Centrocerus urophasianus***	216 in femur	Montana	Uncontaminated site	[55]

* a.w.= ash weight

Table 2. Levels of fluoride in the tissues of wild birds (cont'd).

Species	Mean level in tissue in µg F/g dry weight (d.w.)	Location	Comments	Reference
Sharptail grouse, ***Pedioecetes phasianellus***	97 in femur	Montana	Uncontaminated site	[55]
Spruce grouse, ***Canachites canadensis***	177 in femur	Montana	Uncontaminated site	[55]
Magpie, ***Pica pica***	180 in nestling femurae from control site, 475 in femurae from contaminated site	Wales	Near an aluminum reduction plant and a control site	[42]
Magpie	536 in femur	Montana	Uncontaminated site	[55]
Great tit, ***Parus major***	24-279 a.w.* in eggshells	Germany	Near an aluminum plant	[47]
Starling, ***Sturnus vulgaris***	157-1390 a.w.* in bone	New Zealand	Natural background levels	[46]
Hedge sparrow, ***Prunella modularis***	1021 a.w.* in bone	New Zealand	Natural background levels	[46]
Black-backed gull, ***Larus dominicanus***	986-1689 a.w.* in bone	New Zealand	Country-wide collection	[45]

* a.w.= ash weight

Table 2. Levels of fluoride in the tissues of wild birds (cont'd).

Species	Mean level in tissue in µg F/g dry weight (d.w.)	Location	Comments	Reference
Black-backed gull	754-3140 a.w.* in bone	New Zealand	Natural background levels	[46]
Red-billed gull, ***Larus novaehollandiae***	1404-8003 a.w.* in bone	New Zealand	Country-wide collection	[45]
Red-billed gull	1058-8050 a.w.* in bone	New Zealand	Natural background levels	[46]
Adélie penguin, ***Pygoscelis adeliae***	9600 in femur	Antarctica	Uncontaminated area	[143]
Gentoo penguin, ***Pygoscelis papua***	10290 in femur	Antarctica	Uncontaminated area	[143]

* a.w.= ash weight

Table 3. Effects of Fluoride Exposure on Wild Mammals.

Species	Concentration To Produce Response	Response	Comments	Reference
Silver fox, ***Vulpes vulpes***	97-136 ppm F in diet	Agalactia in vixens, mortality of kits	Foxes kept at fox farms	[132]
White-tailed deer, ***Odocoileus virginianus***	F emissions of 200-250 kg/day	Mild dental fluorosis	Aluminum plant in South Carolina	[59]
White-tailed deer	25-50 ppm F as NaF in the diet for 2 yrs.	Lesions in developing incisors, hyperostoses of femurae	Captive study	[144]
White-tailed deer	F water content 1000-1200 ppm, vegetation 36 ppm	Emaciation, dental fluorosis, osteofluorosis	Unspecified location	[40]
Bison, ***Bison bison***	F content of vegetation, 5-430 ppm	Dental fluorosis, osteofluorosis	Yellowstone National Park, Wyoming	[58]
Elk, ***Cervus canadensis***	F content of water .51-24 mg/L, vegetation 4.9-127 ppm	Dental fluorosis, osteofluorosis	Utah, Wyoming, Montana	[58]
Black-tailed deer ***Odocoileus hemionus columbianus***	F content of browse 44-334 ppm	Tooth wear; chalky, roughened, bone surfaces; thickened humeri	Washington, near an aluminum plant	[136]

Table 3. Effects of Fluoride Exposure on Wild Mammals (cont'd).

Species	Concentration To Produce Response	Response	Comments	Reference
Mule deer, ***Odocoileus hemionus***	5.2-127 ppm F in vegetation	Dental and bone lesions	Uncontaminated site in Western USA	[138]
Mule deer	1500 ppm in pre-molars	Rapid, irregular tooth wear	Area contaminated from a steel mill	[53]
Deer mice, ***Perommyscus maniculatus***	38, 1065, 1355, 1936 ppm F in diet	Weight loss, mortality, bone thickening, tooth wear	Captive study	[145]
Bank vole, ***Clethrionomys glareolus***	47-97 ppm F in a diet of krill, ***Euphausia superba***	Reduced number of litters, high mortality of offspring, degeneration of seminiferous tubules in high F groups	Captive study	[111]
Field vole, ***Microtus agrestis***	minimum of 2500 ppm in the skeleton	Loss of enamel, pitting, discoloration, uneven tooth wear	Aluminum reduction plant	[140]

Table 3. Effects of Fluoride Exposure on Wild Mammals (cont'd).

Species	Concentration To Produce Response	Response	Comments	Reference
Wood mouse, ***Apodemus sylvaticus***	minimum of 2500 ppm in the skeleton	Total loss of enamel, uneven tooth wear	Aluminum reduction plant	[140]
Mole, ***Talpa europaea***	Not stated	Extreme tooth wear, accretion of hard material on canines	Up to 15 km from aluminum plant	[140]
Mink, ***Mustela vison***	111.5 and 287 ppm F on wet weight basis in diet	Some teeth discolored, gross osteofluorosis	Captive study	[146]

Table 4. Effects of Fluoride Exposure on Wild Birds.

Species	Concentration To Produce Response	Response	Comments	Reference
Weaver bird, ***Ploceus phillipinus***	NaF, 5gm/100g body weight, IM injection	Increased cholesterol and ascorbic acid after treatment, reduction in catecholamines, depletion of neurosecretory material	Captive study	[77]
House martin, ***Delichon urbica***	NA	Nesting birds absent around sources of F emission	Czechoslovakia, near an industrial complex	[147]
Black-crowned night heron, ***Nycticorax nycticorax***	540-11,000 µg/g F in femurae (ash wt), environmental levels not given	Increased bone diameter, diminished bone strength in adults and young	Idaho, effluent from a phosphate plant	[44]
Starling, ***Sturnus vulgaris***	6-160 mg F/kg body weight, oral dose to nestlings	Mortality in high dose groups, slower growth in 13, 17 mg F/kg groups	Captive study	[148]
Screech owl, ***Otus asio***	0, 40, 200 ppm NaF added to diet of breeding adults	Lower reproductive success in 200 ppm group	Captive study	[133]

Table 4. Effects of Fluoride Exposure on Wild Birds (cont'd).

Species	Concentration To Produce Response	Response	Comments	Reference
Screech owl	same as above	Lower egg weight and length in 200 ppm group, also hatchlings were smaller, shorter tibiotarsus	Captive study	[134]
American kestrel, ***Falco sparverius***	0, 10, 50, 500 ppm NaF in diet of breeding pairs	Thicker eggshells and higher F content in dosed pairs, higher bone F in dosed birds	Captive study	[149]
American kestrel	fed kestrels cockerels with 62, 4512, 7690 ppm F in the femurae	F content of eggshells increased with F dose, smaller clutch size and lower fertility with increasing dose	Captive study	[135]
Adélie penguin, ***Pygoscelis adeliae***	diet (krill) contains 300-500 mg F/kg w.w.	No apparent impact on health or reproduction	Antarctica	[143]
Mallard duck, ***Anas platyrhynchos***	40, 80 mg NaF/kg/d.	Increased F excretion from the salt gland and cloaca	Captive study	[150]
Snow goose, ***Chen caerulescens***	industrial effluent with 6750 mg/L F	Mortality due to internal haemorrhaging	Boron purification plant, Oklahoma	[43]

Table 5. Effects of exposure to fluoride on reproduction in mammals

Species	Exposure	Effect	Ref.
Domestic cattle, ***Bos taurus***, both sexes	Exposed in the field, exposure not quantified (up to several yrs.), forage contained estimated 40-50 ppm F^-	Sterility in both sexes, agalactia, poor survival of young	[124]
Domestic cattle, both sexes	Up to 880 ppm F^- in feed (weight basis not specified)	Delay of oestrus after parturition, agalactia in high fluoride exposure groups	[125]
Domestic cattle, dams	5,8,12 ppm F^- in drinking water, also with added phosphate over 4 breeding seasons	Reduced fertility, anoestrus in 8,12 ppm F^- groups	[126]
Domestic cattle, bull sperm	30 mM NaF added to preparation	Immobilized sperm, reduced respiration	[151]
Domestic cattle, dams	10,28,55,109 ppm F^- dw in forage for 7.5 yr.	No effects on reproductive efficiency at any treatment level	[108]
House mouse, ***Mus musculus*** testes	10, 20 mg NaF/kg bw/d. for 30 d. orally	Seminiferous tubule diameter decreased with treatment, tubule lumens devoid of sperm. Lumen of the epididymis of treated animals devoid of sperm, denuded of cells. Vas deferens lacked sperm, was decreased in diameter. Lack of differentiation in seminiferous tubules, arrested development of sperm, tubular atrophy and necrosis.	[122]

Table 5. Effects of exposure to fluoride on reproduction in mammals (cont'd).

Species	Exposure	Effect	Ref.
House mouse, testes	10, 500, 1000 ppm NaF in drinking water for 30, 60, 90 d.	No effect of treatment on serum testosterone, testis cholesterol.	[121]
House mouse, males	0, 10, 20 mg NaF/kg bw/d. for 30 d.	Succinate dehydrogenase reduced but recovered after withdrawal of treament. ATPase and sialic acid decreased in epididymes.	[118]
House mouse, males	0, 5.2 mg F/kg bw/d. for 35 d., orally	No effect of treatment on sperm morphology	[152]
House mouse, females	Low F diet and 0, 50 ppm NaF in water for 25 wk.	Mice on low flouride regimen produced significantly fewer litters in the 1st and 2nd generations	[116]
House mouse, females	Low F diet and 0, 50, 100, 200 ppm NaF in water for 25 wk.	Mice on low F regimen produced fewer litters with longer time between litters and later age at first conception for the 2nd generation	[117]
House mouse, females	Low F diet or diet with 2 ppm NaF added for 3 generations	No effect of diet on production of litters which may be attributed to F deficiency. Previously noted effects of F on reproduction may be linked to an iron deficient diet.	[109]
Rat, ***Rattus norvegicus*** both sexes	0, 500, 1000, 1500, 2500 ppm NaF added to the diet for 9 mo.	Reduced reproduction, growth and incidence of mortality in 2 highest dose groups. Threshold level for reproductive effects estimated at 250 ppm NaF added to diet.	[115]

Table 5. Effects of exposure to fluoride on reproduction in mammals (cont'd).

Species	Exposure	Effect	Ref.
Rat, both sexes	0, 430 ppm NaF, 600, 1000 ppm rock phosphate added to the ration for 5 generations	Agalactia, disruption of estrus, possibly through inanition. No treatment had a direct effect upon reproduction	[107]
Rat, both sexes	5, 30 mg F^-/kg as NaF for 1 mo. (route not specified).	Inhibition of spermatozoid function, loss of fertility in males and females	[153]
Rat, males	0, 100, 200 ppm F^- as NaF in drinking water for 6, 16 wk.	Reduction in testicular plasma Zn. Vacuolization of germinal epithelium in both exposure groups after 6 and 16 wks.	[154]
Rat, males	0, 5, 10 mg/kg bw NaF/d. orally for 30 d.	10 mg/kg group exhibited reduced sperm motility, count, fertility rate, succinate dehydrogenase activity, ATPase, sialic acid. No effect on serum testosterone, cholesterol.	[119]
Rat, males	Single injection of 0, 50 µg NaF/50 µL distilled H_2O into vas deferens	No effect on testis weight, protein, cholesterol. Decrease in sperm motility, count, fertility in treated rats, including absence of sperm in tubule lumens.	[120]
Rabbit, ***Oryctolagus cuniculus*** males	0, 20, 40 mg NaF/kg bw/D. for 30 d. orally, then withdrawn	Sperm motility, count decreased with increasing fluoride dose. Fertilizing capacity reduced to 33% and 0% for 20 and 40 mg doses. Reduced activity of epididymal ATPase, SDH.	[123]

Table 5. Effects of exposure to fluoride on reproduction in mammals (cont'd).

Species	Exposure	Effect	Ref.
Fox, ***Vulpes vulpes*** both sexes	98-137 ppm F^- in the diet for several years.	All vixens experienced agalactia with resulting kit mortality.	[132]
Fox, both sexes	Decreasing fluoride in the diet from 108 to 9 ppm.	Transplacentally exposed vixens produced fewer kits. Diet-exposed vixens exhibited limited recovery.	[110]
Sheltie dog, ***Canis domesticus*** both sexes	460 ppm F^- in the diet for 2 yr.	No effects on reproduction which could be attributed to fluoride exposure.	[155]

Table 6. Effects of exposure to fluoride on reproduction in birds.

Species	Exposure	Effect	Ref.
Domestic chicken, ***Gallus gallus*** females	0-1300 ppm NaF in the diet for 112 d.	200 ppm - positive effect on egg prod. 400 ppm - reduction in egg prod Dietary F had no effect on fertility or hatchability	[129]
Domestic chicken females	0-1300 ppm F with 0-1040 ppm Al in the diet for 112 d.	1300 ppm - reduction in egg prod.	[156]
Domestic chicken males and females	0, 100 ppm in the water from 0-20 wk, 20 + wk, 0-20+ wk.	No effects on egg production, fertility duration of fertility hatchability	[128]
Domestic chicken females	0,300,600,9001200 ppm NaF in the diet for 8 wk.	900,1200 ppm produced reductions in egg production	[113]
Domestic chicken females	0-1300 ppm F as NaF for 252 d.	>700 ppm produced significantly fewer eggs	[114]
Domestic chicken females	500 ppm F as NaF with monosodium phosphate (MSP) for 16 wk.	Egg production increased significantly with addition of F and MSP	[157]
Domestic chicken males	440, 800 1200 ppm NaF added to ration for 24 wk.	Leydig cell atrophy, degeneration of germinal layer of seminiferous tubules, reduction of spermatozoa in tubules in 800, 1200 ppm groups	[131]
Screech Owl, ***Otus asio*** both sexes	0, 40 200 ppm NaF added to ration for 5-6 mo.	Hatching success reduced by 40% in 200 ppm group, same group had sig. smaller egg weight and vol., no difference in no. of eggs produced	[134]

Table 6. Effects of exposure to fluoride on reproduction in birds (cont'd).

Species	Exposure	Effect	Ref.
Screech owl, both sexes	0, 40, 200 ppm NaF added to the diet	200 ppm pairs produced significantly fewer fertile eggs, fewer young/clutch, no effect on eggshell thickness	[133]
American kestrel, ***Falco sparverius*** both sexes	Fed cockerels containing 62, 4512 or 7690 ppm F^- in their femurae for 10 d.	Produced fewer eggs/clutch, decreasing fertility with increasing dose	[135]
American kestrel, both sexes	10, 50, 500 ppm NaF mixed in diet	No significant effect on fertility or hatchability	[149]

Chapter 2.

The effects of orally administered sodium fluoride on the reproductive performance of captive male American kestrels

ABSTRACT

In order to determine the effects of fluoride on avian reproductive performance, four groups of 10 male American kestrels (*Falco sparverius*) were exposed to one of three levels of sodium fluoride (1.65, 3.3, 6.6 mg NaF/D) or a control solution of $NaHCO_3$ via oral gavage for 45 days. Upon completion of dosing each male was paired with an untreated female and the number of reproductive behaviours as well as the proportion of fertile eggs produced was recorded. In addition, circulating testosterone levels, gonado-somatic indices and bone fluoride levels were determined. In a second test the same procedures were followed except the treatment period was shifted ahead so that dosing continued for 15 days after the birds were paired. Statistical analysis of femoral fluoride burdens indicated a significant increase with increasing fluoride treatment. Femoral fluoride levels were comparable to those measured in free-living birds collected from fluoride-contaminated environments. However, there was no effect of fluoride treatment on subsequent production of fertile eggs during either test period, although there was a dose-dependent decline in the proportion of fertile eggs produced during the second trial. Circulating testosterone levels were found to vary significantly over the breeding season but not among treatments. Reproductive behaviours recorded during the two test periods were found not to vary significantly among treatments. Similarly there was no difference in gonado-somatic indices related to dose. Although the fluoride burdens induced in this study were comparable to those measured in the wild there was no effect on reproductive performance of captive kestrels. It is unlikely that male American kestrels inhabiting contaminated environments would suffer reduced reproductive success as a result of exposure to these levels of fluoride.

INTRODUCTION

Fluoride is widely distributed in the environment [1], occurring in soil, water and air. As a result, plants and animals taken from fluoride-contaminated environments have been found to contain fluoride burdens elevated above those measured in uncontaminated areas [2-4]. The significance of fluoride exposure to biota has been evaluated and reported in a number of reviews [1,5-9]. The suite of symptoms associated with chronic exposure to fluoride (referred to as fluorosis or fluoride toxicosis) include lameness, swelling of joints, inanition, mottling of the dentition and general loss of condition [10,11]. These responses have been extensively documented in domestic cattle exposed to fluoride through the consumption of contaminated fodder and mineral supplements.

In addition to reports regarding domesticated animals, numerous authors have documented levels of fluoride in the calcified tissues of various wildlife species collected in the vicinity of anthropogenic sources of fluoride [2,12-14]. The effects commonly associated with exposure in wildlife are consistent with those found in domestic animals.

Laboratory studies investigating the beneficial properties of dietary fluoride on domestic fowl documented reduced egg production and sperm quality as a result of exposure to fluoride [15-17]. Generally, the contribution of male reproductive dysfunction to lowered egg fertility in caged layers has not been extensively investigated. Similar results were obtained in several laboratory rodent studies, the first of which was conducted in 1925 and noted reproductive impairment in rats exposed to fluoride added to the ration [18]. More recently, numerous studies have demonstrated various impacts resulting from fluoride exposure, including deterioration in sperm quality and number and histological damage to the testes in male laboratory rodents exposed to fluoride [19-21].

While a number of effects of fluoride exposure on free-living wildlife have been

documented, the results obtained have been biased towards endpoints which may be easily measured during routine necropsy of moribund individuals. In addition, numerous studies have documented elevated fluoride levels in the skeletons of individuals removed (primarily by trapping) from suspected contaminated areas. However, these sampling methods are not conducive to the investigation of reproductive effects on exposed individuals or breeding pairs. There are numerous logistical and economic considerations which make reproductive assessments of free-living populations difficult to carry out. As a result, while the impacts of fluoride exposure on a variety of reproductive endpoints have been well established in laboratory and domestic species, the significance or even the occurrence of these lesions in free-living wildlife are poorly documented.

In addition to sampling biases, the emphasis of fluoride research on animals was originally centered on cattle and effects associated with consumption of fluoride-contaminated forage or mineral supplements. While the principal endpoints investigated were feed conversion, agalactia and lameness, several authors [22-24] also discussed potential impacts on reproduction from an animal husbandry perspective (i.e. the number of services required for conception). Accordingly, when the first investigations of fluoride effects on wildlife were conducted in the 1950's, discussion focussed primarily on endpoints previously investigated in domestic animals: osteofluorosis and mottling and erosion of the dentition.

The potential impacts of exposure to fluoride on reproduction in free-living wildlife are extremely difficult to demonstrate conclusively. Many factors, including diet, age, body condition and exposure to other xenobiotic compounds are known to impair reproductive performance in birds. Furthermore, demonstrating exposure to fluoride through tissue analysis and measuring reduced production of fertile eggs in the wild does not prove causality but merely a correlation between two

seemingly related events. Studies designed to assess impacts of fluoride exposure on reproduction would, by design, be more costly and complex than merely removing animals from a contaminated site. Thus, demonstration of causality could prove to be problematic.

As a result of the logistical and financial considerations inherent in conducting long-term field studies and the traditional emphasis placed on endpoints related to calcified tissues, the effects of exposure to fluoride on reproductive success of free-living wildlife are virtually unknown.

In order to determine if wildlife species are at risk, several studies involving wild birds held in captivity have been conducted. The limited results obtained to date indicate that wild birds, when exposed to fluoride, experienced reduced reproductive performance. In each of these studies both members of the breeding pair were exposed to fluoride in the diet. The emphasis was to document impacts on the production of fertile eggs by the female. However, results obtained in research on rodents (and to a limited extent on domestic fowl) indicate the male's fertilizing capacity may be compromised as a result of chronic fluoride exposure. While reduced egg fertility may, in fact, reflect compromised male reproductive performance, this possibility has never been investigated.

Using the American kestrel (*Falco sparverius*) as a model species, the purpose of this research was to determine the contribution of the male of a breeding pair to potential reproductive failure resulting from exposure to fluoride. Various aspects of the male reproductive system were investigated including fertilizing capacity, levels of circulating sex steroid levels, reproductive behaviours and gonado-somatic indices. Unlike previous studies, all tested males were exposed to a known amount of fluoride. Information obtained as a result of this study will permit more effective evaluation of the risk experienced by wildlife inhabiting areas known to be contaminated with fluoride of anthropogenic or natural origin.

MATERIALS AND METHODS

In two separate years 40 male American kestrels were randomly selected from the pedigreed colony maintained at the Avian Science and Conservation Centre of McGill University. Each winter males were housed communally in large flight pens (6x9x2.4 m) holding approximately 30 birds at ambient photoperiod and temperature. Birds were fed day-old cockerels at the rate of 1.5 cockerels/kestrel/day. Three times per week a pre-mixed vitamin and mineral supplement (SA-37, Rogar/STB) was smudged onto the exposed breast muscle of the chicks. On 15 March of the first year and 1 April of the second the experimental males were placed in individual holding pens where dosing with several levels of fluoride or a control solution was initiated. In both years fluoride administration continued for 45 days. In the first year dosing was completed prior to pairing while in the second year males continued to receive fluoride for 2 weeks after pairing. The holding pens were maintained under ambient natural photoperiod with light supplied by incandescent bulbs.

Treatments

Due to a shortage of one year old birds, older males were included in the experimental design for the first trial. The older age class birds were randomly assigned to all treatments. During the second trial only one-year-olds were used in all dose groups. The age structure of each treatment group for both years of the study is given below. In order to eliminate the potentially confounding effects of prior experience, only females which had been previously paired were incorporated into the study.

Treatment-year	n	Age (number of birds)
Control-trial 1	10	1(6), 6(1), 8(1), 9(2)
1.65 mg NaF/D-trial 1	10	1(4), 2(2), 3(1), 4(1), 8(1), 10(1)
3.3 mg NaF/D-trial 1	10	1(10)
6.6 mg NaF/D-trial 1	10	1(7), 3(1), 7(1), 10(1)
Control-trial 2	10	1(10)
1.65 mg NaF/D-trial 2	10	1(10)
3.3 mg NaF/D-trial 2	10	1(10)
6.6 mg NaF/D-trial 2	10	1(10)

The range of doses used in this study were selected to be non-lethal but adequate to potentially impact reproductive performance based on the studies of Pattee *et al.* [25] who exposed eastern screech owls (*Otus asio*) to fluoride by adding up to 200 ppm NaF to their daily food ration. Adult kestrels consume approximately 25-30 g of food per day which is similar to the screech owls in the captive study. At that rate the kestrels would have consumed 6 mg F per day. Because of the reported higher toxicity of oral doses versus dietary administration [26] the levels used in this study were halved. In addition, a pre-trial exposure study involving six male kestrels per treatment was conducted using the above regime as well as a 13.2 mg NaF/D dose. There was no significant weight loss or mortality in any of the treated birds except the high dose group where two males died. Since the objectives of the study were to investigate sub-lethal effects of fluoride exposure, the 13.2 mg NaF/D treatment was dropped from subsequent experiments. Based on a mean weight of 111 g [27] and actual fluoride consumption of 0.75 to 3.3 mg per day, kestrels in this study were dosed at a maximal rate of approximately 27 mg/kg or half the 16 day LD_{50} calculated for common quail (*Coturnix coturnix*) by Fleming *et al.* [28] where chicks were administered fluoride in the same manner used here.

In order to overcome biases inherent in mixing fluoride in food, feeding to prey species and/or differences in food consumption among experimental birds, all doses were administered orally by gavage to each bird daily. While this method was labour-intensive it confirmed the dose received by each bird and reduced significantly the variability within and among treatment groups when determining actual fluoride exposure. However, fluoride administered in an aqueous vehicle does not simulate a natural route of exposure for wild animals which would normally be via ingestion of contaminated tissues, water or particulates adhering to vegetation, soil or pelage.

When the males were placed in the holding pens each was administered a daily dose of sodium fluoride using a stainless steel, curved gavage tube mounted on a 3 ml plastic syringe. Dosing occurred in the morning approximately one hour after the birds were given food. In this way the fluoride solution would mix with the food in the stomach, thereby reducing contact with the intestinal mucosa.

The amount of sodium included in each treatment was equalized to prevent any potentially confounding effects occurring as a result of differences in sodium uptake. Males in the control group received 13.19 mg $NaHCO_3$ mixed in 0.5 ml double distilled water daily. The 1.65 and 3.3 mg NaF treatments also contained added sodium bicarbonate to equal the amount of sodium in the high fluoride treatment. The high fluoride treatment contained only sodium fluoride. The dose groups are referred to by the weight of NaF in the solution. However, the actual mass of fluoride may be obtained by dividing the weight of the salt by the ratio of the atomic weights of NaF and F (2.2 in this case). The composition of the four treatments and the amount of elemental fluoride contained in each is given below. All dosing solutions were made in volumes adequate for the entire study and were stored in plastic containers. Fluoride concentrations were confirmed using a fluoride electrode [29].

Dose	NaF (mg)	F (mg)	$NaHCO_3$ (mg)	Total Sodium (mg)
Control	0	0	13.189	3.614
Low	1.65	0.75	9.892	3.614
Middle	3.3	1.5	6.595	3.614
High	6.6	3.3	0	3.614

Beginning at the initiation of dosing and continuing throughout the study in both years, weekly blood samples were taken for subsequent determination of circulating testosterone levels. A one ml sample was removed from the brachial vein of each male in the late morning, between 9 and 12 a.m. Testosterone has been shown to exhibit diurnal variability in the domestic fowl [30,31], and the Japanese quail [32]. While this has not been demonstrated for the American kestrel (or any other raptor), all blood samples used in this study were taken at the same time of day to minimize any potential effects of collection time on testosterone levels.

Twenty birds were bled per day on two consecutive days. All samples were centrifuged and the plasma stored frozen for subsequent determination of testosterone. In trial 1 each male was weighed at the beginning of the study and again at necropsy. In trial 2 birds were weighed weekly, concurrent with blood collection.

Treated males and untreated females were paired randomly while avoiding related matings. Both birds were placed in the pen simultaneously and observed periodically for the rest of the day and were separated if aggressive encounters were noted.

Pairs were placed in breeding pens on 1 May for the first trial and 6 May of the next year for the second trial. Pens were constructed of plywood with heavy gauge wire mesh ceilings and floors and were 30 cm off the ground. Half the ceiling was also covered with rigid plastic to provide protection from the rain and sun. Pens measured 2.4 x 1.2 x 2.4 m (LxWxH) and were equipped with a nestbox, two rope perches and a wooden perch. The front of each pen had an access door and a 10 x 30 cm one-way glass observation window. Pens were built in two rows of 20 with a common corridor. While birds in separate pens were not in visual contact they could hear each other.

Behavioural data were collected during both years of the study and were based on the repertoire of reproductive behaviours described by Willoughby and Cade [33]. Four behaviours routinely observed and associated with kestrel reproduction were selected: (1) nest box activities where the male enters the box and by vocalizing and rearranging the nesting material, tries to attract the female to the box; (2) number of copulations; (3) vocalizations, typically a loud rolling 'chirr' usually performed by the male; and (4) food transfers where the male holds a piece of food in his beak and, while perching beside the female on a perch, offers it to her.

The observer stood on a wooden platform in front of the pen in a darkened hallway and observed the birds through the one-way glass window. The birds were always visible except when inside or on top of the nestbox. Emphasis was placed on recording the activities of the male. Comments on the female were restricted to noting any unusual behaviours or antagonistic interactions. The treatment order was randomly established each day. Pens were then randomly selected, one from each treatment and observed for a 3 minute period during the first trial and a 10 minute period during the second. The observation period lasted typically for 1 1/2 to 2 hours. Observations were often conducted after the birds were fed in the morning, as the presence of food increased the likelihood of

observing reproductive behaviours, and throughout the day thereafter. During the first trial birds were observed twice per day for 16 days. In the subsequent year observations were conducted once per day for 21 days. The change from two to one period per day was to decrease the proportion of the recording time where the pair appeared to be adjusting to the presence of the observer. During both years observations commenced after the birds were paired and ended when females had begun clutch formation (anywhere from 8 to 21 days later). If it was obvious to the observer that the birds were aware of his presence the observation period was stopped and the subsequent period was focused on another pair.

Nestboxes were checked daily and freshly laid eggs were individually marked, measured and recorded on data sheets. Four days after production of the last egg (usually the fifth) the males were removed from the pens and sacrificed. Kestrel eggs require at least four days of natural incubation prior to being placed in an artificial incubator to maximize subsequent hatchability (D. Bird, unpublished data). Upon removal of the male, eggs were measured and placed in modified Marsh Farms Roll-X incubators at 37.5°C and 55% relative humidity. Fertility of incubated eggs was assessed and recorded at day 14 of incubation using a candling light.

All males were weighed and killed by cervical dislocation. Testes were removed, weighed and measured, cut into 1mm slices and placed in Bouin's fixative. Gonadosomatic indices (GSI) were calculated for each bird by dividing combined testes weights by body weights and multiplying by 100.

Fluoride determination

In the first year of the study fluoride levels were determined for the diaphyseal section of the left femur of each bird following the method of Singer and Armstrong [29]. At dissection both femurae were removed and cleaned of all

attached tissue, weighed, and length and width measurements taken. The diaphyseal sections of the left femurae were placed in pre-weighed crucibles and ashed in a muffle furnace at 600°C for 12 hours. Femurae were then re-weighed to obtain ash weights. The ashed diaphyses were then placed in labelled tubes containing 6 ml of 0.5M $HClO_4$. The dissolved diaphysis were divided into 3 subsamples of 2 ml $HClO_4$. Ten ml of TISAB (total ionic strength adjusting buffer) were added to each subsample which brought the pH up to approximately 5.4. This buffer is used to ensure there is minimal interference from other ions in the solution. TISAB was made from with the following recipe: To 57 mL glacial acetic acid, add 58 g NaCl and 1.0 g CDTA (cyclohexane diamine tetraacetic acid). Dilute to 500 mL with H_2O. Adjust the solution to pH 5.8 with 5 M NaOH and complete to 1 L with H_2O.

A series of standard fluoride solutions was prepared using serial dilutions of Orion 100 ppm F^- standard solution. Standards of 100, 10 and 1 ppm were prepared, containing equal parts TISAB (deionized double-distilled water was used throughout). An Orion fluoride electrode and a digital mv/pH meter were used to determine the fluoride concentration in each of the standard solutions. The electrode was placed in each of the 3 standard solutions and a millivolt reading taken when the meter had stabilized (15-40 minutes). A standard curve was then created based on the mV readings obtained from the fluoride electrode. In this way an equation could be developed which predicted the fluoride concentration in a solution based on the mV reading from the electrode. New standard curves were created each day.

Each of the three subsamples was read three times, so that each sample had nine separate mV readings. These were averaged to obtain one mean mV value which was used to calculate the fluoride concentration of the solution from the standard curve. The total amount of fluoride in the solution was assumed to originate from the bone sample. The concentration of fluoride in the bone could

be determined using the absolute amount of fluoride contained in the solution.

Testosterone determination

Circulating plasma testosterone levels in male American kestrels were measured using a direct serum, double antibody radioimmunoassay procedure modified by Sanford [34] based on a procedure reported by Schanbacher and D'Occhio [35]. This method was validated for the American kestrel by Rehder *et al.* [36]. Duplicate 50 µl plasma samples were cleaned up with charcoal to remove endogenous steroids and placed in reference tubes. Testosterone antibody (produced in rabbits to testosterone-7 -butyrate-bovine serum albumin conjugate), which was known to cross react with dihydrotestosterone (35%), was then added to the tubes followed by 100 µl of labelled testosterone in PBS buffer. After incubation, free and bound testosterone fractions were separated by addition of 100 µl of anti-rabbit gamma globulin serum. Following further incubation and centrifugation at 2000Xg, the pellet was resuspended and radioactivity contained in the pellet counted in a Beckman liquid scintillation spectrometer. The minimum detectable level of testosterone was 0.19 ng/ml for the first trial and 0.20-0.23 ng/ml for the second. Intra- and inter-assay coefficients of variation were 3.98 and 5%, respectively, for trial 1 and 6.03 and 4.71%, respectively, for trial 2.

Statistical analysis

A Fisher's exact test [37] was used to measure differences in the number of pairs producing eggs among treatments and between years. Egg fertility data were expressed in proportions and transformed using the Freeman-Tukey binomial transformation [38], a modification of the arcsin square root transformation. Data were then analysed using analysis of variance (ANOVA) in the GLM procedure of the SAS® software program [39]. Dunnett's test [40] was then used to determine the effect of dose on significant main effects.

Data to calculate the time to produce the first egg were transformed using natural logs to approximate a normal distribution and standardize variance among treatment means. Differences between treatments were analysed using ANOVA in the GLM procedure. Correlation indicated the relationship between the time to produce the first egg, number of eggs produced and the proportion of fertile eggs.

The testosterone data were tranformed using natural logs and analysed using a repeated measures ANOVA for main effects and a two way ANOVA for within-week effects where warranted.

Behavioral data were log-transformed as required. However, as variances were not equal and data were not normally distributed, differences among behaviours were measured using Fisher's exact test. Cumulative data were log-transformed, ranked and analysed using ANOVA on ranks.

Linear regression (REG procedure in the SAS® program) was used to analyse the relationship between left and right testicular masses. Analysis of variance was used to determine the relationship between body weight, GSI and dose. Analysis of covariance (ANCOVA) measured the effect of time from pairing to necropsy and dose on gonad mass. Multiple linear regression was used to calculate the effects of the two continuous variables, time to necropsy and body weight on testis mass and GSI.

Bone fluoride levels were log transformed and analysed using ANOVA and, where necessary, analysis of covariance was used to account for differences in age of the males within treatments. Spearman rank correlation coefficients (r_s) were calculated for bone fluoride levels and reproductive behaviours using the SAS® FREQ procedure.

RESULTS

Although 10 males were assigned to each treatment group and each was subsequently paired with an untreated female, the number of pairs within and among dose groups which produced eggs was variable (Table 1). However, a Fisher's exact test revealed no significant differences among treatments for both years.

Reproductive success

A total of 304 eggs was laid by all treatment groups over the two years of the study (140 the first year, 164 the second year). The number of eggs produced between years did not vary significantly ($p=0.15$), although the production of the untreated pairs was lower during the first year (32 eggs) than the second (44 eggs). However, the 'dose' effect of the ANOVA model was significant. A Dunnett's test revealed that, among the treated, paired males, only the 1.65 mg NaF/D group varied from the control group by laying significantly more eggs (Table 2). Although the number of pairs producing eggs did not vary significantly, the 1.65 mg NaF/D treatment had the highest number of pairs producing eggs in both years (10 in Trial 1 and 9 in Trial 2). Accordingly, the number of eggs produced per active pair among treatments was analysed and also found to be not significantly different (Likelihood Ratio Chi-Square $p=.8$, $p=.84$ for Trial 1 and Trial 2, respectively).

The time taken to produce the first egg was noted and the mean value calculated for each treatment (Table 3). The number of days to produce the first egg after introduction of the pair into the breeding pen, of those pairs producing eggs, was significantly negatively correlated with the number of eggs produced (Pearson $r= -.33$, $p=.0061$). In order to determine if fluoride treatment resulted in a delay in the time to produce the first egg an ANOVA was performed which revealed that neither dose ($p= .14$) nor year ($p= .95$) had an effect on this variable.

(Fig. 3).

During trial 2 body weights were recorded weekly for all treated males and are presented in Fig. 4. Mean body weights recorded on the first day of dosing are significantly different among treatments. The control and low fluoride groups gained weight over the first 3 weeks of the study and then their weights decreased slowly through week 5, while the 3.3 and 6.6 mg NaF/D treatments gained slightly until the second week and then lost weight until week 5. All birds were paired, after weighing, on week 5.

In the first trial, initial male body weights were not significantly different ($p=0.144$) among treatments. However, in the second year, while the significant differences persisted into the second week, by week 3 and thereafter there were no differences in treatment body weights. A repeated measures ANOVA revealed that the 'within-subjects' effect, time, was significant. However, 'dose' did not have a significant effect on male body weights. As mentioned above, there was a significant difference in treatment group body weights during the second week. An ANOVA performed on the week 2 body weights with the first week used as a covariate (to adjust for the non-equal body weights in week 1) showed no significant effect of fluoride dose during the second week on body weight.

At necropsy the left testis was usually larger than the right and the masses were significantly correlated ($r_s=0.64, p<0.005$)(Fig.5). Accordingly, statistical analyses were conducted on combined testis weight. The relationship between combined testis mass and body mass was examined for both sample collection periods. Although there was a significant correlation between these two variables when data from both years were combined ($p=.004$), all correlations among treatments were not significant. Analysis of covariance revealed that treatment and year, when adjusted for body weight, had no significant effect on combined testes

weight. An analysis of variance of the main variables, year and treatment, on the gonadosomatic index, which expresses testicular mass as a function of body weight, indicated no significant effect.

The potential confounding effects of the time elapsed between pairing and sacrifice on testis mass were examined. A multiple regression model measured the effect of the two continuous variables body weight and time to sacrifice, on combined testis weight. While body weight had a significant effect on testis mass (Fig. 6), as would be predicted, time to sacrifice was not significant (Fig. 7).

Bone fluoride levels

Bone fluoride levels were calculated for all male kestrels in the first trial. Since quantification of bone fluoride levels is the standard method to assess exposure in biota, measurement of fluoride in the femurae of the males in this study permitted comparisons with levels measured in animals in captivity as well as in the wild. In addition, statistical analysis of bone fluoride levels measured in this study indicated the success of the treatment method in reducing variability in fluoride burdens within and among dose groups. Since the same dose rates and exposure periods were used during both trials the analysis was not repeated after the second year. As would be predicted, the addition of fluoride via oral gavage resulted in significantly elevated levels of fluoride in the calcified tissues of the treated birds. The mean levels of fluoride measured in the diaphyses of the femur presented on an ash weight basis are illustrated in Fig.8. Summary statistics are presented in Table 5.

The 1 year old birds in the control group had a mean bone fluoride level of 827 ppm, while the 6.6 mg/D group mean for 1 year-olds was 3195 ppm on an ash weight basis. Although each bird within a treatment was administered the same amount of fluoride, there was considerable variability in bone concentrations. Several males in the 3.3 mg/D treatment had femur fluoride levels which varied

by more than a factor of two. Within a treatment, younger birds often had higher femur fluoride concentrations than older birds. A 5 year-old male in the 1.65 mg/D group had 1300 ppm while a 2 year-old in the same treatment measured over 3000 ppm in the diaphysis of the femur. The coefficient of variation (cv) for each treatment is given in Table 5. The cv's for the 3.3 and 6.6 mg NaF/D groups are considerably lower than the control and 1.65 mg NaF treatments. In addition, the presence of older birds in several of the treatment groups affected the mean levels of bone fluoride in those groups. An analysis of covariance revealed that the 'age' factor, when added to the model as a covariate, was significant ($p = 0.009$). The presence of the older birds in the control and 1.65 mg NaF/D groups inflated the femur fluoride levels upward while reducing it marginally in the high fluoride exposure group.

Measured concentrations in bone were recalculated using the age estimate from the general linear model. The results indicate hypothetical concentrations which would have been measured if the birds in each treatment were 1 year old. The means for each group are presented in Figure 9.

In both years all birds were exposed to fluoride for 45 days. Fluoride doses administered to male kestrels increased by a factor of two. However, mean bone levels, adjusted for age, increased by 75% from the control to 1.65 and 1.65 to 3.3 mg /D and by 21% from 3.3 to 6.6 mg /D.

Reproductive Behaviours

Results of analyses of reproductive behaviours for both the first and second trials indicate that exposure to fluoride did not have a significant impact on the parameters measured (despite the fact that data were collected somewhat differently over the two years). Initially, observations were conducted in the morning and again later in the day during 3 minute bouts. During the second trial

each pair was observed once for 10 minutes in the late morning or early afternoon, usually within 1.5 hours of being fed. The number of behaviours observed during a standardized 10 minute period in trial 1 ranged from approximately 0.3 to 0.6. In the second year comparable figures ranged from 0.3 to 0.9. In the first year of the study all treatment groups exhibited similar levels of activity with the control group slightly more active and the 1.65 mg NaF/D group having the lowest level of reproductive behaviours. During trial 2 this trend was somewhat reversed with the low fluoride exposure group recording the most behaviours (see Figs. 10 and 11). In both years the number of copulations observed was relatively constant between groups (more copulations were observed for the 1.65 mg/D treatment during the second year, although the difference was not significant, $p=0.32$) . There were no statistically significant dose dependent trends observed for any reproductive behaviours for either year of the study. In addition, when all reproductive behaviours were combined and analysed, no significant differences between treatments were detected for trial 1 or 2 ($p=0.67$ and $P=0.21$, respectively). As would be predicted, reproductive behaviours were positively correlated with the proportion of fertile eggs produced with the exception of food transfers ($p=0.13$). However, no significant correlations were found between these behaviours and bone fluoride levels (Table 6).

Testosterone levels

Weekly testosterone levels are presented as treatment means for both years of the study and are shown in Figs. 12 and 13. A repeated measures ANOVA revealed that while time had a significant effect on log-transformed testosterone levels in both years, fluoride treatment did not. However, when the two years of data were combined, the effect of year of collection had a significant effect on weekly testosterone levels ($p<0.0001$). During the second trial circulating testosterone levels were very similar except for the 3.3 mg NaF/D group which

was elevated considerably. The significantly ($p=0.0009$) higher weekly mean for this treatment may be attributed to one individual.

In both years circulating testosterone increased after the males and females were paired. In 1987 the increase was more dramatic but not significantly so. Generally, levels dropped during egg production with the exception of the 3.3 mg NaF/D group in trial 1 which rose significantly ($p = 0.046$) in the last week of blood collection. In the first year of the study there was a large increase in circulating testosterone levels during the week prior to pairing which continued for the two successive weeks. During the second trial the increase in testosterone did not occur until the males were paired with females in breeding enclosures.

DISCUSSION

When considering the impacts of environmental contaminants on the reproductive success of a population of free-living animals, the production of healthy young, as measured by hatching success, is the most significant endpoint to measure. If the ability to produce offspring has been compromised, the determination of the mechanism of action of the compound or compounds causing the injury becomes necessary if its effects are to be mediated. While the potential for ingested fluoride to cause reproductive failure in laboratory animals has been recorded in the literature, the body of evidence for similar effects on wildlife is not as extensive nor as clear. Male American kestrels, a representative avian predator, were exposed to fluoride to determine if reproductive output and several of its correlates would be affected.

Despite exposing male American kestrels to a significant level of ingested fluoride for 45 days there was no indication that their ability to form a pair bond with the female, copulate with her and fertilize a clutch of eggs was significantly diminished.

Egg production and fertility

As was expected (the females being untreated) there was no significant difference in the number of pairs producing eggs, although the numbers were somewhat variable among treatments. As there was no dose-dependent trend in the occurrence of non-productive pairs, an effect on the males resulting from fluoride exposure which impaired their ability to form a pair bond may be ruled out. While several of the males used in the first trial were experienced breeders, a female kestrel in captivity will produce a clutch of eggs in the absence of a male, indicating previous experience is not a significant factor when considering the likelihood of laying.

As all birds were paired on the same day in each year and were placed in

identical pens, it would appear likely that environmental variables related to captive management are not the cause of some pairs failing to produce eggs. Carriere *et al.* [41] fed 24 pairs of captive American kestrels varying levels of dietary fluoride, of which 23 produced eggs. In a similar study involving administration of dietary fluoride to captive kestrels, all of 14 pairs produced eggs [42]. When 48 pairs of captive kestrels were exposed to dietary lead prior to the breeding season [43] only 1 pair did not produce eggs. Twenty-two of 25 pairs of kestrels housed in captivity produced a clutch of eggs [44], while Porter and Wiemeyer [45] reported egg production by all of 16 captive pairs. However, Rehder *et al.* [36], in a captive study, found that when 10 American kestrels of both sexes were paired, 3 failed to produce any eggs. The lower proportion of successful pairings documented here and by Rehder *et al.* [36] are not an artifact of the captive management methods common to these studies since the same facilities were used by Carriere *et al.* [41] and Bird and Massari [42] who reported a high ratio of pairs producing eggs.

Numerous variables such as changes in weather, food availability and disturbance may affect the likelihood of a pair producing eggs. Meijer and Schwabl [46] found that free-living European kestrels (*F. tinnunculus*) paired but did not produce eggs during years of low food availability. Disturbances from a number of sources may have contributed wholly or in part to the increase in the number of non-productive pairings observed in this study. In addition, as there was no mate choice on the part of either sex the potential for incompatible pairings to take place is higher than under natural conditions. Furthermore, the production of eggs by a female cannot be interpreted as a confirmation of a pair bond since unpaired female American kestrels, provided with a nest cavity, can lay a complete clutch of eggs [47].

The number of eggs laid was not affected by fluoride administration nor by the

year of the study. While Carriere *et al.* [41] assessed egg production in kestrels exposed to fluoride no statistics on differences between treatments were reported. Similarly, Bird and Massari [42] exposed breeding pairs of kestrels to dietary fluoride but did not report statistical analysis of the number of eggs produced per treatment. Regardless, in both studies there were no obvious correlations between egg production and fluoride dose.

The proportion of fertile, incubated eggs when analysed by year and treatment was not significantly different. The fertility of eggs resulting from random pairings at the Avian Science and Conservation Centre over several years was calculated by Bird and Laguë [48] to be 75% when incompatible pairs were excluded. This is comparable to the levels achieved by the control groups in both years of the study but higher than any of the fluoride-exposed treatments. Similar statistics recorded by Carriere *et al.* [41] and Bird and Massari [42], using kestrels from the same breeding facility, indicate that fertility of eggs produced by pairs exposed to fluoride was comparable to, or higher than the 75% value determined by Bird and Laguë [48]. Pattee *et al.* [25] found no significant reduction in fertility of eggs produced by pairs of screech owls fed fluoride in their diet (fertility did drop in a dose-dependent manner). Similarly, van Toledo and Combs [17] working with domestic hens found no reduction in fertility when exposing hens with up to 1200 ppm NaF in the diet. The fertility of eggs produced by White Leghorn hens was unaffected by the addition of 100 ppm NaF in the drinking water over a 15 month period [49]. Higher doses of fluoride (up to 800 ppm in the diet) also had no effect on fertility of eggs produced by domestic laying hens [50].

When estimating the risk experienced by an animal in the wild, the number of fertile eggs produced by the breeding pair is quite significant. In the first year, dosing was terminated once the birds were placed in breeding pens. Both the proportion of eggs which were fertile, as well as the number of fertile eggs

produced per active pair, was highest in the control group and increased with increasing fluoride dose (so that the high fluoride treatment was comparable to the control group). In the second year of the study, while dosing of the male kestrels continued throughout the pair formation period and into egg production, the number of fertile eggs produced per pair as well as fertility was highest in the control group and declined with increasing fluoride dose, although not significantly.

An explanation for the apparent discrepancy between the two years of fertility data collected in this study may be that the effects of fluoride on reproduction are somewhat transitory and the males in the first trial, treated only prior to pairing, may have partially recovered from any fluoride-induced toxicity prior to breeding . In fact, results obtained by Chinoy and Sequeira [51] studying the reversibility of fluoride-induced changes in the histoarchitecture of exposed mice indicate that effects on fertility are transitory and reversible.

The significance of producing fewer fertile eggs per clutch for a pair of free-living birds cannot be overstated. Using the second year data as a model, the 6.6 mg NaF/D treatment produced almost 2 fewer fertile eggs per clutch than the control group (3.9 vs. 2.2). Mortality occurring from hatch through fledging is quite low, estimated to be 13% for the American kestrel [52]. However, mortality experienced by first year birds (post-fledging) in many avian species is quite high, estimated at 31% for male sparrowhawks (*Accipiter nisus*) [53] and approximately 50% for American kestrels [54]. Accordingly, in areas where kestrels were exposed to significant burdens of fluoride during early pair formation and copulation, it is possible that they may subsequently recruit only one individual into the breeding population from a full clutch of eggs.

While the basis for the increase in fertility with increasing fluoride exposure documented in the first year is currently unknown, it is unlikely to be related to

treatment. There are no references in the literature demonstrating increases in fertility in any species with exposure to fluoride. Messer *et al.* [55] maintained that mice on a low fluoride diet (0.1 - 0.3 ppm) developed signs of fluorine deficiency as well as progressive development of infertility. Mice on a higher fluoride diet (50 ppm in the drinking water) produced more litters and at an earlier age. However, when the study was repeated by Tao and Suttie [56] the results indicated that the original diet was deficient in iron and that supplementation with fluoride increased the efficiency of iron uptake from the diet.

Attempts to demonstrate the essentiality of fluoride have been equivocal, in part because of the difficulty in producing fluorine-free diets [6,9,57,58]. While the kestrels in the control group did not receive added fluorine, there is no indication that their diet was deficient in this element. All birds received day-old cockerels as part of their diet to which vitamin supplements were added on a regular basis. Carriere *et al.* [41] measured the fluorine content of the day old cockerel femurae to be 62 ± 51 ppm on a dry, fat-free basis. Diaphyseal sections of femurae measured in adults in this study indicate that fluoride levels are comparable to those measured in the wild and are not considered to represent a deficient condition.

Behaviour

The results of behaviour observations conducted on all paired kestrels support the finding of no significant difference in the proportion of fertile eggs produced for each treatment. Although reproductive behaviours were quite variable, there were no significant dose-dependent trends among treatments in either year of the study. However, in the first year the sum of all reproductive behaviours followed a similar trend to the proportion of fertile eggs produced. A Spearman rank correlation coefficient test revealed that the two parameters were positively, but not significantly correlated ($p=0.083$). In 1987, when fertility of eggs decreased with increasing exposure to fluoride there were no significant

correlations with any behavioural endpoints. While the high fluoride dose group had the lowest proportion of fertile eggs and the fewest fertile eggs per pair, it was intermediate between the 1.65 and 3.3 mg NaF/D treatments in terms of the number of behaviours observed.

Correlating the number of copulations observed with the number of fertile eggs produced illustrates the weakness in associating these two events. Four pairs in 1985 alone were observed copulating at least once and went on to produce a clutch of infertile eggs. In fact, the most active pair (8 copulations observed) produced 5 infertile eggs and was in the control treatment. It would appear that the transfer of semen is relatively inefficient in American kestrels and multiple copulations are necessary to insure the fertility of the clutch. Balgooyen estimated 690 attempts by the male resulted in the fertilization of one clutch (based on 15 times per day for 46 days)[59]. However, only one attempt was necessary when artifically inseminating female kestrels to produce a fertile clutch of eggs [60]. Accordingly, when assessing the impact of exposure to fluoride on free-living kestrels, counting the number of copulations may be of little value in predicting the likelihood of significant impacts on reproduction.

While there are no published studies which describe the effects of exposure to fluoride on reproductive behaviours,the results of exposure to pesticides and industrial contaminants on similar endpoints are well documented. One member of each of five pairs of laughing gulls (*Larus atricilla*) was exposed to a single dose of parathion. Incubation behaviour was significantly reduced over a 48 hour period [61]. Similarly, female red-winged blackbirds given a single oral dose of methyl parathion were slower to return to the nest and spent less time incubating [62]. However, these behavioural changes did not affect subsequent hatching or fledging. Japanese quail (*Coturnix coturnix*) dosed orally with carbaryl daily were found to exhibit fewer reproductive and aggressive behaviours than vehicle-dosed control birds [63].

Fox *et al.* [64] documented reduced nest defence in herring gulls (*L. argentatus*) exposed to environmental contaminants, while Fox and Weseloh [65] noted that gulls exposed to PCBs and dioxins spent less time incubating resulting in increased embryonic mortality. Laboratory studies on ring doves revealed that pairs exposed to PCBs, DDE and mirex performed fewer reproductive behaviours and spent less time incubating than unexposed pairs. However, there was no significant difference in the number of young produced per egg laid [66]. In each case above it was hypothesized that exposure to contaminants resulted in modification of steroid hormone profiles resulting in reproductive dysfunction.

In a similar manner, if fluoride exposure were to impact reproductive behaviours, there should be a correlation between the proportion of fertile eggs produced, the number of reproductive behaviours observed and bone fluoride levels. Initially, the relationship between the number of fertile eggs laid and reproductive behaviours was examined. In fact, all reproductive behaviours, except food transfers, were significantly positively correlated with arc sine-transformed proportions of eggs laid which were fertile (Table 7). There was however, no significant relationship between male bone fluoride levels and reproductive behaviours (Table 7). Similarly, when the proportion of fertile eggs laid per egg-laying pair was correlated with the fluoride content of the males' femurae, no significant relationship was found ($p=0.70$).

These results support the previous finding of no significant relationship between fluoride dose and reproductive performance. The behaviours chosen proved to be a significant indicator of the number of fertile eggs produced and, as a result, did not correlate well with trends in bone fluoride among males. While there was no correlation between femoral fluoride burden and reproductive effects, that, in part, may reflect the body's ability to sequester and thus render ingested fluoride somewhat harmless. In fact, the skeleton can store considerable amounts of fluoride with no significant pathological change [67], so much so that bone

fluoride is essentially detoxified [68].

Testosterone levels

Testosterone levels measured in this study and analysed using a repeated measures ANOVA were not significantly different among treatments. While there are no published studies on birds, testosterone levels in rats orally exposed to fluoride (5, 10 mg/kg bw) for 30 days were not significantly different [69]. Chinoy and Sequeira [19] fed mice 10 and 20 mg NaF/D for 20 days and found no difference in serum testosterone levels.

Although there were no significant differences among treatments, testosterone levels did vary significantly over time. Testosterone secretion stimulates sexual behaviour in birds [70-72]. In this study testosterone levels peaked during the period of egg laying. Other studies have reported maximal levels of T during the period of territorial acquisition and mate defense. These results were confirmed for captive American kestrels by Rehder et al. [36]. However, in these studies the birds were paired well in advance of mating. Due to restrictions imposed by the fluoride dosing methodology, males and females were not paired until later in the breeding season (early May). The lack of a peak in circulating T prior to egg production is not likely related to the treatment but rather to a number of factors (primarily captive management practices) which, in concert, created an environment which did not adequately stimulate the males early in the study.

As males were not paired until later in the season, the lack of a female may have precluded a peak in testosterone. Moore [73] found that sexually active females caused an increase in circulating testosterone when paired with males on long days. Similar results have been documented for ring doves (*Steptopelia risoria*) [74] and pigeons (*Columba livia*) [75]. In addition, all males were housed in large, communal cages prior to fluoride administration. Zebra finches (*Poephila guttata*) were found to have lowered levels of androgen when maintained with conspecific

males than when kept singly or with a mate [76]. Finally, when all males were placed in breeding pens concurrent with females, they were visually (but not auditorially) isolated from other such males. It has been suggested that the presence of territorial males may increase testosterone levels above that necessary to maintain reproductive function [77]. The presence of an early testosterone peak corresponding to the presence of territorial males has been demonstrated in Eurasian kestrels but, interestingly was not observed in captive males [46]. The lack of a similar peak in the birds in this study may reflect an inadequate stimulus normally provided by visual contact with adjacent males. Maintaining breeding birds in captivity may, in itself, result in an overall reduction in levels of circulating steroids as has been documented by Meijer and Schwabl [46] and Wingfield [77].

Body weights

Initial body weights of male kestrels from the first trial from all treatment groups were not significantly different ($p=0.14$). However, the control and low fluoride group were significantly lighter than the two higher fluoride treatments in trial 2 ($p=0.015$). As all birds used in the study were selected randomly using data cards containing only a band number as an identifier, no biases in selection could have occurred as a result of differences in size, weight or appearance. It is possible that groups of birds from one treatment may have largely originated from one pen with a less hospitable over-winter environment than other pens (i.e. colder, more air currents, etc.) which resulted in a higher rate of weight loss. The kestrels used by Rehder *et al.* [36] were selected from the same facility and showed substantial initial weight differences, although no explanation for this difference was given.

Chinoy *et al.* [69], working with mice exposed to fluoride orally, found a dose-dependent reduction in body weight to be indicative of impending toxicity. While initial weights may have varied somewhat, body weights of male kestrels overall

were not significantly affected by the administration of fluoride but did change significantly over time. However, based on the time trend of body weights, the changes occurred in the first 3 weeks and variation after that was relatively minor and insignificant. All treatments exhibited weight loss over the test period in both years. In a study examining testosterone levels in captive male American kestrels, Rehder *et al.* [36] documented similar weight loss profiles to the males in the control and 1.65 mg NaF/D groups during the second trial where an initial increase in April was followed by a gradual decrease in weight until June. Similar decreases in body mass of captive male raptors during the breeding season have been documented by Meijer and Schwabl [46] and Pattee *et al.* [25] and for passerines in the wild by Wingfield and Farner [78] and Wingfield [79]. Mice orally administered a sub-lethal dose of NaF (5.2mg F/kg BW) for 35 days exhibited significant weight loss after day 19 [80]. In trial 2 the time*dose interaction term in the model was significant, which confirms that there were initial differences among doses. However, if the first week weight for all treatments is treated as a covariate to eliminate the effect of the different initial treatment weights, there was no significant difference in weights attributable to fluoride dose in the second or third weeks. These results indicate that the spread in weight data among treatments which occurred between weeks 1 and 3 is not related to fluoride exposure.

While there was no significant relationship between fluoride dose and body weight at necropsy, a regression of body weight on femur fluoride level for trial 1 revealed a significant ($p = 0.0052$) negative correlation (Fig. 14). However, the r^2 was quite low (0.019), indicating that the model accounted for only a small proportion of the variability in the data. The relationship between treatment and body weight was not significant because of the overlap in weights among dose groups. When the 'dose' effect was removed and bone fluoride was used as an indicator of exposure, there was a decrease in body weight associated with higher bone fluoride levels.

Gonadosomatic indices

Testes weights at necropsy were measured for all males for both years of the study. The mean ratio of left to right testicular weights was found not to vary with fluoride exposure and ranged between 1.5 and 1.8. This is consistent with the literature where, in the majority of species, the left testis is larger than the right [81]. Fluoride dose was also found to not have an effect on the mass of the testis when measured as a function of body mass (GSI). Further, when analysed separately, neither the left nor the right testis mass was significantly affected by the treatment ($p=0.12$ and $p=0.09$ respectively). Chinoy *et al.* [20] injected microdoses of NaF directly into the vas deferentia of mice and found no effect on testicular mass. Chinoy and Sequeira [19] found no difference in testes weights when mice were exposed to fluoride in the diet. Morrisey [82] evaluated numerous variables commonly measured in reproductive assessments for their ability to discriminate known reproductive toxins and found, among others, testis weight to be the most statistically powerful endpoint. However, there are few published studies which examine the effects of fluoride exposure on gonadal mass in an avian or mammalian species. Mehdi *et al* [16] and Al-Azawi *et al.* [83] reported on the impacts of fluoride exposure on the reproductive system of male chickens but did not discuss testicular mass. Chinoy and Sequeira [51] and Kour and Singh [84] examined the histoarchitecture of the testes of mice exposed to fluoride but did not report testicular masses.

Bone fluoride levels

Bone fluoride measurements were carried out in this study not to confirm the effectiveness of the method of administration but rather to permit comparison of exposure levels between treatments and with other research conducted on fluoride levels in free-living birds. Furthermore, bone fluoride has been demonstrated to be indicative of fluoride retention in the body as a whole [85]. The oral administration of fluoride used in this study proved to be relatively effective in causing a significant increase in inter-treatment bone levels, although

the deviation about the treatment means was comparable to results reported where the fluoride was administered in the diet. There are several possible explanations for the variability in bone fluoride levels. All birds were dosed 1-1.5 hours after feeding. If a male had not yet eaten, the fluoride may have been absorbed more efficiently than those birds with food in their stomachs. Second, male kestrels may have regurgitated the solution. This was noted on two occasions, but since the birds were observed only periodically, it could have occurred more frequently. There was a linear relationship between mean fluoride concentration in the femur and dose, with a 30 - 50% increase in bone fluoride when the dose was doubled. In the two studies where kestrels were dosed with fluoride in the diet, the first documented comparable bone levels in two groups, one which received 5 times more fluoride [42]. In the other study, fluoride-contaminated chicks were fed to kestrels [41]. Although the fluoride concentration of the femurae of the high dose chicks exceeded the lower dose by 50%, the femurae of the kestrels consuming chicks from both dose groups were quite similar (450 vs. 472 ppm). Pattee *et al.* [25] fed screech owls 40 and 200 ppm NaF in the diet and measured only 100 ppm more fluoride in the femurae of the high dose group (1,720 vs. 1,600) despite a five-fold increase in dietary fluoride.

In the first year of the study the age distribution of the experimental males was not uniform, although the majority of birds (27 of 40) were one year old. The mean fluoride concentration in the femoral diaphyses of this group was 827 ppm. Bird and Massari [42] measured mean concentrations in the same tissue of their control group at 515 ppm, somewhat lower than this study. However, their sample included one and two year old birds, which may account for the difference observed. The results obtained in the other study involving kestrels are not directly comparable as they were measured on a dry fat-free basis which yields a lower bone fluoride concentration.

While the maximal femur fluoride concentration measured in this study was 4,748 ppm ash weight in an individual from the 6.6 mg NaF/D group, this is well below the 'saturation point' of 15,000 - 20,000 ppm ash weight calculated by Phillips and Suttie [86].

Bone fluoride levels are usually reported on an ash weight or dry weight basis. As measurements in this study were calculated on an ash weight basis, published results presented as dry weights can be converted using a regression developed by Henny and Burke [87]. While the range of doses used in this study were calculated based on their likelihood of causing a reduction in reproductive performance, they resulted in femur burdens comparable to levels measured in birds collected from anthropogenically-contaminated sites or areas with high natural background levels of fluoride. For example, fluoride concentrations measured in black-crowned night herons collected from a fluoride-contaminated site [87] ranged from 5,400-6,000 ppm ash weight, almost double the mean of the high fluoride exposure group in this study. However, bone levels in birds of prey collected as carcasses from uncontaminated sites ranged from 870 [88] to 4,775 [89] ppm ash weight. Adelie penguins (*Pygoscelis adeliae*), known to eat a high fluoride diet, had 9,600 ppm F^- ash weight in their femurae [90].

It is important to note that elevated levels of bone fluoride measured in wild birds do not necessarily equate with a concommitant increase in toxicity. While numerous studies have surveyed bone fluoride levels in many avian and mammalian receptors (see literature review), there is no information regarding the toxic implications of those burdens. However, the elevated fluoride concentrations documented in Adelie penguins [90] were not associated with any ill effects.

While the oral route of exposure used in this study was effective in assuring that each treatment group was exposed to a known amount of fluoride, it is not an

ecologically relevant manner to administer a substance. Fleming *et al.* [26] fed starling chicks (*Sturnus vulgaris*) oral doses of fluoride for 16 days and found this route to be more toxic than dietary exposure due primarily to the significantly higher bioavailability of sodium fluoride in solution. Generally unbound fluoride in water will be more easily absorbed in the gut than organic fluoride bound in protein [91]. However, the bone fluoride levels achieved in this study mirror those documented in wild birds surveyed at a number of contaminated and uncontaminated sites. While the method of administration was more effective in delivering a known quantity of fluoride than the dietary route, the total exposure, using bone fluoride as a measure, was comparable to that documented for wild birds.

Kestrels in this study experienced a significant fluoride exposure (in excess of 3,000 ppm in the femur on an ash weight basis). While these levels are comparable to calcified tissue levels in animals removed from contaminated sites [2,3,12], the vehicle of exposure was quite different. However, skeletal burdens represent fluoride that has been absorbed and incorporated into the body irrespective of the form the fluoride was in when ingested. Kestrels, like most, if not all birds of prey produce pellets containing undigested material, including bones. While the skeleton contains the majority of the body's fluoride load [57], birds of prey surveyed in Britain were found to have appreciable fluoride burdens [88,92], indicating that skeletal fluoride is at least somewhat bioavailable to raptors. Eurasian kestrels, which consume similar prey to the American kestrel, were found to contain bone fluoride levels equal to those measured in the high exposure group in this study [92]. Unfortunately there is no indication if this level of exposure in wild birds results in reproductive toxicity. Based on the results of this research it is possible that birds of prey with similar bone fluoride burdens would also experience reduced reproductive success as documented during the second year of this study.

The American kestrels used in this study were exposed to a known amount of NaF for 45 days. It was anticipated that exposure to a significant fluoride burden would compromise the reproductive capability of the male kestrels to the extent that an effect would be seen in terms of production of young. In both years there was no significant dose-dependent reduction in the number of offspring produced as a function of the number of eggs laid. Furthermore, no significant differences were observed in various reproductive endpoints including circulating androgen levels or reproductive behaviours. The fluoride was administered in a highly bioavailable manner which would not likely be encountered by animals living in contaminated environments. Measurement of femur fluoride concentrations confirmed that the birds were significantly exposed over the test period. As a result, it is unlikely that kestrels in the wild would be exposed to environmental levels of fluoride which would result in a significant reduction in their reproductive capacity without experiencing direct toxicity.

While the decrease in reproductive success of the high fluoride exposure group documented in the second year of the study was not statistically significant, the implications of producing 2.2 fertile eggs versus 3.9 to a pair of birds attempting to recruit as many offspring into the population as possible could be quite important. Based on this, research should be carried out on free-living populations breeding in fluoride-contaminated areas to determine if they too are experiencing reduced reproductive performance as a result of exposure to fluoride.

REFERENCES CITED

1. **Smith, F.A. and H.C. Hodge.** 1979. Airborne Fluorides and Man: Part 1. *C. R. C. Crit. Rev. Environ. Control* 8:293-371.

2. **Kay, E.** 1974. An inquiry into the distriubution of fluoride in the environment of Garrison, Montana. *Fluoride* 7:7-31.

3. **Tourangeau, P.C., C.C. Gordon and C.E. Carlson.** 1977. Fluoride emissions of coal-fired power plants and their impact on plant and animal species. *Fluoride* 10:47-62.

4. **Murray, F.** 1985. Cycling of fluoride in a mangrove community near a fluoride emission source. *J. Appl. Ecol.* 22:277-285.

5. **Roholm, K.** 1937. *Fluorine Intoxication: A Clinical-hygenic Study with a Review of the Literature and Some Experimental Investigations.* H.K. Lewis and Co. Ltd. London.

6. **National Academy of Sciences.** 1974. *The Effects of Fluorides in Animals.* National Research Council. Washington,D.C.

7. **Rose, D. and J.R. Marier.** 1977. *Environmental Fluoride 1977.* NRCC 16081. Publications, NRCC. Ottawa.

8. **Smith, G.E.** 1985. A surfeit of fluoride? *Sci. Prog. Oxf.* 69:429-442.

9. **Walton, K.C.** 1988. Environmental fluoride and fluorosis in mammals. *Mammal Rev.* 18:77-90.

10. **Suttie, J.W.** 1977. Effects of fluoride on livestock. *J. Occup. Med.* 19:40-48.

11. **Shupe, J.L.** 1980. Clinicopathologic features of fluoride toxicosis in cattle. *J. Anim. Sci.* 51:746-758.

12. **Wright, D.A., A.W. Davidson and M.S. Johnson.** 1978. Fluoride accumulation by Long-tailed field mice (*Apodemus sylvaticus* L.) and Field voles (*Microtus agrestis* L.) from polluted environments. *Environ. Pollut.* 17:303-310.

13. **Newman, J.R. and J.J. Murphy.** 1979. Effects of industrial fluoride on Black-tailed deer (preliminary report). *Fluoride* 12:129-135.

14. **Cooke, J.A., S.M. Andrews and M.S. Johnson.** 1990. Lead, zinc, cadmium and fluoride in small mammals from contaminated grassland established on

fluorspar tailings. *Water Air Soil Pollut.* 51:43-54.

15. **Merkley, J.W.** 1981. The effect of sodium fluoride on egg production, egg quality, and bone strength of caged layers. *Poult. Sci.* 60:771-776.

16. **Mehdi, A.W.R., K.A. Al-Soudi, N.A.J. Al-Jiboori and M.K. Al-Hiti.** 1983. Effect of high fluoride intake on chicken performance, ovulation, spermatogenesis and bone fluoride content. *Fluoride* 16:37-43.

17. **Van Toledo, B. and G.F. Combs Jr.** 1984. Fluorosis in the laying hen. *Poult. Sci.* 63:1543-1552.

18. **Schulz, J.A. and A.R. Lamb.** 1925. The effect of fluorine as sodium fluoride on the growth and reproduction of albino rats. *Science* 61:93-94.

19. **Chinoy, N.J. and E. Sequeira.** 1989. Fluoride induced biochemical changes in reproductive organs of male mice. *Fluoride* 22:78-85.

20. **Chinoy, N.J., M.V. Rao, M.V. Narayana and E. Neelakanta.** 1991. Microdose vasal injection of sodium fluoride in the rat. *Reprod. Toxicol.* 5:505-512.

21. **Krasowska, A. and T. Wlotowski.** 1992. The effect of high fluoride intake on tissue trace elements and histology of testicular tubules in the rat. *Comp. Biochem. Physiol.* 103C:31-34.

22. **Mitchell, H.H. and M. Edman.** 1952. The fluorine problem in livestock feeding. *Nutrit. Abst. Rev.* 21:787-804.

23. **Udall, D.H. and K.P. Keller.** 1952. A report on fluorosis in cattle in the Columbia River Valley. *Cornell Vet.* 42:159-184.

24. **Shupe, J.L., M.L. Miner, D.A. Greenwood, L.E. Harris and G.E. Stoddard.** 1963. The effect of fluorine on dairy cattle II. Clinical and pathologic effects. *Am. J. Vet. Res.* 24:964-984.

25. **Pattee, O.H., S.N. Wiemeyer and D.M. Swineford.** 1988. Effects of dietary fluoride on reproduction in Eastern Screech-Owls. *Arch. Environ. Contam. Toxicol.* 17:213-218.

26. **Fleming, W.J., C.E. Grue, C.A. Schuler and C.M. Bunck.** 1987. Effects of oral doses of fluoride on nestling European starlings. *Arch. Environ. Contam. Toxicol.* 16:483-489.

27. **Dunning, J.B.J. and E.P. Odum** 1993. *CRC Handbook of Avian Body Masses.* CRC Press Inc. Boca Raton, Fl.

28. **Fleming, W.J. and C.A. Schuler.** 1988. Influence of the method of fluoride administration on toxicity and fluoride concentrations in Japanese quail. *Environ. Toxicol. Chem.* 7:841-845.

29. **Singer, L. and W.D. Armstrong.** 1968. Determination of fluoride in bone with the fluoride electrode. *Anal. Chem.* 40:613-614.

30. **Bolton, N.J., A. Chadwick, H.M. Chapman, T.R. Hall and C.G. Scanes.** 1974. Diurnal fluctuations in hormone levels in pituitary and plasma in the juvenile domestic cockerel. *J. Endocr.* 63:63P-64P.

31. **Schanbacher, B.D., W.R. Gomes and N.L. VanDemark.** 1974. Diurnal rhythms in serum testosterone levels and thymidine uptake by testes in the domestic fowl. *J. Anim. Sci.* 38:1245-1248.

32. **Ottinger, M.A. and H.J. Brinkley.** 1979. Testosterone and sex related physical characteristics during the maturation of the male Japanese quail (*Coturnix coturnix japonica*). *Biol. Reprod.* 20:905-909.

33. **Willoughby, E.J. and T.J. Cade.** 1964. Breeding behavior of the American kestrel (sparrow hawk). *Living Bird* 3:75-96.

34. **Sanford, L.M.** 1985. Evidence that estrogen regulation of testosterone secretion in adult rams is mediated by both indirect (gonadotropin dependent) and direct (gonadotropin independent) means. *J. Androl.* 6:306-314.

35. **Schanbacher, B.D. and M.J. D'occhio.** 1982. Validation of a direct radioimmunoassay for testosterone in unextracted serum from five species: application to study of the hypothalamic-pituitary-gonadal axis in males. *Am. J. Androl.* 3:45-51.

36. **Rehder, N.B., D.M. Bird and L.M. Sanford.** 1988. Plasma androgen levels and body weights for breeding and nonbreeding male American kestrels. *Condor* 90:555-560.

37. **Sokal, R.R. and F.J. Rohlf.** 1995. *Biometry : the principles and practice of statistics in biological research.* W.H. Freeman and Co. New York.

38. **Haseman, J.K. and L.L. Kupper.** 1979. Analysis of dichotomous response data from certain toxicological experiments. *Biometrics* 35:293

39. **SAS Institute Inc.** 1988. *SAS/STAT User's Guide.* SAS Institute Inc. Cary, N.C.

40. **Dunnett, C.W.** 1955. A multiple comparisons procedure for comparing several treatments with a control. *J. Am. Stat. Assoc.* 50:1096-1121.

41. **Carriere, D., D.M. Bird and J.W. Stamm.** 1987. Influence of a diet of fluoride-fed cockerels on reproductive performance of captive American kestrels. *Environ. Pollut.(Ser.A)* 46:151-159.

42. **Bird, D.M. and C. Massari.** 1983. Effects of dietary sodium fluoride on bone fluoride levels and reproductive performance of captive American Kestrels. *Environ. Pollut. (Ser. A)* 31:67-76.

43. **Pattee, O.H.** 1984. Eggshell thickness and reproduction in American kestrels exposed to chronic dietary lead. *Arch. Environ. Contam. Toxicol.* 13:29-34.

44. **Porter, R.D. and S.N. Wiemeyer.** 1970. Propagation of captive American kestrels. *J. Wildl. Manage.* 34:594-604.

45. **Porter, R.D. and S.N. Wiemeyer.** 1972. Reproductive patterns in captive American kestrels (Sparrow Hawks). *Condor* 74:46-53.

46. **Meijer, T. and H. Schwabl.** 1989. Hormonal patterns in breeding and non-breeding kestrels, *Falco tinnunculus*: field and laboratory studies. *Gen. Comp. Endocrinol.* 74:148-160.

47. **Bird, D.M., P.C. Laguë and R.B. Buckland.** 1976. Artificial insemination vs. natural mating in captive American kestrels. *Can. J. Zool.* 54:1183-1191.

48. **Bird, D.M. and P.C. Laguë.** 1982. Fertility, egg weight loss, hatchability, and fledging success in replacement clutches of captive American kestrels. *Can. J. Zool.* 60:80-88.

49. **Merkley, J.W. and T.J. Sexton.** 1982. Reproductive performance of white Leghorns provided fluoride. *Poult. Sci.* 61:52-56.

50. **Guenter, W.** 1979. Fluorine toxicity and laying hen performance. *Poult. Sci.* 58:1063

51. **Chinoy, N.J. and E. Sequeira.** 1989. Effects of fluoride on the histoarchitecture of reproductive organs of the male mouse. *Reprod. Toxicol.* 3:261-267.

52. **Craighead, J.J. and F.C.J. Craighead** 1969. *Hawks, Owls and Wildlife.* Dover Publications Inc. New York.

53. **Newton, I.** 1988. Age and Reproduction in the Sparrowhawk. In Clutton-Brock, T.H. eds., *Reproductive Success. Studies of individual variation in contrasting breeding systems.* University of Chicago Press, Chicago, pp. 201-219.

54. **Roest, A.I.** 1957. Notes on the American sparrow hawk. *Auk* 74:1-19.

55. **Messer, H.H., W.D. Armstrong and L. Singer.** 1973. Influence of fluoride intake on reproduction in mice. *J. Nutr.* 103:1319-1326.

56. **Tao, S. and J.W. Suttie.** 1976. Evidence for a lack of an effect of dietary fluoride level on reproduction in mice. *J. Nutr.* 106:115-1122.

57. **Shupe, J.L., A.E. Olson and R.P. Sharma.** 1972. Fluoride toxicity in domestic and wild animals. *Clin. Toxicol.* 5:195-213.

58. **Underwood, E.J.** 1977. *Fluorine.* Academic Press. New York.

59. **Balgooyen, T.G.** 1976. Behavior and ecology of the American kestrel (*Falco sparverius* L.) in the Sierra Nevada of California. Univ. Calif. Publ. Zool. 103:1-85.

60. **Bird, D.M. and R.B. Buckland.** 1976. The onset and duration of fertility in the American kestrel. *Can. J. Zool.* 54:1595-1597.

61. **White, D.H., C.A. Mitchell and E.F. Hill.** 1983. Parathion alters incubation behaviour of laughing gulls. *Bull. Environ. Contam. Toxicol.* 31:93-97.

62. **Meyers, S.M., J.L. Cummings and R.S. Bennett.** 1990. Effects of methyl parathion on red-winged blackbird (*Agelaius phoeniceus*) incubation behaviour and nesting success. *Environ. Toxicol. Chem.* 9:807-813.

63. **DeRosa, C.T., D.H. Taylor, P. Farrell and S.K. Seilkop.** 1976. Effects of sevin on the reproductive biology of the Coturnix. *Poult. Sci.* 55:2133-2141.

64. **Fox, G.A., A.P. Gilman, D.B. Peakall and F.W. Anderka.** 1978. Behavioral abnormalities of nesting Lake Ontario herring gulls. *J. Wildl. Manage.* 42:477-483.

65. **Fox, G.A. and D.V. Weseloh.** 1987. Colonial waterbirds as bio-indicators of environmental contamination in the Great Lakes. *ICBP Tech Publ.* 6:209-216.

66. **McArthur, M.L.B., G.A. Fox, D.B. Peakall and B.J.R. Philogène.** 1983. Ecological significance of behavioral and hormonal abnormalities in breeding ring doves fed an organochlorine chemical mixture. *Arch. Environ. Contam. Toxicol.* 12:343-353.

67. **Hodge, H.C. and F.A. Smith** 1965. *Fluorine Chemistry.* Academic Press. New York.

68. **Machoy, Z. and A. Machoy-Mokrzynska.** 1990. Mechanisms of fluoride elimination and detoxification in living organisms. *Fluoride* 23:151-153.

69. **Chinoy, N.J., P.K. Pradeep and E. Sequeira.** 1992. Effect of fluoride ingestion on the physiology of reproductive organs of male rat. *J. Environ. Biol.* 13:55-61.

70. **Wingfield, J.C. and D.S. Farner.** 1978. The annual cycle of plasma irLH and steroid hormones in feral populations of the white-crowned sparrow, *Zonotrichia leucophrys gambelii. Biol. Reprod.* 19:1046-1056.

71. **Balthazart, J.** 1983. Hormonal correlates of behaviour. In D.S. Farner, J.R. King and K.C.Parkes eds., *Avian Biology.* Academic Press, New York, pp. 221-365.

72. **Wingfield, J.C.** 1991. Mating systems and hormone-behavior interactions. Conference Proceeding. Twentieth International Ornithological Congress. pp.2055-2062.

73. **Moore, M.C.** 1983. Effect of female sexual displays on the endocrine physiology and behaviour of male White-crowned sparrows, *Zonotrichia leucophrys. J. Zool., Lond.* 199:137-148.

74. **Haase, E., E. Paulke and P.J. Sharp.** 1976. Effects of seasonal and social factors on testicular activity and hormone levels in domestic pigeons. *J. Exp. Zool.* 197:81-88.

75. **Feder, H.H., A. Storey, D. Goodwin, C. Reboulleau and R. Silver.** 1977. Testosterone and "5a-dihydrotestosterone" levels in peripheral plasma of male and female ring doves (*Streptopelia risoria*) during the reproductive cycle. *Biol. Reprod.* 16:666-677.

76. **Sossinka, R., E. Prove and K. Immelmann** 1980. Hormonal mechanisms in avian behavior. In Epple, A. and M.H. Stetson, eds., *Avian Endocrinology.* Academic Press, New York, pp. 533-547.

77. **Wingfield, J.C.** 1984. Environmental and endocrine control of reproduction in the song sparrow, *Melospiza melodia*. II. Agonistic interactions as environmental information stimulating secretion of testosterone. *Gen. Comp. Endocrinol.* 56:417-424.

78. **Wingfield, J.C. and D.S. Farner.** 1978. The Endocrinology of a natural Breeding Population of White- crowned Sparrow (*Zonotrichia leucophrys pugetensis*). *Physiol. Zool.* 51:188-205.

79. **Wingfield, J.C.** 1984. Environmental and endocrine control of reproduction in the song sparrow, *Melospiza melodia*. I. Temporal organization of the breeding cycle. *Gen. Comp. Endocrinol.* 56:406-416.

80. **Pillai, K.S., A.T. Mathai and P.B. Deshmukh.** 1988. Effect of subacute dosage of fluoride on male mice. *Toxicol. Lett.* 44:21-29.

81. **Lake, P.E.** 1981. Male Genital Organs. In King, A.S. and J. McLelland, eds., *Form and Function in Birds.* Academic Press, London, pp. 1-61.

82. **Morrisey, R.E.** 1989. Association of sperm, vaginal cytology, and reproductive organ weight data with fertility of Swiss (CD-1) mice. In Working, P.K. ed., *Toxicology of the male and female reproductive systems.* Hemisphere Publishing Corp. New York, pp. 199-216.

83. **Al-Azawi, S.H., K.A. Al-Soudi and A.W.R. Mehdi.** 1986. Effect of fluoride intake on the structural-functional aspects of the reproductive system of the male domestic fowl. *J. Agric. Water Reso. Res.* 5:285-300.

84. **Kour, K. and J. Singh.** 1980. Histological finding of mice testes following fluoride ingestion. *Fluoride* 13:160-167.

85. **Wright, D.A. and A. Thompson.** 1978. Retention of fluoride from diets containing materials produced during aluminum smelting. *Br. J. Nutr.* 40:139-147.

86. **Phillips, P.H. and J.W. Suttie.** 1960. The significance of time in intoxication of domestic animals by fluoride. *A.M.A. Arch. Ind. Health* 21:343-345.

87. **Henny, C.J. and P.M. Burke.** 1990. Fluoride accumulation and bone strength in wild Black-crowned Night-Herons. *Arch. Environ. Contam. Toxicol.* 19:132-137.

88. **Seel, D.C. and A.G. Thompson.** 1984. Bone fluoride in predatory birds in the British Isles. *Environ. Pollut. (Ser. A)* 36:367-374.

89. **Stewart, D.J., T.R. Manley, D.A. White, D.L. Harrison and E.A. Stringer.** 1974. Natural flourine levels in the Bluff area, New Zealand. *N. Z. J. Sci.* 17:105-113.

90. **Culik, B.** 1987. Fluoride turnover in Adélie penguins (*Pygoscelis adeliae*) and other bird species. *Polar Biol.* 7:179-187.

91. **Rao, G.S.** 1984. Dietary intake and bioavailability of fluoride. *Ann. Rev. Nutr.* 4:115-136.

92. **Seel, D.C., A.G. Thompson and R.E. Bryant** 1987. Bone fluoride in four species of predatory bird in the British Isles. In Coughtrey, P.J., M.H. Martin and M.H. Unsworth, eds., *Pollutant Transport and Fate in Ecosystems. Special Publication Number 6 of the British Ecological Society.* Blackwell Scientific Publications, Oxford, pp. 211-221.

Table 1. Number of eggs produced by female American kestrels when paired with males receiving one of three fluoride doses or a control solution for 45 days. Trial 1 and 2 involved different birds and occurred in separate years.

	Control		**1.65 mg NaF**		**3.3 mg NaF**		**6.6 mg NaF**	
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
Birds/treatment	10	10	10	10	10	10	10	10
Pairs with eggs	7[a]	9	10	9	8	8	7	8[b]

a 1 female which laid two broken eggs on ground was excluded.

b After being paired 1 male died of causes unrelated to treatment.

Table 2. Number of eggs produced and fertility from pairs of captive American kestrels where the male was exposed to one of three fluoride treatments or a control solution for 45 days. Trial 1 and 2 involved different birds and occurred in separate years.

Dose	No. of eggs produced	No. eggs/pr.	No. fertile eggs	% fertility	No. fertile eggs /pair.[1]
Trial 1-Control	32	4.6	23	72	3.3
Trial 1-1.65 mg NaF	39	3.9	17	44	1.7
Trial 1-3.3 mg NaF	35	4.4	19	54	2.4
Trial 1-6.6 mg NaF	34	4.86	23	59	3.29
Trial 2-Control	44	4.9	35	80	3.9
Trial 2-1.65 mg NaF	45	5	31	69	3.4
Trial 2-3.3 mg NaF	36	4.5	25	69	2.78
Trial 2-6.6 mg NaF	39	4.9	18	46	2.25

[1] only pairs which produced eggs are included.

Table 3. Time (in days) for captive American kestrels, treated with fluoride to produce the first egg of the clutch after pairing (mean ± std. dev.). Trials 1 and 2 were conducted in successive years.

	Control	**1.65 mg NaF/D**	**3.3 mg NaF/D**	**6.6 mg NaF/D**
Trial 1	13.5 ± 3.1	14.8 ± 4.5	12.7 ± 3.9	12.3 ± 4.4
Trial 2	12.8 ± 4.1	14.9 ± 3.5	11.1 ± 2.2	14.5 ± 3.4

Table 4. Body and testis weights of captive male American kestrels treated with three levels of fluoride or a control solution for 45 days. Trial 1 and 2 involved different birds and occurred over two years.

Trial/dose	No. of birds	Mean body weight (g)	Mean combined testes weight (g)	Mean gonado-somatic index (GSI)[a]	Left/right testis weight
Trial 1- Control	10	108	0.14	0.13	1.5
Trial 1- 1.65 mg NaF	10	106	0.13	0.12	1.8
Trial 1- 3.3 mg NaF	10	107	0.12	0.11	1.6
Trial 1- 6.6 mg NaF	10	101	0.10	0.10	1.8
Trial 2- Control	9	104	0.11	0.11	1.6
Trial 2- 1.65 mg NaF	9	107	0.14	0.13	1.5
Trial 2- 3.3 mg NaF	9	107	0.07	0.07	1.5
Trial 2- 6.6 mg NaF	9	108	0.11	0.11	1.8

a GSI=(combined testes wt./body wt.)*100

Table 5. Summary statistics of bone fluoride levels measured in the femurae of captive male American kestrels exposed to three levels of fluoride or a control vehicle daily for 45 days. Data are presented on an ash weight basis.

Treatment	**n**	**Mean, ppm NaF (± std. dev.)**	**CV**	**Mean age (yrs.)**
Control	10	1152 (± 589)	51	4.0
1.65 mg NaF/D	10	1691 (± 901)	53	3.5
3.3 mg NaF/D	10	2482 (± 919)	37	1
6.6 mg NaF/D	10	3187 (± 720)	23	2.6

Table 6. Correlations between various reproductive behaviours, production fertile eggs and bone fluoride levels (males only) of pairs of captive American kestrels where the males were exposed to fluoride. Spearman r (p value in brackets). Trials 1 and 2 combined.

	Reproductive Behaviours				
	copulations	vocalizations	nest box activities	food transfers	sum of behaviours
proportion of fertile eggs	0.4204 (0.0079)[1]	0.332 (.039)[1]	0.330 (0.040)[1]	0.25 (0.13)	0.421 (0.015)[1]
bone fluoride	-0.166 (0.354)	0.040 (0.824)	0.126 (0.481)	-0.230 (0.198)	-0.059 (0.743)

[1] - significant at $p<0.05$

Fig. 1. Mean final body weights of captive male American kestrels exposed to three levels of fluoride or a control solution for 45 days. Columns are not significantly different at p=0.05.

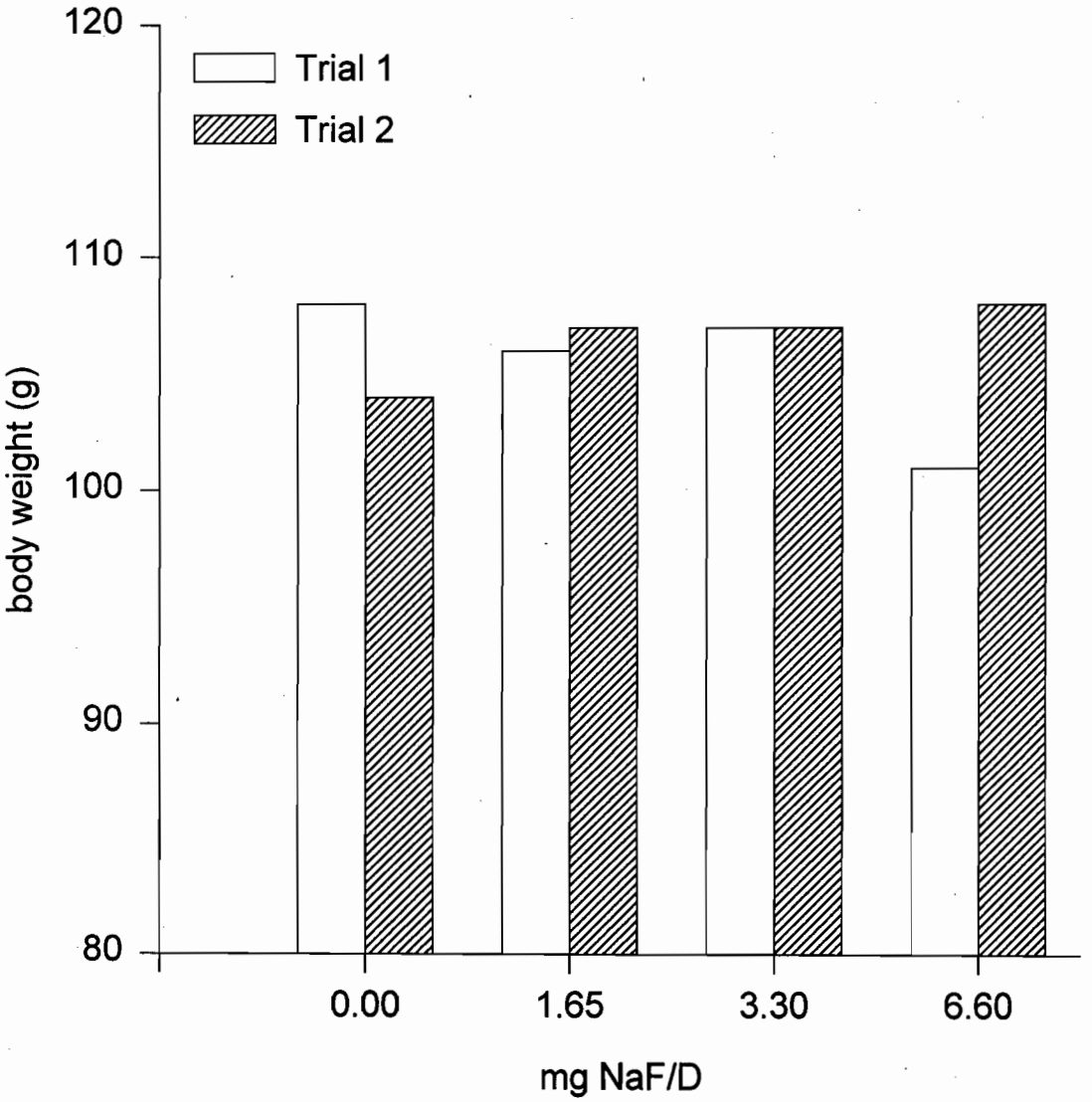

120
110
100
90
80
Trial 1
Trial 2
body weight (g)
0.00
1.65
3.30
6.60
mg NaF/D

Fig. 2. Average weight loss of captive male American kestrels from trial 1 after being treated with three levels of fluoride or a control solution for 45 days (expressed as a function of total body weight). Bars represent treatment mean± 1 standard error. Columns are not significantly different at p=0.05.

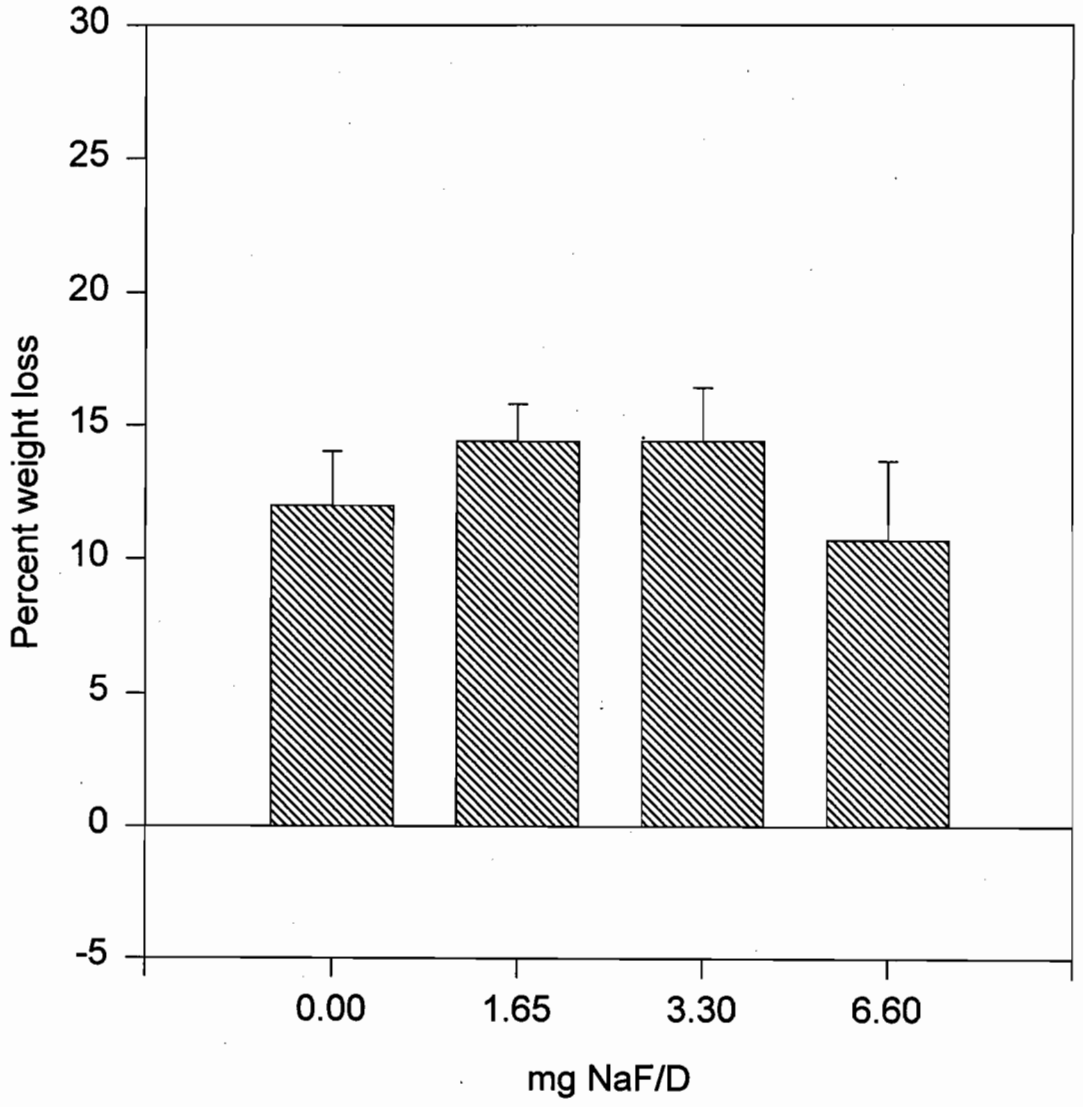

30
25
20
15
10
5
0
-5
Percent weight loss
0.00
1.65
3.30
6.60
mg NaF/D

Fig. 3. Mean weight loss of captive American kestrels from trial 1 treated with three levels of fluoride or a control solution for 45 days. Bars represent mean individual weight loss for each treatment ± 1 standard deviation.

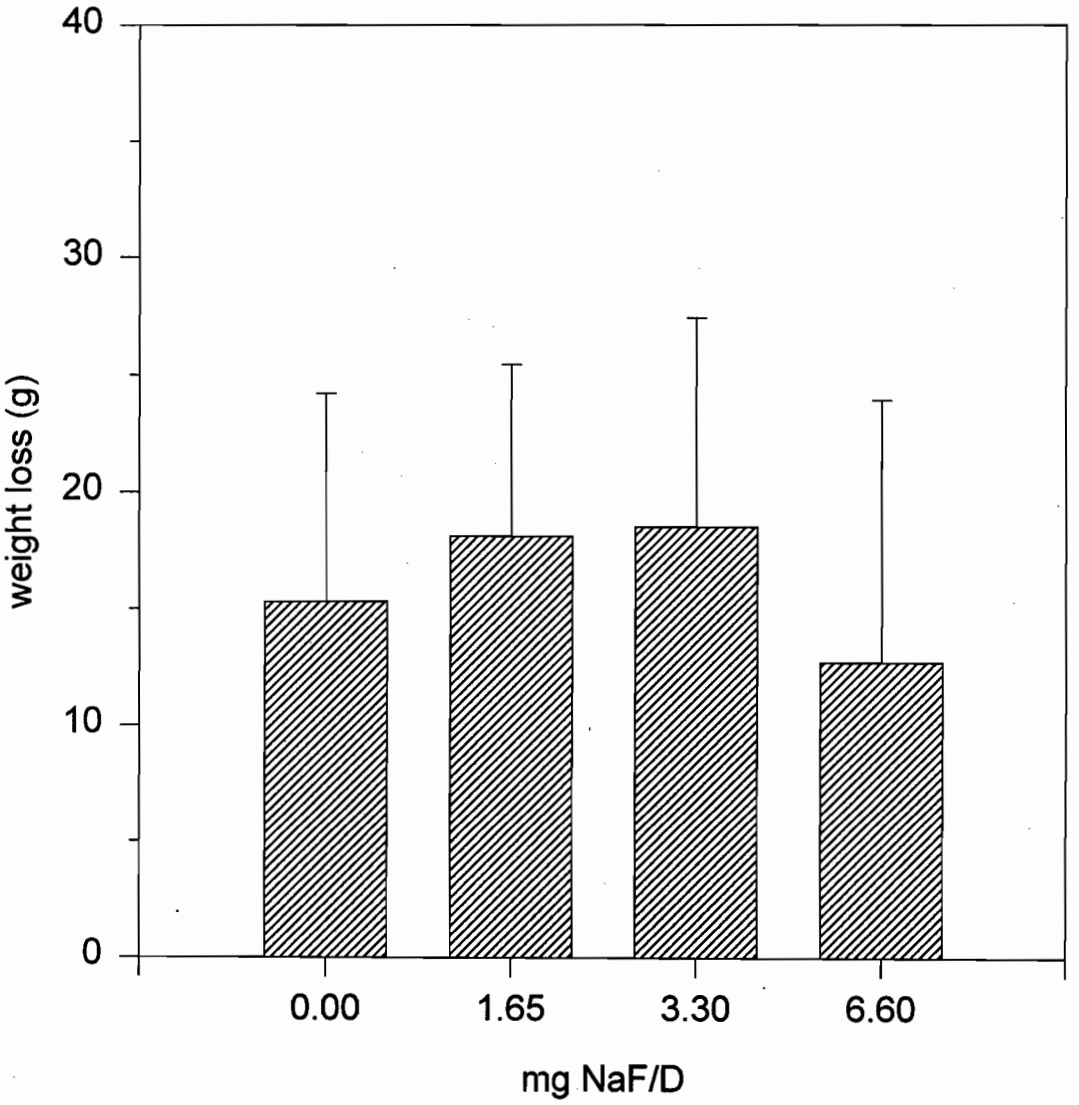

40
30
20
10
0
weight loss (g)
0.00
1.65
3.30
6.60
mg NaF/D

Fig. 4. Body weight weight change by week for captive male American kestrels from trial 2 exposed to three levels of fluoride or a control solution for 45 days. Within groups, columns with the same letter are not significantly different at p=0.05.

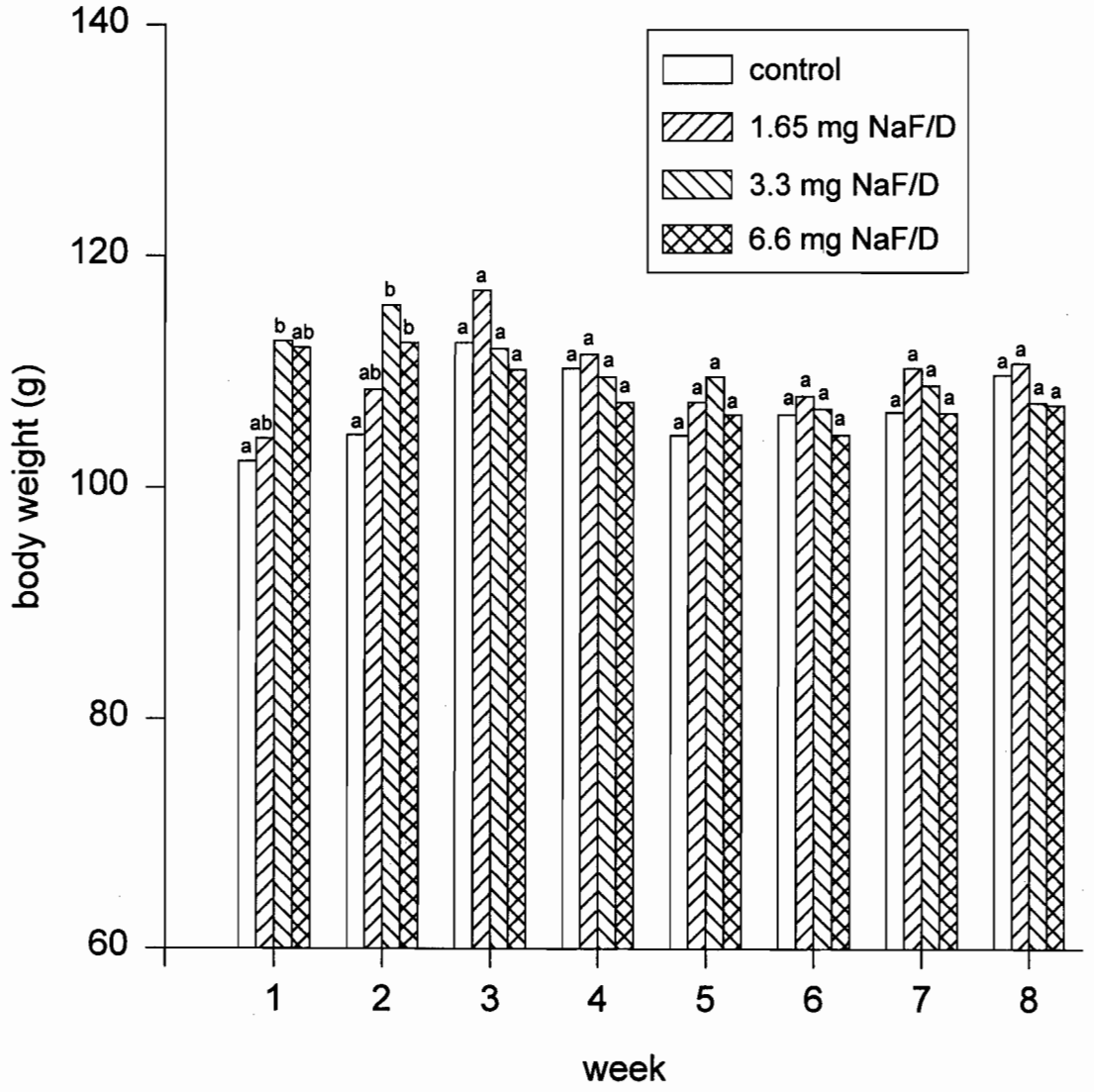

140
120
100
80
60
body weight (g)
control
1.65 mg NaF/D
3.3 mg NaF/D
6.6 mg NaF/D
1
2
3
4
5
6
7
8
week

Fig. 5. Correlation between left and right testis weights for American kestrels exposed to three levels of fluoride or a control solution for 45 days. Data are combined from trials 1 and 2.

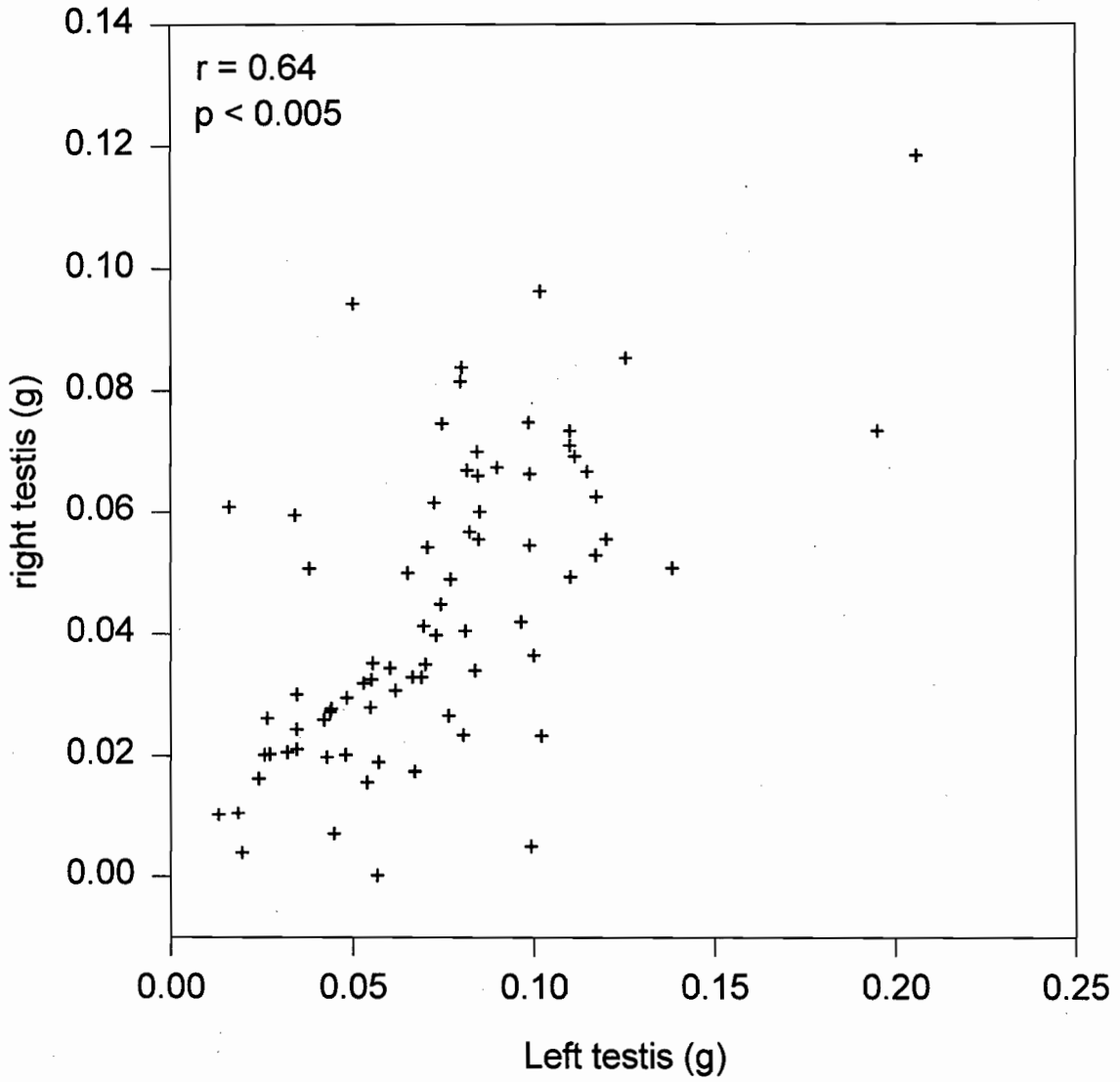

r = 0.64
p < 0.005
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
right testis (g)
0.00
0.05
0.10
0.15
0.20
0.25
Left testis (g)

Fig. 6. Regression of combined testes weight on body weight (± 95% confidence interval) for American kestrels treated with three levels of fluoride or a control solution. Data from both years of the study and all treatment groups are combined.

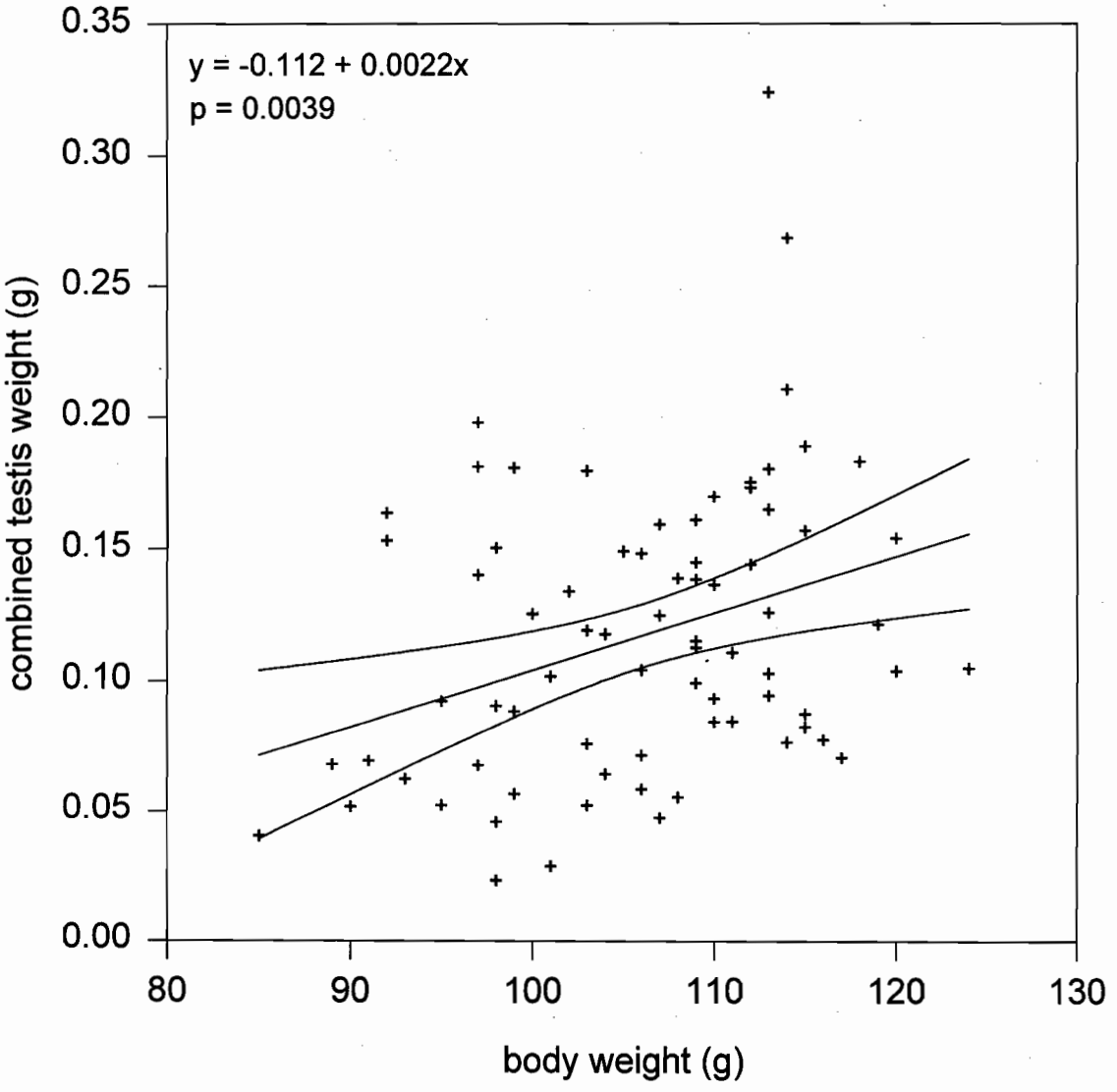

y = -0.112 + 0.0022x
p = 0.0039
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
combined testis weight (g)
80
90
100
110
120
130
body weight (g)

Fig. 7. Regression of combined testes weights on time to sacrifice from pairing for American kestrels exposed to fluoride. Data (± 95% confidence interval) are combined from both years of the study and all treatments.

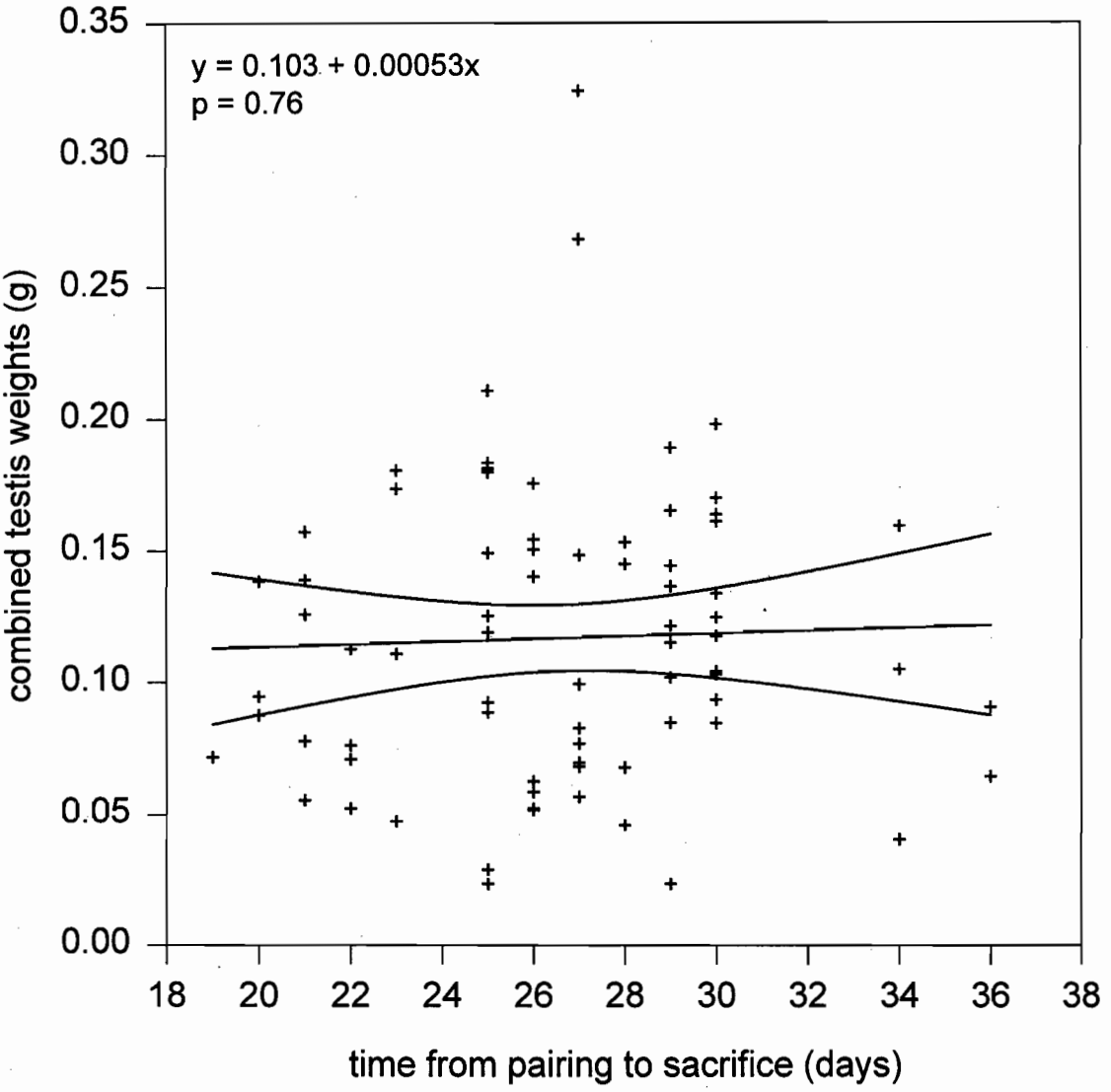

y = 0.103 + 0.00053x
p = 0.76
combined testis weights (g)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
18
20
22
24
26
28
30
32
34
36
38
time from pairing to sacrifice (days)

Fig. 8. Fluoride content of the femurae of captive male American kestrels from trial 1 after being exposed to three levels of fluoride or a control solution for 45 days. Columns represent treatment means ± 1 standard error. Columns with the same letter are not significantly different at p=0.05.

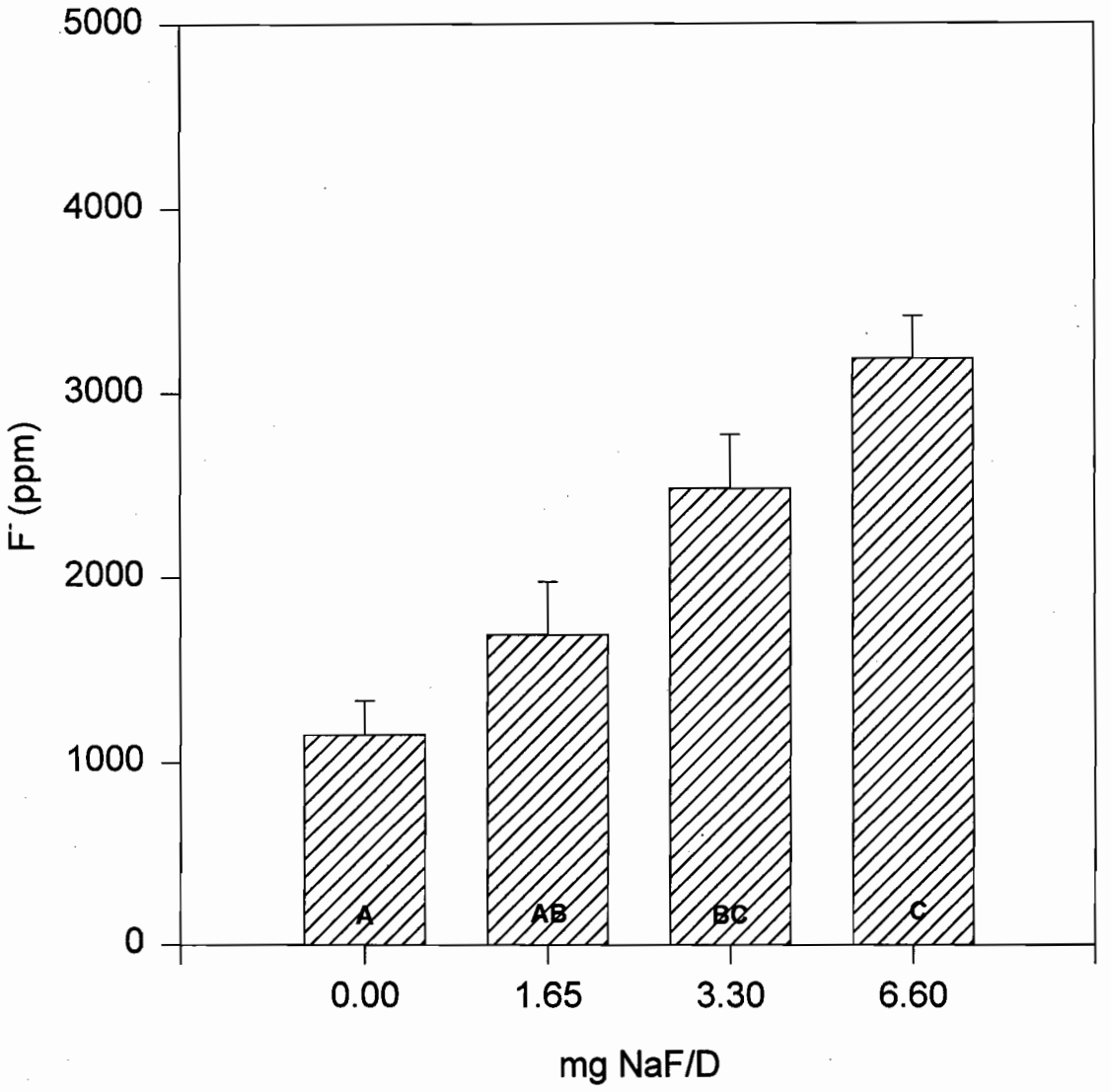

5000
4000
3000
2000
1000
0
F⁻ (ppm)
A
AB
BC
C
0.00
1.65
3.30
6.60
mg NaF/D

Fig. 9. Actual male American kestrel femoral fluoride levels versus predicted levels if the age of all birds were adjusted to 1 yr.

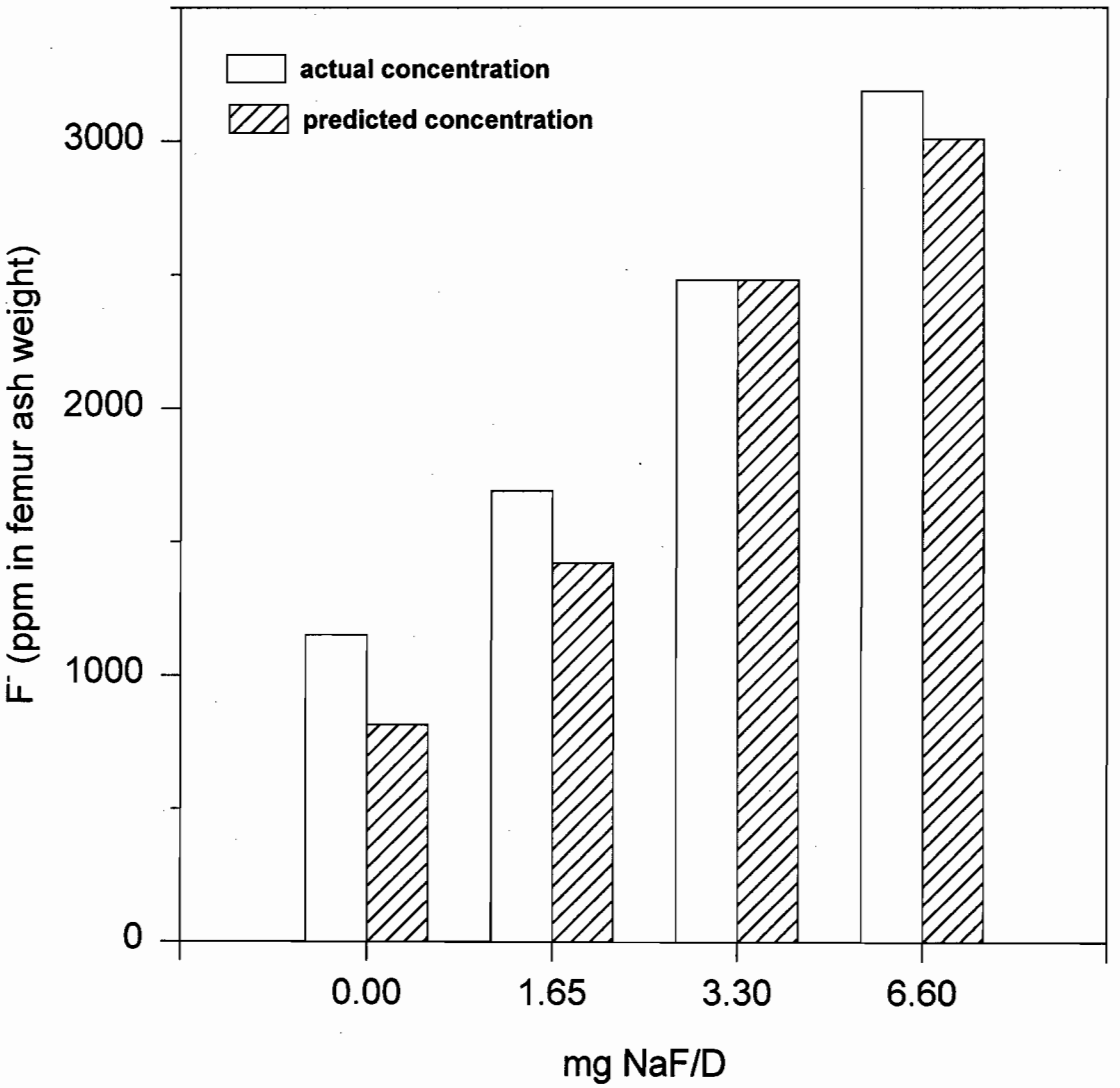

actual concentration
predicted concentration
3000
2000
1000
0
F⁻ (ppm in femur ash weight)
0.00
1.65
3.30
6.60
mg NaF/D

Fig 10. Reproductive behaviours observed during trial 1 of captive male American kestrels exposed to three levels of fluoride or a control solution for 45 days. Columns represent the mean number of behaviours per 10 minute observation period for each treatment. There was no significant difference among treatments for any of the behaviours recorded.

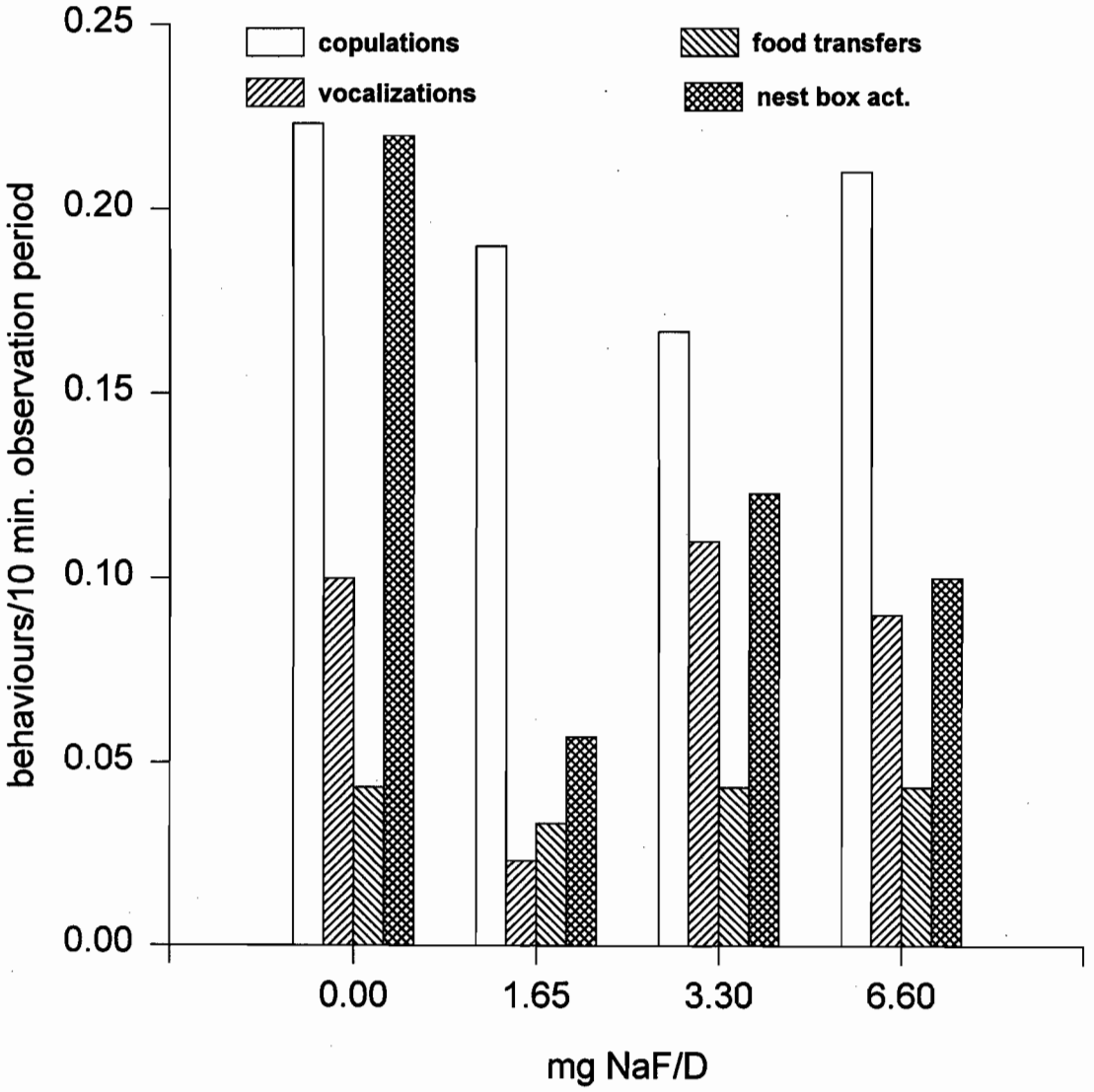

0.25
0.20
0.15
0.10
0.05
0.00
behaviours/10 min. observation period
copulations
vocalizations
food transfers
nest box act.
0.00
1.65
3.30
6.60
mg NaF/D

Fig 11. Reproductive behaviours observed during trial 2 of captive male American kestrels exposed to three levels of fluoride or a control solution for 45 days. Columns represent the mean number of behaviours per 10 minute observation period for each treatment. There was no significant difference among treatments for any of the behaviours recorded.

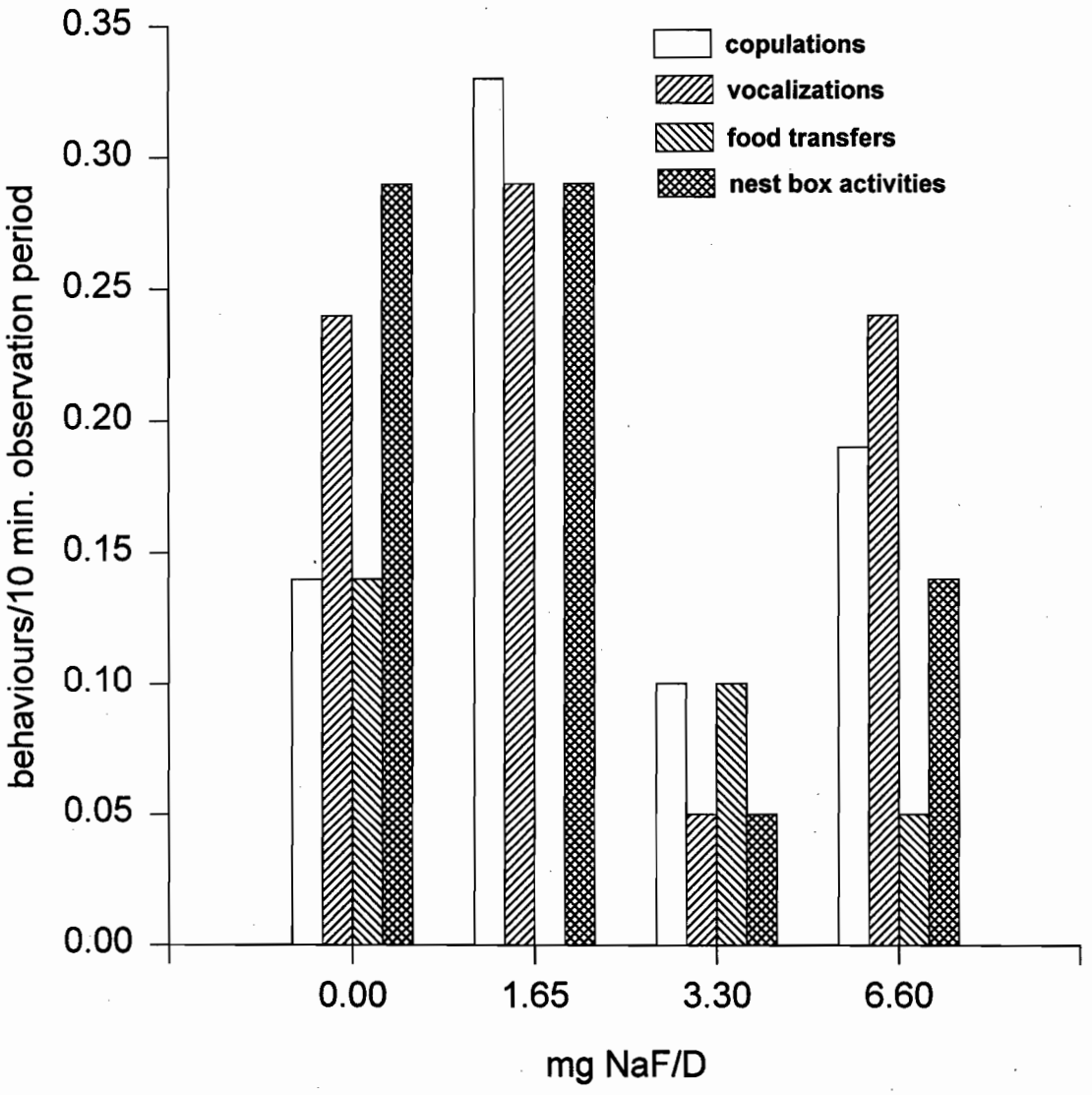

0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
behaviours/10 min. observation period
copulations
vocalizations
food transfers
nest box activities
0.00
1.65
3.30
6.60
mg NaF/D

Fig. 12. Mean weekly testosterone levels of captive male American kestrels exposed to fluoride or a control solution measured during trial 1. Each point represents a weekly mean of 10 individuals ± 1 standard deviation.

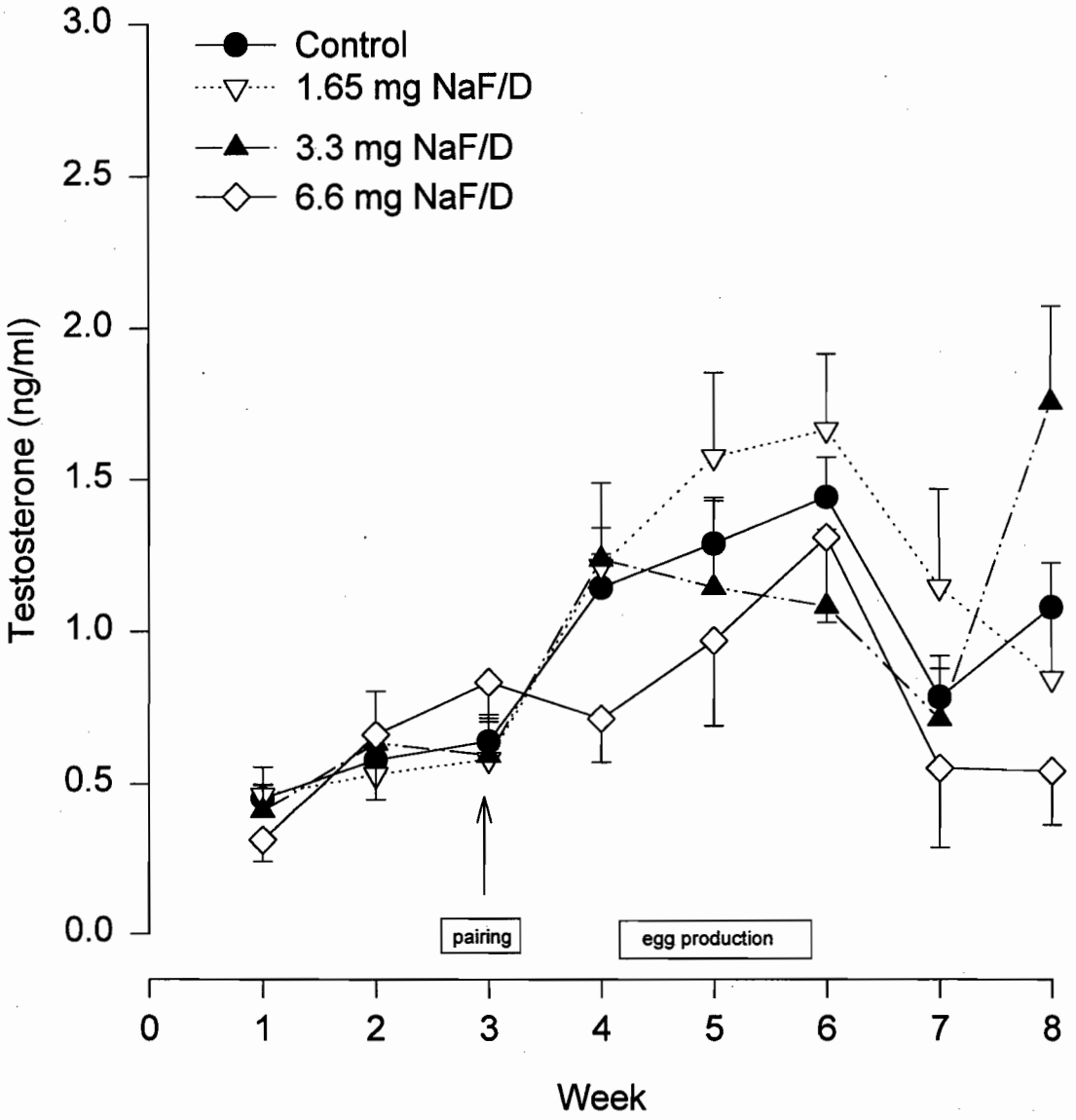

Control
1.65 mg NaF/D
3.3 mg NaF/D
6.6 mg NaF/D
Testosterone (ng/ml)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
pairing
egg production
0
1
2
3
4
5
6
7
8
Week

Fig. 13. Mean weekly testosterone levels of captive male American kestrels exposed to fluoride or a control solution, measured during trial 2. Each point represents a weekly mean of 10 individuals ± 1 standard deviation.

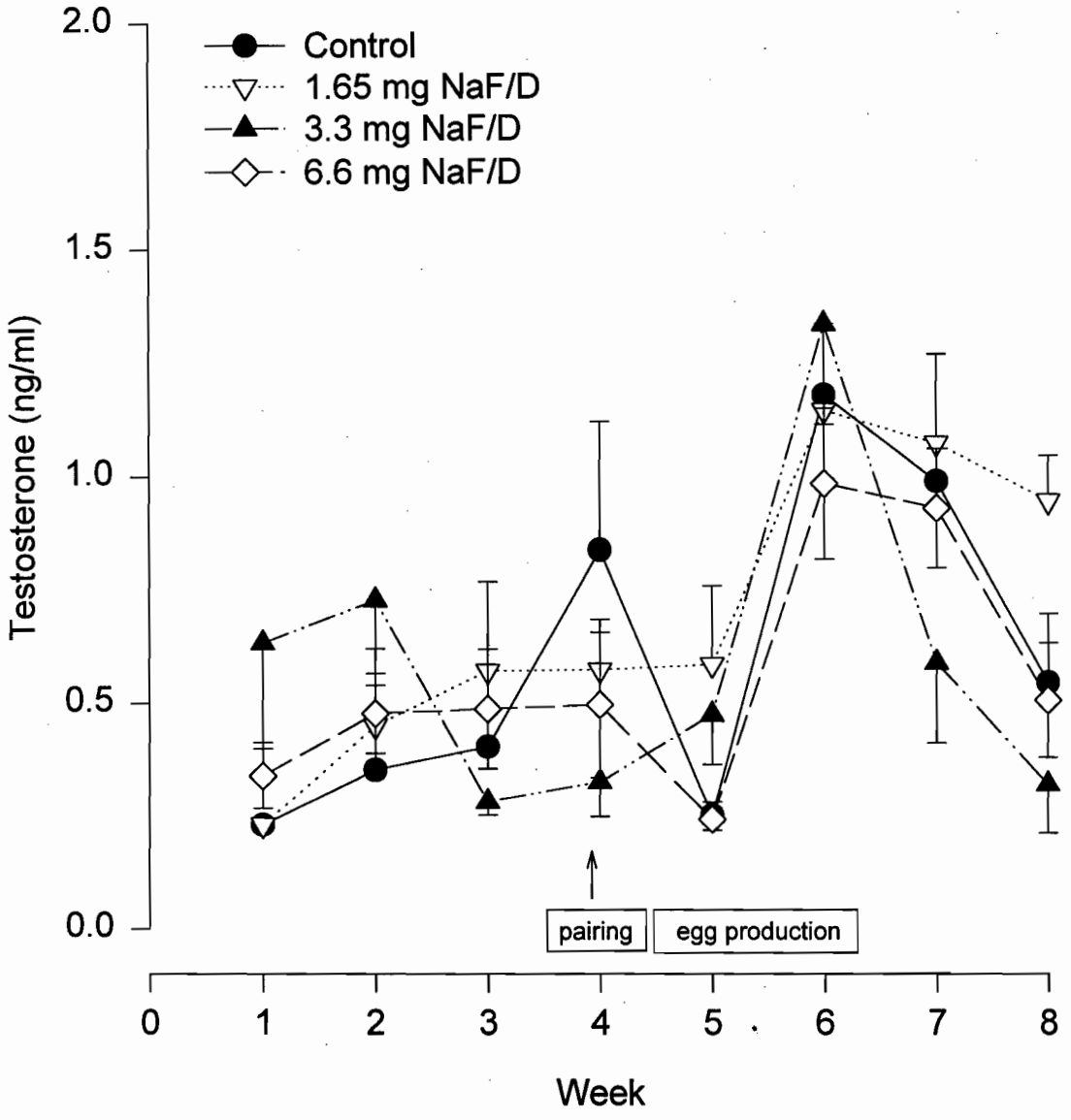
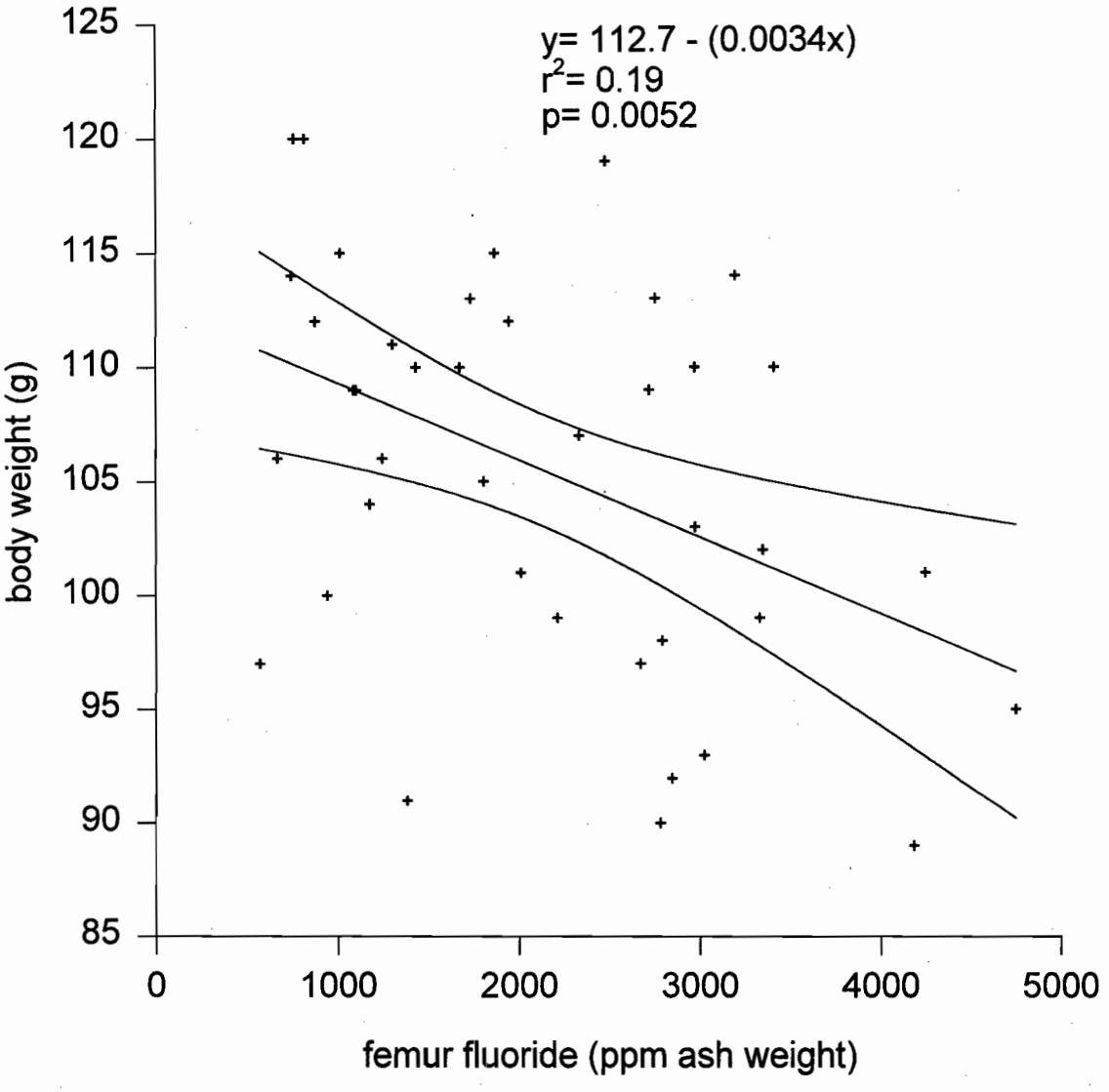

Control
1.65 mg NaF/D
3.3 mg NaF/D
6.6 mg NaF/D
Testosterone (ng/ml)
2.0
1.5
1.0
0.5
0.0
pairing
egg production
0
1
2
3
4
5
6
7
8
Week

Fig. 14. Regression of body weight on corresponding bone fluoride concentration of male American kestrels exposed to three levels of fluoride or a control solution. Data were collected during trial 1.


y= 112.7 - (0.0034x)
r2= 0.19
p= 0.0052
125
120
115
110
105
100
95
90
85
body weight (g)
0
1000
2000
3000
4000
5000
femur fluoride (ppm ash weight)

CONNECTING STATEMENT

2 male American kestrels were exposed to sodium fluoride and their
t reproductive performance evaluated. This species was chosen as a
ıe first part of this research since it would likely be exposed to fluoride
l during the breeding season if found nesting in fluoride-contaminated
ıts. In addition, the kestrel is easily managed in captivity and has a
ell documented breeding biology.

semen quality

l component of this study was to evaluate the impacts of fluoride
ı)
n semen quality and fertilizing capacity, ideally in the American
ı American kestrel has been used for artificial insemination studies
re the collection of semen as well as in evaluations of the effects of
Mirex on semen quality. Accordingly, it seemed appropriate to use this
ıvaluate the effects of fluoride exposure on various aspects of semen
le it is possible to collect semen from kestrels and use those samples
inseminate females, several factors make it quite difficult to conduct
ıpeatable examinations of semen quality. Inherent limitations include
f ejaculate volumes (7-8 µl) and samples frequently (24-100%)
ıd with urates and cellular debris, which makes microscopic
ıroblematic. In addition, sperm concentrations (31,000/µl) compared
ated avian species are quite low. Finally, kestrels can be unreliable
ırs making serial sampling of individuals difficult. After attempting to
:rel for this aspect of the study with limited success because of the
ıed above, it was decided to use Japanese quail (*Coturnix coturnix*
ıtead. This species is also easily maintained in captivity and reliably
ımen with a high sperm concentration. Perhaps most important, a
ction methodology has been developed. Accordingly, Chapter 3
ıe effects of fluoride exposure on various semen quality parameters
g capacity of male Japanese quail.

ABSTRACT

In order to determine the effects of fluoride on semen quality and fertilizing capacity in an avian species, 10 male Japanese quail (*Coturnix japonica*) per group were treated with one of three levels of fluoridated water (125, 250, 500 ppm NaF) or a control solution ($NaHCO_3$). In the first component of the study male quail were exposed to $NaHCO_3$ or 250 ppm NaF for 64 days. During the treatment period the test birds were briefly paired with untreated females on five separate occasions. The frequency of male reproductive behaviours, the duration of fertility and the proportion of fertile eggs produced was monitored for each pairing. In the second component of the study male quail were exposed to one of three levels of fluoridated water or the control solution for 132 days. Weekly semen samples were obtained from all treatment groups and evaluated for semen volume, sperm concentration, live/dead ratios and motility. Upon completion of dosing all males were sacrificed and the testes and femurae removed for examination and fluoride determination, respectively. Analysis of bone samples indicated that the treatment resulted in significant increases in fluoride burdens with increases in the fluoride content of the drinking water. However, there was no difference between treatments in the proportion of eggs produced which were fertile or in the duration of time over which fertile eggs were produced subsequent to pairing. Similarly, regression analysis revealed no significant difference among treatments for any of the semen quality parameters. It was concluded that, due to the extended length of exposure and to the highly bio-available form of fluoride used, male Japanese quail were at low risk of experiencing reproductive failure as a result of exposure to fluoride.

METHODS

There are three distinct components to this study. Twenty male and 20 female Japanese quail were monitored to estimate food and water consumption. In addition, these birds were subsequently paired and the duration of fertility determined. Twenty males from this group were exposed to fluoride and re-paired to determine the impact of fluoride exposure on the duration of survival and viability of sperm maintained in the sperm storage tubules located at the junction of the shell gland and vagina . Finally, a second group of males was exposed to three levels of fluoride and serially collected semen samples were evaluated for numerous endpoints including total sperm per ejaculate and sperm motility. During the semen evaluation period all males were weighed and water consumption measured.

Subjects

Japanese quail used in the study were obtained from a breeding colony maintained at the Avian Science and Conservation Centre of McGill University. The breeding stock originated from a commercial strain developed for meat production. Birds were randomly selected from large groups of mixed sex at six weeks of age and placed in individual batteries where they were maintained during all test procedures. The parentage of individual birds was not known.

Batteries consisted of 10 adjoining cages wall-mounted in a heated, windowless room. Sexes were randomly assigned to batteries and single birds within sexes to pens. Individual pens measured 30x30x20cm (LxWxH) and were constructed of 2.0x2.0 cm welded wire. Each pen was equipped with a gravity-fed water bowl and with access to a common food trough which spanned the front of each battery of cages. Eggs rolled through a gap in the front of the pen to a tray where they were collected each morning. Birds could see and hear all other birds but could not maintain intimate contact with those in adjacent pens.

Air temperature was maintained between 20 and 24° C and ambient air temperatures were recorded daily. Photoperiod was maintained at 16L:8D with lights coming on at 7:00 a.m. A 21% protein turkey laying ration and water were available *ad libitum*. Because of the design of the batteries food was easily scattered out of the trays making estimates of consumption difficult. As a result, after the initial estimation of food consumption, the rations were no longer weighed. However, all birds were weighed weekly during the fertility testing and periodically during the semen evaluation study. Water was kept in 10L plastic jugs which flowed into the water bowls of each battery of 10 pens. Water consumption was measured by calibrating the jugs and measuring the change in volume over time (usually once per week). As a result, consumption was measured in terms of 10 birds and then divided to obtain estimates of individual consumption rates.

Determination of length of fertility

In order to determine the duration of fertility of the females, two pre-experiment trials were performed. Twenty 6-week old male and 20 6-week old female Japanese quail were placed in individual quail batteries for a 3- week acclimation period during which food and water consumption were measured.

All males were paired with their corresponding female penmate for 30 min, then returned to their cages and re-paired within 12 d. Previous research [76] found that 83% of male quail copulate with a female within 5 min of being placed together. Eggs were collected each morning thereafter and placed in an artificial incubator at 37.5°C and 55% relative humidity for 6 d at which point all eggs were removed and candled. Each egg was classified as fertile, infertile or addled and then discarded. After 12 d the procedure was repeated.

Assessment of fertilizing capacity

In order to determine the effect of fluoride exposure on duration of fertility the 20

males discussed above were divided into two groups: controls which received distilled water with $NaHCO_3$ and treated birds which were given distilled water with 250 ppm NaF added. Sodium fluoride was diluted in 10L doubly distilled water to make 250 ppm and then added to plastic jugs connected to the quail water bowls. Water consumption was monitored by calibrating and graduating the jugs. Males were also weighed weekly starting the day prior to dosing.

Birds were randomly paired (by placing the female in the pen with the male) in the morning for 3.5 h and every 12 - 15 d thereafter for a total of 5 matings, each time with the same female. Observations of reproductive behaviours were made during the first hour at 5 min intervals for each pair at each mating. During a 1 h observation period pens were scanned 13 times and the behaviours noted and classified. Dosing commenced the day following the first pairing and continued for the duration of the study. The five pairings and subsequent egg collections took place over a 65 day period. Eggs were collected each morning and placed in artificial incubators as described above.

Reproductive behaviours were noted as described by Beach and Inman [77] and Farris [78]: neck grabs, where the male grasps the feathers on the back of the female's neck with his beak prior to mounting were recorded, as well as copulations. The latter were recorded only if the male's tail was down and a brief period of rigidity was noted during mounting.

Semen quality assessment

A total of 40 six week-old male Japanese quail was randomly assigned to one of four treatments (10 birds per group): deionized doubly distilled water; 125 ppm NaF in doubly distilled water; 250 ppm NaF in doubly distilled water; 500 ppm NaF in doubly distilled water. Sodium fluoride (Fisher Scientific, analytical grade) was dissolved in the appropriate volume of deionized double distilled water with an initial fluoride concentration of <0.1 ppm. Birds were maintained singly in

battery pens as described above. All environmental conditions were maintained as stated previously. Prior to dosing males were acclimated to the semen collection method. Initially, semen was collected from males two to three times per week until dosing commenced. Food was removed from birds to be massaged 12-16 hours prior to collection [60]. Semen was collected from groups of 10 males between 8:00 and 10:00 a.m.

The semen collection method followed that of Marks and Lepore [63] with several modifications. The bird is placed breast down in the palm of the right hand with the head closest to the wrist. Foamy material is manually expressed from the cloacal gland using the thumb and forefinger of the left hand. Pressure is maintained dorsally on the gland with the fingers of the left hand while the abdomen of the bird about the pelvis is stroked ventrally with the forefinger and adjacent finger of the right hand. Stroking toward the cloaca continues with increasing pressure until a drop of yellow, viscous semen appears at the tip of the cloacal papillae. Using a micropipette fitted on the end of a length of rubber hosing with a mouthpiece, the semen is collected and the bird returned to its pen.

During the acclimation period a number of different diluents were tested to determine which provided the most suitable environment for the quail sperm. Wilcox phosphate buffer [79] was found to be the most effective at maintaining the integrity and viability of the sperm. Antibiotics were not added to the diluent since the semen was not to be used for insemination and the diluted semen samples were analysed within 30 min of dilution. Since the pH of quail semen was measured to be 8.0-8.4 the pH of the Wilcox buffer (normally 7.2) was increased to 8.2 with the addition of NaOH.

Upon collection of the semen sample the volume was estimated by measuring the filled portion of the micropipette. The sample was emptied into a 1 ml

microcentrifuge tube and mixed with Wilcox buffer (10 parts buffer: 1 part semen) maintained at room temperature. Two drops of approximately 10 µl of diluted semen were placed at either end of a microscope slide under cover slips. Motility was scored on a subjective scale of 1 to 5 according to the method of Bird and Laguë [52] where 1 designated no movement and 5 indicated rapid and vigorous activity. To assess the proportion of dead sperm in a sample, 5 µl of eosin B and nigrosin were added to 10 µl of dilute semen in a Neubauer hemacytometer and approximately 200 sperm were counted. Any that had taken up the stain were considered dead. The number of dead sperm in the sample were presented as a proportion of the number of live sperm.

Upon addition of fluoride to the drinking water, all males were sampled once a week, 10 birds per sampling session. In this way 10 semen samples were collected and then analysed immediately. Two sets of 10 birds were sampled daily over two days.

Male Japanese quail were exposed to fluoride in their drinking water beginning 6 May and ending 15 September for a total exposure period of 132 days. These birds were approximately 22 weeks old at the start of dosing and were maintained in single battery pens. Room temperature was maintained between 19 and 24° C with a 16L:8D light regime. One week after the initiation of dosing the heating control system failed, causing the temperature in the room to rise to 41° C, killing all the control birds. The control birds were most affected presumably as a result of their proximity to a wall-mounted steam radiator. The remaining males were transferred to another unheated facility with large screened windows providing natural light. Light was artificially supplemented to 16L:8D. All birds were replaced in their original pens on the same feed and water system. Ten 6-week old males were added to the control pens and massaged according to previously described methods twice weekly.

The length of the study was extended to allow the control males ample time to produce consistent semen samples. In addition there are numerous references in the literature discussing the semen characteristics of the Japanese quail. These data could be used to confirm that the control birds in this study were producing normal sperm samples.

Addition of fluoride to the drinking water commenced upon completion of the acclimation period and ran for a period of 132 days. The duration of fluoride exposure was based, in part, on the length of time necessary to obtain a sufficient number of semen samples and the time required for a significant burden of fluoride to accumulate in the femurae. Lin *et al.* [80] estimated the duration of one cycle of the seminiferous epithelium in the Japanese quail to be 2.69 days. Amann [43] recommended that subchronic studies span at least six cycles of the seminiferous epithelium (spermatogenesis in the Japanese quail requires 4.3 to 4.7 cycles). In this way an agent that acted on type A-spermatogonia would be detected when measuring mature spermatozoa. Exposure of the quail to fluoride for this duration ensures that any effects on sperm quality would be detected.

Statistical analysis

Body weight data were tested for normality using a test for normality described in D'Agostino and Belanger [81] using SAS software [82]. If necessary, data were log transformed. Effects of fluoride exposure on body weight were determined using a repeated measures ANOVA using Systat software [83]. Due to the method in which water was administered to male quail, it was not feasible to measure individual rates of consumption. Accordingly, it was not possible to estimate the variability in consumption rates between individuals with the result that the water consumption data were analysed among groups over time and presented as estimated individual consumption rates.

Changes in the number of reproductive behaviours observed over the five matings were analysed using a repeated measures ANOVA.

The number of fertile eggs laid and sperm motility scores were arcsin transformed using the Freeman-Tukey binomial transformation [84] prior to analysis.

Analyses of semen volume, motility, and concentration were problematic because of the number of missing values which were recorded when a male was massaged but no semen sample was obtained. Accordingly, a repeated measures analysis of variance was not used since any observations containing missing values are omitted from the analysis. Since the majority of the quail in the study failed to produce a semen sample at least once during the sampling period, virtually all the data would have been omitted in a repeated measures design. As a result, for each variable, individual regression equations were developed. The coefficients were then grouped by treatment and analysed using an ANOVA and a series of contrasts when main effects were found to be significant. In some instances individual ANOVAs were performed on data collected during a specific week. Where warranted, the intercepts of the individual regressions performed by treatment were analysed to determine if significant differences existed among treatments.

Fluoride determination

At the end of the dosing period all males were weighed and sacrificed by cervical dislocation. Both testes were removed and weighed and the length and breadth were measured to the nearest 0.01 cm with digital calipers. They were then diced into 1-2 mm chunks and placed in Bouin's fixative. The left femur of each male was dissected out and all attached tissue removed for subsequent fluoride determination. Carcasses were stored frozen.

Testicular volumes were calculated using the volume of an ellipse, $V= 4/3 \text{ pi } ab^2$ where a=½ length and b=½ width. This formula has been used elsewhere to calculate testicular volumes [85].

The cleaned femurae were dried at 80°C for 19 hours, weighed and ashed in a muffle furnace at 600°C for 4 hours. The samples were re-weighed to determine the proportion of ash and ground to a fine powder using a mortar and pestle. Fluoride determination followed the method of Singer and Armstrong [86]. All samples were dissolved in hydrochloric acid and adjusted to a pH 5-5.5 using an adjusting buffer (TISAB). The concentration of fluoride in the solution was measured using a fluoride ion specific electrode (Orion Research Inc., Boston, MA). Standard curves were created daily using serial dilutions of analytical grade sodium fluoride (Fisher Scientific). Each sample was read three times and the average of the readings used as the final concentration.

RESULTS

Food and water consumption

Food and water consumption were measured for the 6-week old male quail prior to administration of fluoride to the drinking water. Due to the design of the feed and water trays it was not practical to measure individual consumption rates. Each value for 10 birds was divided to obtain estimates of individual consumption for illustrative purposes. However, as there was no way to estimate the variability associated with these estimates, statistical treatment was confined to inter-group differences in consumption. Food was placed in a single trough which spanned 10 pens. In addition, there was considerable spillage when the birds ate. As a result differences in the weight of food from day to day reflect what was consumed as well as that scattered from the trough. Accordingly, food removed is labelled as disappearance to reflect both consumption and spillage.

At the first weighing the 20 males were 7 weeks old and weighed an average of 173g. By week 5 of the study their mean weight had increased to 190g (Fig. 1). A repeated measures ANOVA indicated this increase over time to be significant ($p<0.05$) and reflects growth of the young birds. Food disappearance over this time was variable but averaged 202g/10 males/day (S.D. 44.6 g) (Fig. 2). Similarly, water consumption was also quite variable with 10 males drinking approximately 214 ml/day (S.D. 44 ml) (Fig. 3).

Duration of fertility

In order to determine the duration of fertility male quail were paired briefly with females and then removed. Egg production and fertility were monitored for the following 8 d based on the results of previous studies [60,87]. Initially, eggs were incubated for 6 d prior to candling. As a result there was a time lag before incubated eggs could be assessed for fertility. Birds were repaired on the ninth day and eggs were collected and incubated for the subsequent 12 d.

Subsequent to the first pairing 19 females laid at least one egg (Table 1). At the time the birds were re-paired 9 d later, 6 of 18 layers were still producing fertile eggs, indicating that duration of fertility exceeded 8 days. Females were re-paired and egg fertility was monitored for the following 12 d. The maximum duration of fertility was 10 d. This 10-d interval was used subsequently when investigating the effects of fluoride exposure on fertility. Egg production by the 20 females generally ranged from 12 to 18 with the exception of the fourth day when the total dropped to 8 eggs. As in the first trial, 19 of 20 birds produced at least one egg (Table 1). Eggs which were broken by the female or were in any way defective, i.e. deformed, thin-shelled or shell-less, were excluded from further analysis.

The number of eggs laid per day and the proportion which were fertile are presented in Fig. 4 with the highest proportion (75%) occurring on the fourth day of the second trial. Fertility gradually subsided until day 11 when all eggs incubated were infertile.

During the second pairing, reproductive behaviours were recorded. All males were observed copulating except in pen 8. Eggs subsequently produced by that female were infertile. Interestingly, five additional females, observed copulating with separate males, went on to lay infertile eggs.

The fertility of all incubated eggs is reported in Table 1. The slightly higher rate of fertility documented from the first trial may be related to the shorter duration (8 d). In both cases, the first day after pairing has been excluded from analysis [63] since the egg laid would have been too late to have been fertilized .

Effects of fluoride exposure on body weight

Body weights were taken for all males prior to and for the duration of the test

investigating the effects of fluoride exposure on fertilizing capacity. Prior to treatment, the designated control males were lighter than the birds that received fluoride (Fig. 5), although not significantly so ($p = 0.12$). A repeated measures ANOVA revealed that while body weights did increase over time ($p < 0.05$), there was no effect attributable to treatment ($p = 0.11$). In case the pre-treatment difference between groups was having an effect on subsequent weights, a repeated measures analysis of covariance was carried out using the first week (pre-treatment) as a covariate. In this case, while the effect of the weight differential in the first week on subsequent weeks was significant ($p<0.05$), treatment was not ($p=0.69$).

Water consumption was also monitored for the duration of the fertility trials. The males receiving fluoridated water consistently drank more than those receiving untreated water (Fig. 6). However, control males also weighed less than those exposed to fluoride. In order to determine if the weight differential between the treatments was responsible for the lower consumption rate observed in the controls, weekly mean consumption rates were divided by corresponding treatment body weight means. Males exposed to fluoridated water drank more than control males when consumption was adjusted for body weight (Fig. 7).

Effects of fluoride administration on reproductive behaviours

The first observation period represents reproductive behaviours observed in both groups prior to treatment. The number of copulations was nearly identical for the two groups (40 vs 42 for the pre-control and 250 ppm treatments). However, the mean number of copulations observed dropped thereafter in both treatments but more so for the males receiving the fluoridated water (Fig. 8). Several of the matings during the second and third trials were not carried out because of the asynchrony among the females. In order to maximize the use of available data these two periods were combined for subsequent statistical analysis. A repeated measures ANOVA revealed that while the number of behaviours did drop

significantly over time ($p<0.0005$), the effect of treatment on that reduction was not significant ($p=0.12$).

The number of neck grabs did not vary in a dose-dependent manner as copulations did. While both treatments were the same prior to dosing (29 counted for 10 breeding pairs over 1 hour), totals varied inconsistently thereafter (Fig. 9) . Not surprisingly, there was no correlation between the two behaviours for the control ($r_s=-0.29$, $p=0.64$) or the 250 ppm NaF group ($r_s=-0.14$, $p=0.87$).

Effect of fluoride on egg fertility

Fluoride was added to the males' drinking water beginning 21 February and continued until 26 April. During this period birds were paired five times with the initial pairing occurring the day prior to fluoride being administered to the males. The proportion of fertile eggs produced ranged from 18 to 45% (including the first egg laid after mating). As can be seen from Fig. 10, fertility did not vary between groups except after the second pairing. The fertility data were arcsin transformed and analysed using a repeated measures ANOVA. Due to the number of missing values occurring during trials 2 and 3, the data were combined for the purpose of this analysis. Although the control males produced more fertile eggs, there was no significant effect of the fluoride treatment on the proportion of fertile eggs produced by treated and untreated males ($p=0.79$). Although fertility increased in both groups over time, this increase was marginally non-significant ($p=0.055$). If the repeated measures analysis is confined to the pre-treatment trial and the final trial, the effect of the treatment is non-significant ($p=0.72$) as is the effect of time ($p=0.30$). The proportions of fertile eggs were calculated by including the first egg produced after pairing. Since this egg is usually well into the oviduct at the time of pairing it is highly unlikely that it would be fertilized and thus, it is often excluded from analysis. In this case the maximum proportion of fertile eggs increases to 50% (Fig. 11).

In order to assess if fluoride treatment had an impact on survivability of sperm in the sperm storage tubules the proportions of fertile eggs produced on the ninth day post-copulation were analysed. Results are presented in Table 2. There were no significant differences between the treatments ($p=0.78$).

Effect of fluoride exposure on semen quality

The effect of the heat stress and resulting change in environment caused the 30 males to stop producing semen and lose weight. A repeated measures ANOVA indicated that the weight loss recorded over this period was highly significant ($p<0.005$) and was consistent among treatments ($p=0.15$) (Fig. 12). Results from the analysis of semen quality indicate that the change of environment had no apparent long-term effect on the males. Body weights had recovered within four weeks during which time semen samples were collected but not included in subsequent analyses. Since the time to produce a viable sperm from a spermatogonium in the quail is about 15 days, all sperm which were potentially affected by the heater malfunction would have been replaced before data collection commenced. Most important, the majority of semen characteristics exhibited little variability over time and were not significantly different from the controls.

There was insufficient time between the initiation of dosing and moving the three dose groups to new facilities as a result of the heating malfunction to assess water consumption rates.. However, consumption rates were determined for all treatments during the two month period from early June to the end of July. Several of these data points are shown in Fig. 13. The control males were 6 weeks old when added to the pens on 21 May. The first estimates of consumption in Fig. 13 were made for the period 10-12 June. Overall, water consumption rates did not change dramatically, however all four groups exhibited a decline during the period 5-9 July. The control group drank considerably more than the remaining treatments even though the birds weighed considerably less.

While the other groups were also quite variable over time, only the 500 ppm NaF treatment declined steadily throughout the test period. An analysis of variance indicated a significant difference in consumption rates among the four treatments ($p=0.0017$).

Results of a *post hoc* multiple comparison test are presented in Table 3. The control group was not significantly different from the 125 ppm NaF treatment, but they drank significantly more than the two high fluoride groups. None of the treatments receiving fluoride was significantly different.

The three treatment groups had been entrained to the semen collection method for several weeks prior to the initiation of dosing. As a result, the proportion of males for which semen samples were obtained was high and relatively constant throughout the sampling period. Typically, 92 to 98% of males gave samples of somewhat variable quality. Initially this proportion was lower as the newly introduced control birds, young and unfamiliar with the semen collection method, did not regularly yield semen samples. Results of weekly semen collections are presented in Table 4.

Effects of fluoride on semen volume

All treatment groups produced semen consistently throughout the fluoride exposure period with anywhere from 83 to 100% of males providing samples large enough to analyse (>4.0 µl). Semen volume increased in the control group over the study period (Fig. 14). Initially, all males produced a mean of 9.13 µl (250 ppm NaF) to 10.29 µl (125 ppm NaF) (Table 5). The initial volume of semen produced did not vary among treatments ($p=0.24$). However, the control males showed a subsequent drop in semen production during weeks 2 to 6. The initial high value was from four active males and was not sustained during subsequent weeks. Regression analysis of individual male's semen production for the

duration of the study followed by an ANOVA of the regression coefficients by treatment indicated that the fluoride treatment did have an effect on semen production (p=0.0227). Contrasts of mean regression coefficients are presented in Table 5.

The untreated males were the only group which produced increases in semen volume over the study period while the 125 ppm NaF treatment, with a negative slope, decreased. The remaining treatments essentially did not change over the 15 weeks. The only significant difference detected was between the control and 125 ppm NaF groups. After 18 weeks of consuming fluoridated or non-fluoridated water individual semen volumes were not significantly different among treatments (p=0.59)

Effects of fluoride on sperm motility

Sperm motility was assessed for all semen samples collected throughout the study. Results are presented as means by treatment in Fig. 15. Each point represents the results of one collection of up to 10 individuals. Table 4 indicates that the number of males from which semen samples were successfully obtained was somewhat variable. The high value documented for the control males reflects the number of samples which were too small to analyse collected during the first month of the study. As with semen volume, regressions were developed for changes in motility over time for each male. An ANOVA was used to measure differences in regression coefficients among treatments. Mean motility scores and results of statistical analysis are presented in Table 6.

Mean motility scores declined in a dose-dependent manner from a high of 3.76 (control) to 2.63 (500 ppm NaF). However, there were no consistent responses over time with the exception of the control males where a general increase in motility was noted (regression coefficient = 0.12) (see Fig. 15). There appeared to be a drop in motility during weeks 7-10 for the fluoride-exposed males which

was least pronounced in the 250 ppm NaF treatment. The three treatments receiving fluoride varied from week to week with motility scores tending to increase toward the end of the study. When the regression coefficients were analysed the model was found to not account for a significant proportion of the variability in the data ($p=0.07$).

Effects of fluoride on sperm live/dead ratios

The number of live versus dead sperm was assessed for each sample collected throughout the treatment period. Unlike other semen parameters, live/dead ratios for the control group were relatively constant over time with the exception of a low value recorded during the first week (Fig. 16). The three treatments receiving fluoride showed a decline in this ratio from approximately week 7 onward. This decrease in the proportion of live sperm during the latter half of the study resulted in a mean reduction in this variable when calculated over the entire study period (Table 7).

There was no significant difference in live/dead ratios among treatments at week 7. However, at the end of the study all treatment groups were significantly lower than the control males (Table 7), although there were no differences among the three fluoride doses. These results were confirmed by analysis of the regression coefficients which found the slopes of all treated groups to be not significantly different from each other but different from the controls.

Effects of fluoride on sperm concentration

Sperm concentration was calculated using a Makler counting chamber. Each calculated sperm concentration represents a mean of five individual counts. Data are presented in Fig. 17 as a mean of the number of samples collected on a given day ± 1 standard error.

As was the case with sperm motility and volume, the number of sperm per mm^3

tended to increase with time for the control group and stay relatively constant for the groups receiving fluoride with the exception of the 250 ppm NaF group where it declined (Fig. 17). These trends are reflected in the positive regression slope for the control males and the near-zero slopes for each fluoride treatment. However, an ANOVA of these coefficients found the slopes of all treatments to be not significantly different at p=0.05.

The data were also analysed by examining sperm concentrations measured two times during the study. Sperm concentrations calculated in the first week (p=0.0051) and last week (p=0.0026) were significantly different (Table 8). Both the control and 500 ppm NaF treatments recorded significantly lower concentrations than the remaining dose groups. By the last week of treatment the sperm concentration for the 250 ppm NaF males was significantly lower than the control or 125 ppm NaF groups while the 500 ppm NaF males were intermediate between the two (Table 8).

Effects of fluoride on the number of sperm per ejaculate

The number of sperm per ejaculate was determined by multiplying semen volume by sperm concentration for individual samples (Fig. 18). Again the control group was found to have increasing numbers with time while the three fluoride-treated groups did not vary over the exposure period (Table 9). The treatment mean indicated that the control males produced fewer sperm per ejaculate than the 125 ppm NaF group. This is a function of the lower initial values which gradually rose as the experiment proceeded. The slopes of the regressions within treatments confirmed that the control alone was significantly different from the remaining groups. An analysis of variance revealed that the regression intercept for the 125 ppm NaF treatment was significantly larger than the other groups. This result is a function of the larger area under the curve as well as the negative slope of the regression line. The negative slope may be attributed to the declines in sperm numbers which were observed during the first four weeks.

Fluoride concentrations in bone

Fluoride was measured in the femurae of all treated and control male quail. As would be expected, bone fluoride levels increased but not directly in proportion to fluoride dose. Results of fluoride analyses are presented in Table 10.

The data were tested and found to be normally distributed. A subsequent analysis of variance with a Tukey's test to locate differences between treatment means found all treatments to be significantly different. The variability within treatments was found to be significant but comparable to what was previously documented in American kestrels [41,88]. Coefficients of variation calculated for these data were lower than similar values measured in American kestrels as part of this research. The difference may be attributed, in part, to the uniform age of the quail in this study. The bone fluoride levels in the control males appear to be somewhat elevated. In addition these birds were considerably younger than the treated birds (24 vs 36 weeks). While the fluoride doses increased two-fold, the difference between fluoride treated femurae within groups was considerably less (25% between the 125 and 250 ppm NaF treatments and 34% between 250 and 500 ppm NaF treatments).

Effects of fluoride on gonad size and mass

Both the left and right testes of all quail in the study were weighed and measured at necropsy. The combined testes weighed between 1.36 and 7.22 g with the left usually the larger. Summary statistics are presented in Table 11.

There was no effect of treatment on testicular mass when measured as combined testes weights or when expressed as a function of body mass (gonadosomatic index, GSI). When all data were combined and a regression of combined testicular mass on body mass was performed the relationship was not significant ($p=0.34$)(Fig. 19). However, when individual regressions were plotted (Fig. 20), the slopes for the control and 125 ppm NaF treatments were quite

different from the remaining groups. Due to the variability of the data within each treatment, none of the regressions was significant at the 0.05 level. Similarly, testicular volume which ranged from 3348 (control) to 6773 mm^3 (125 ppm NaF) (Fig. 21) was not significantly different among treatments (p=0.27).

DISCUSSION

Weight gain

Clearly the 6-week old Japanese quail used in this study had not reached their adult mass since there was a significant increase in body weight between 7 and 12 weeks of age. However, there was no corresponding increase in the rate of food disappearance, indicating that wastage was significant, thus masking the concomitant increase in food consumption documented elsewhere [54]. Wilson *et al.* [54] reported that Japanese quail reach sexual maturity at 5-6 weeks of age and a mature body size at 8 weeks. Their males reached adult body weights of 110g, while the males in this study weighed approximately 190g. Similarly, water consumption of undosed birds was variable and showed no consistent trend over time despite the increase in body mass of the birds. The changes in water consumption from day to day may also reflect fluctuations in room temperature or variation in the time period over which consumption was measured. Finally, different genetic stocks of this species have been selected for large body mass [89] including the quail used here. The impacts of artificial selection on other variables measured in this study may make comparison with other investigations using this species difficult.

Quail body weights were unaffected by the fluoride treatment, however numerous studies where fluoride was administered to fowl documented reductions in body weight. The doses of fluoride (added to the ration) which result in weight loss tend to be quite high, ranging from 700 (82) to 1200 ppm NaF (24). In fact, 600 ppm NaF delivered in the ration of hens in the latter study results in a daily intake equivalent to 250 ppm in the drinking water of quail. There was no significant reduction in body weight of the hens at this exposure rate. In the only experiment where fluoride was added to the drinking water, Merkley and Sexton (79) found 100 ppm NaF did not have a significant effect on body weight of day-old white leghorn chicks compared to unexposed birds. In an

experiment to assess the impact of a toxicant on semen quality, significant reductions in body weight could be indicative of acute toxicity which may have indirect consequences on semen production. Accordingly, doses used here were selected to ensure that overt toxicity would not result.

Egg production and fertility

Since several of the eggs produced on the last day of the first 8 day fertility trial were fertile, the second trial was extended to 12 days. The initial choice of 8 days' duration was based on studies by Sittman and Abplanalp [61] who found the mean duration of fertility resulting from random matings to be 6.5 days and by Wentworth and Mellen [60] who reported the maximum length of fertility to be 6.2 days. Results of the second trial indicated that the maximum duration of fertilizing capacity was 10 days since none of the eggs produced during the eleventh and twelfth days was fertile. These results agree fully with those of Birkhead and Fletcher [57]. Ogasawara and Huang [90], also working with Japanese quail, artificially inseminated females and measured fertility from day 2-11 post-insemination. Only in one case out of 33 was the egg laid on the 11^{th} day fertile.

The fertility of eggs produced is comparable with other published studies involving Japanese quail. The fertility values calculated here exclude all broken, deformed and otherwise defective eggs and do not include the eggs laid on the first day post-copulation in both trials or the last two days of trial 2 (days 11 and 12) when only infertile eggs were produced. The fertility of eggs calculated by McFarquhar and Lake [91] produced between days 2 and 10 after the termination of mating was 42.5%, comparable to 42.4% measured in this study and higher than the 30.9% figure by Ogasawara and Huang [90] using artificial insemination, calculated between days 2 and 11 post-pairing. However, using a similar experimental design Sittman and Abplanalp [61] reported 57.1% fertility between days 2 and 10 after mating but excluded all completely infertile pairings.

A comparable fertility rate for the data produced here would be 74%. Taji and Ikeda [62] mated a male to a single female once per day and noted an 80.6% fertility rate.

The lower fertility rate reported here may be attributable to the shorter time the males were allowed to mate with the females. The method which fluoride was to be administered to the males required that they be housed separately from the females. When paired they were aggressive toward the females and pecked them repeatedly on the head and nape of the neck, causing lesions in several cases. Accordingly, males were paired for a 1/2 hour to 3 hour period prior to the initiation of a test. The higher fertility reported above (55) may reflect the fact that the birds were paired continuously prior to the initiation of the study. Although the males were paired for a briefer time than those reported above, Schein *et al* (70) working with Japanese quail, found that 83% of males placed in a pen with a female will succeed in copulating within the first 5 minutes. Thus, 30 minutes was ample time for those males with the intention of copulating to do so. Using the fertility rates obtained here as an indicator, the methods used to house the birds and techniques used in pairing were at least comparable to other studies.

The levels of fluoride selected for this study were based on those administered to domestic fowl. In the majority of these cases, fluoride was administered as sodium fluoride in the food at concentrations ranging from 100 [92] to 800 ppm [93,94] and as high as 1300 ppm [95]. Merkley [21] administered up to 300 ppm sodium fluoride in drinking water to white leghorn pullets and found no effect on egg production. Males were not included in the study. In the only published study where Japanese quail were exposed to fluoride, Fleming and Schuler [72] added fluoride (up to 1000 ppm F^-) to the food or via intubation at rates up to 160 mg F^-/kg bw for 16 days. Quail intubated with up to 36 mg F^-/kg bw survived the study while higher doses administered by stomach tube produced significant mortality.

None of the birds administered fluoride in the diet died, suggesting increased toxicity of fluoride when administered at elevated concentration in a liquid vehicle. Unlike the methods of Fleming and Schuler [72], fluoride was administered in the drinking water in this study. Because large portions of food rations were spilled by the birds on the ground, calculating the mean fluoride intake rate per quail would have been extremely difficult. In addition, adding the fluoride to the water ensured that the compound was evenly distributed in the vehicle.

Effects of fluoride on the duration of egg fertility

To evaluate the effects of fluoride on the duration of fertility, sodium fluoride was added to the drinking water at the rate of 250 ppm NaF. Male quail drank approximately 20-25 ml/day, resulting in an estimated daily intake of 31 mg NaF/kg bw or approximately 15 mg F^-/kg. Since the experimental design required the birds to be exposed for a considerably longer time than the 16 days discussed above (birds were given fluoridated water for 64 days), the dose was halved to insure the survival of the quail.

The addition of 250 ppm NaF to the drinking water of male Japanese quail clearly had no effect on their ability to produce fertile eggs. In all cases, whether the first egg after copulation was excluded from analysis or not , the difference in fertility between the dosed and control birds was not significant. Merkley [21] exposed white Leghorn hens to 100 ppm F in their drinking water and found no effect on egg production but did not measure fertility. However, Merkley and Sexton [92] added 100 ppm F to the drinking water of white Leghorn males and found no decrease in fertility or duration of fertility when paired with similarly dosed hens. Mehdi *et al* [22] added up to 600 ppm of NaF to the ration of male and female chickens and measured egg production, but not fertility. Production declined slightly in a dose-dependent fashion, although the significance of the decline cannot be evaluated as no statistical analyses were provided.

By measuring the duration of fertility after separating the male from female quail the fitness of sperm in the sperm storage tubules may be measured. In this way changes in the fertilizing capacity of the sperm over time resulting from fluoride exposure may be assessed. Various aspects of the physiology and biochemistry of the sperm storage tubules of the Japanese quail have been discussed in the literature [57,73,74]. These ducts typically allow female birds of a number of species to store sperm in a viable state for several days or even weeks, lengthening the period of time over which eggs may be fertilized without copulating with a male [96]. The maximum duration of fertility of eggs for the Japanese quail measured once the male is separated from the female is 10 days as corroborated in this study (see also [57,60,61]).

While fluoride treatment did not affect the proportion of fertile eggs produced, there was also no change in the temporal distribution of fertile eggs. Nine of 45 eggs produced by the control group were fertile on day nine (20%) as were 10 of 42 (24%) produced by the 250 ppm NaF treatment. Fluoride treatment may have had sublethal effects on sperm fitness (32,36), including energy metabolism, development and structural integrity. While these changes may not have had an impact on fertilizing capacity immediately after ejaculation, temporally-induced reductions in motility and viability may have resulted. While the effects of fluoride administration on avian sperm have not been extensively studied, reductions in motility and increases in the proportion of dead sperm have been noted (68). While it is possible that these factors were compromised here as well, these effects were clearly not significant enough to result in decreases in fertility over a 64-day exposure period.

Effects of fluoride on reproductive behaviours

The reproductive behaviours of the male Japanese quail were monitored each time they were placed in a pen with a female. These behaviours have been well characterized in the literature [76,78,97]. The lack of significant difference

between the two treatments for these variables (copulations and neck grasps) supports the findings of the egg fertility results. Although more copulations were observed for the control group, the number of neck grasps was generally higher in the 250 ppm NaF treatment. Except for the pre-treatment period, the number of copulations correlated quite well with the proportion of fertile eggs. The higher occurrence of copulations during the pre-treatment period coupled with a lower proportion of fertile eggs may reflect the inexperience of a given quail resulting in a lower proportion of successful copulations. The number of neck grasps did not follow the same trend as the proportion of fertile eggs. Since copulations often, but not always follow neck grasps [76], the additional variability inherent in the frequency of the latter results in poorer correlations with egg fertility. In fact, it appears to be sufficient to record the number of copulations alone when performing behaviour observations in this context.

When discussing the results of the semen evaluation component of the study, the effect of the unfortunate loss of the control males one week into the treatment period must be considered. Although other sexually mature birds were available, they were 6 weeks old at the time of inclusion, they had to adapt to considerable environmental change as they were moved from flock housing in a heated room to individual pens in an unheated room, and perhaps most importantly, they had not been entrained to the semen collection method. As a result, every parameter measured in the semen samples subsequently collected shows a positive slope for the control males as they matured and became accustomed to manipulation. The three treatment groups, which suffered no mortality (presumably because of their location away from the damaged heating unit), were unaffected by the above. They had been entrained to the semen collection method for 8 weeks prior to the initiation of the study and were 20 weeks old at the initiation of dosing. As a result, the control results cannot strictly be treated as such since the above mentioned variables were not held constant between the untreated and treated birds.

Since the three fluoride-treated groups were also moved to unfamiliar, unheated facilities, there was a significant reduction in body weight for all treatments. Because of the major changes in environment, semen samples were not evaluated until body weights had begun to increase, i.e. about the end of May. The control birds' weights were considerably lower but were comparable to treated birds within several weeks.

Effects of fluoride on water consumption

The amount of water consumed by treated and control males during the semen evaluation experiment varied significantly. All groups receiving fluoride drank less than the undosed males, but the 250 and 500 ppm NaF treatments drank significantly less. Interestingly, the 250 ppm NaF treated males in the egg fertility study drank more than the control males while the reverse was the case for the birds used in the semen evaluation experiment. Since the water given to the undosed birds was the same in both cases, other environmental factors may have been the cause of the discrepancy. Males were held inside in a heated facility for the duration of the egg fertility study. However, as a result of the heater malfunction and subsequent death of the control males, the semen evaluation research was moved to an unheated room with direct access to the outdoors providing natural light and air. After the change of environment, the untreated birds drank at a higher rate while those drinking fluoridated water consumed less. The addition of fluoride to the diet of laying hens frequently causes depression of feed intake [93,95]. Although several authors exposed fowl to fluoridated water [21,92], water consumption rates were not reported. Since it is likely that addition of fluoride to the drinking water would cause a reduction in consumption rates as was found in the unheated facility, some unknown aspect of the environment in the indoor pens may have caused the control males to drink less.

One result of reduced consumption was to cause a decline in the amount of

fluoride ingested. Assuming the treated groups were drinking at the same rate as the controls, the 500 ppm group was actually ingesting 2.5 mg less fluoride per day than predicted because of reduced water consumption. Similarly, the 250 ppm NaF treatment ingested approximately 4.8 mg NaF/day, not the predicted 5.75 mg and finally, the 125 ppm males drank 2.5 mg NaF/day, not the 2.9 mg that was anticipated. These changes in consumption rates probably did not have a significant effect among groups since the two-fold increase in fluoride concentration between doses was preserved (2.5, 4.8 and 9.2 mg NaF/day for the 125, 250 and 500 ppm NaF treatments, respectively). However, over the 122 day exposure period these reductions in total fluoride consumed could be significant. Since other reports do not routinely discuss actual versus predicted consumption rates it is difficult to interpret these findings except to acknowledge that this factor must be considered when discussing the results of this research. These results are in disagreement with the findings of Krasowska and Wlostowski [31], the only reference citing water consumption rates, who found no difference in drinking rates in rats exposed to 100 and 200 ppm F in their water of up to 16 weeks.

Effects of fluoride exposure on semen quality

Analysis of semen samples is an effective method to assess changes in male reproductive potential [98-100]. However, depending on the species, this may not be possible as animals from which serial samples can be obtained tend to be large and expensive to maintain (i.e. rabbits, beagle dogs). Commonly used laboratory species such as the rat are not amenable to collection of semen [101]. Japanese quail were selected for this study because they are small and easily housed at relatively high densities. As well, methods for the collection of semen have been determined [58,60,63], although there are no reports discussing the frequency at which longitudinal samples may be collected. The ability to collect serial semen samples from the same individuals permitted an analysis of the effects of chronic fluoride administration on various aspects of semen quality.

Ejaculated semen volume increased dramatically in the controls and is almost certainly a reflection of acclimation of the young birds to the collection procedure. It stayed relatively constant in those birds exposed to fluoride. Wentworth and Mellen [60] reported the volume of semen collected from Japanese quail to be 10 µl, in close agreement with the mean range of 9.1-10.3 µl documented in this study but somewhat higher than the range of 3.9-6.9 µl measured by Buxton and Orcutt [102]. The fluoride-treated males showed no effect of dosage on semen volume. This is reflected in the lack of difference in the regression coefficients for the three fluoride-treated groups which indicates the slope of the regression of change in semen volume over time. Although there were no significant differences in semen volume, there was a decrease with increasing fluoride dose. There are no references in the literature which discuss the effect of fluoride administration on any aspect of semen quality of serially sampled birds or mammals. Several authors have investigated sperm morphology of exposed fowl [22,75], however samples were obtained after euthanasia making estimation of ejaculate volume impossible. Semen volume is one of the recommended endpoints to measure when evaluating risks to male reproduction [99,103,104] although there are errors associated with its measurement [105]. Bird *et al.* [106] measured semen volume in American kestrels exposed to PCBs and mirex and noted differences among treatments. Furthermore, measurement of semen volume is necessary for the calculation of total sperm per ejaculate, an important indicator of semen quality [43,102].

Sperm motility was highly variable and an analysis of variance of individual regression coefficients indicated no effect of treatment on the four groups. While the model did not account for a significant proportion of the variability in the data, there were obvious differences between the control and fluoride-treated males. As mentioned previously, initial age and lack of experience probably accounted for the increase in sperm motility observed in the control group. Clulow and Jones [107] assessed motility in the genital ducts of Japanese quail and found

up to 82.9% of sperm to be motile. Relative motility was also recorded but unfortunately not reported. Cheng *et al.* [108] discussed the motility of quail sperm but made no quantitative analysis. While motility is commonly measured in routine semen analysis and has proven valuable when assessing the quality of sperm [42,47], subjective measurement of movement may not be useful when estimating the effect of treatment on fertilizing capacity [44]. The variability associated with the values reported here may be related to the difficulty in accurately and repeatedly quantifying the swimming vigour of thousands of sperm. While these measurements were made on living samples, the use of computer-aided video recording has greatly reduced the variability associated with motility measurements [44].

Live/dead ratios measured in this study did not vary among fluoride treatments but were all significantly lower than the control samples. There was one reference to live/dead ratios of Japanese quail sperm in the literature. Shrivastava *et al.* [58] documented a range of 4.1-7.2% dead sperm collected from untreated quail of various ages. These results compare favorably to the mean control value of 4.3% recorded here. While there was no difference in this variable at week 7, the decline by week 15 observed in the treated males from the untreated level was significant. Numerous studies have documented various negative impacts of fluoride exposure on sperm viability. Chinoy *et al.* [35] noted reductions in the number of viable sperm and an increased rate of abnormalities in the testes of rats injected with 50 µl of NaF. Reductions in sperm count and viability were also noted in rats orally exposed to 5 and 10 mg NaF/kg bw for 30 days [32] and rabbits dosed with 20 and 40 mg NaF/kg bw for 30 days [34]. The effect of 250 ppm NaF on the duration of fertility was not significant. However, the experiment terminated at approximately the time when the proportion of live sperm began to decline in the semen characterization study. As a result, since there was no difference between the 250 and 500 ppm NaF groups, it is possible that a decrease in fertility would have been observed if the egg fertility test had

been extended by two to four weeks. In addition, species which produce semen with a lower sperm concentration would be more likely to suffer reduced fertility from a depressed live/dead ratio than those which produce relatively concentrated semen (ie. the Japanese quail). American kestrels are known to produce semen with a low sperm concentration. It is not known if the fluoride-exposed male kestrels evaluated as part of this research exhibited increases in the proportion of dead sperm because of the difficulties associated with microscopic evaluation of semen samples.

Several authors have reported sperm concentrations for Japanese quail. Values ranged from a mean of 1.2 $x10^6/mm^3$ [60] to a range from 0.75-1.57 $x10^6/mm^3$ [58]. Buxton and Orcutt [102] calculated a mean of 4.7 $x10^6/mm^3$, a value considerably higher than the previous reports and higher than the range found for the untreated males in this study of 1.2-1.7 $x10^6$ sperm/mm^3. Sperm concentrations were relatively constant over time and among treatments. After 15 weeks of fluoride administration values ranged from 1.3 to $1.6x10^6$ sperm/µl of semen. While concentrations did decline it was not in a dose-dependent manner with the 250 ppm NaF group having a lower value than the 500 ppm NaF group. Perhaps more importantly the slopes of the regressions calculated for each treatment were not significantly different indicating that the effect of fluoride exposure on sperm concentration over time did not vary among treatments. Chinoy *et al.* [32] noted reductions in sperm counts of rats administered 5 and 10 mg NaF/kg bw orally for 30 days. If the doses in this study were converted to a body weight basis and using actual amounts of water consumed, the 125 ppm NaF treatment equates to 12.5 mg NaF/kg bw followed by 24 and 46 mg NaF/kg bw for each successive dosage. The lack of effect of this treatment regime on Japanese quail may relate to the relative insensitivity of birds to the effects of fluoride compared to mammals [18,22].

Sperm number per ejaculate was calculated for each sample by multiplying

semen volume by the corresponding sperm concentration. Sperm numbers per ejaculate were calculated to range from 1.1-4.5 $x10^7$ [102] with a mean of 2.6 $x10^7$ and 1.2 $x10^7$ by Wentworth and Mellen [60]. The values measured for the control males in this study, 1.48 $x10^7$sperm/ejaculate, are in closer agreement with the latter. The elevated estimates presented by Buxton and Orcutt [102] may reflect the unusually low ejaculate volumes they recorded. Many of the studies examining sperm characteristics of Japanese quail removed the sperm surgically, making determination of ejaculate volume impossible. The slopes of all response curves derived in this study were similar except for the control males, presumably because the effects of age and experience resulted in an increase in total sperm/ejaculate over time. Since both these parameters declined in a dose-dependent fashion for the fluoride-treated males (but not significantly in all cases) a corresponding reduction in the intercepts of the regressions of sperm/ejaculate versus time was noted. This decline in intercepts with dose (1.69, 1.36, 1.2$x10^6$sperm/µl for 125, 250, 500 ppm NaF, respectively) was significant for all paired dose-group analyses. Since the slopes of the curves were not significantly different from zero, it is safe to assume that differences in intercepts would translate into lower sperm per ejaculate over the treatment period.

The total number of sperm ejaculated is a useful parameter to assess when evaluating semen quality . The fewer sperm released, the less likely the egg will be fertilized. However, this is only true to a point. Normally the number of sperm produced is well in excess of what is needed to ensure fertilization. In fact, rats whose sperm production had been reduced by 90% did not experience a reduction in fertility [45]. However, the ratio of sperm ejaculated versus the threshold number below which reduced fertility is observed is variable among species. In rats and bulls the safety factor is large, perhaps 1400-fold while in humans it is quite small (i.e. 2-4 fold [44]). This factor has not been determined for Japanese quail. Clulow and Jones [107] measured sperm production rates in

this species per gram of testicular tissue and, compared to rats, quail produce four times as many sperm but with considerably less survival time in the reproductive tract. Although males produce large quantities of sperm it is difficult to predict with confidence what the impact of a 40-50% reduction in the number of sperm per ejaculate would be until the effects on fertility have been evaluated.

Overall, with the exception of the reduction in live/dead ratios, there were no significant effects of fluoride administration on semen quality. These results are supported by the lack of effect of 250 ppm NaF administered to male Japanese quail on the number of eggs fertilized or duration of fertility of eggs produced over a 12 d period. Unlike measurements of damage to testicular ultrastructure and other histological effects, measurements of semen quality are a more direct assessments of a male's ability to fertilize an egg. Reductions in the number of Sertoli cells, Leydig cells or other indicators of testicular damage may or may not impact on sperm number, motility and viability. Because of the difficulty associated with collecting serial semen samples in many laboratory animal models, coupled with the difficulty in making consistent, repeatable quantitative assessments of semen parameters, this component of the reproductive system has been somewhat neglected [109]. Although there are numerous published reports on the tendency of fluoride to cause degeneration of the testicular ultrastructure there is no information on what, if any, effect exposure had on the animals' ability to produce viable sperm as determined by fertilizing capacity.

The use of Japanese quail in this study has demonstrated that up to 500 ppm NaF administered in a highly available form over an extended period of time had no significant effect on their ability to maintain semen quality. Furthermore, the ultimate test of semen quality, fertilizing capacity, was confirmed by the lack of effect on egg fertility between males treated with 250 ppm NaF and control males. Confirmation of exposure by analysis of femoral fluoride burdens confirmed that elevated levels of fluoride were ingested and assimilated with little

or no measurable impact on reproduction. The ability to obtain serial semen samples from this species should increase its appeal as a model species in toxicological examinations of sperm quality.

Effect of fluoride on testicular mass

When evaluating the impacts of a chemical on the male reproductive system analysis of organ weights can be a useful indicator of toxic insult [99,101]. Several studies have reported testicular weights of different genetic lines of Japanese quail [89,110]. Gonadosomatic indices (GSI) were calculated to be between 0.13 and 1.05 (testes weight/body weight *100) for 36 day old males. Comparable values reported here for unexposed birds ranged from 1.9 to 3.7 with a mean of 2.5. These differences may be attributed to the genetic strain investigated [110] and the age of the animal, since testicular mass increases with age up to maturity in many species [101]. The effect of numerous compounds on testicular weights of Japanese quail have been described in the literature.

Hill and Soares [68] noted reductions in testicular weights of quail subchronically exposed to organic and inorganic mercury. In an investigation of the toxicity of PCBs, Biessmann [64] reported non-significant reductions in testicular masses of male quails. Eroschenko [111] exposed Japanese quail to chlordecone, an organochlorine insecticide, and found significant reductions in GSI between control and treated birds. Gonad weights of quail receiving DDT in their ration were found to vary inversely with dosage [65]. While there are no references in the literature discussing the effects of fluoride administration on avian testicular tissue, information is available for mammalian studies. Chinoy *et al.* [35] injected 50 µg NaF in the vas deferentia of rats. Although reproductive effects were noted, there was no effect on the mass of the testes. Chinoy *et al.* orally administered 5 and 10 mg/kg bw NaF to rats and measured effects on testicular mass but did not report these results [32]. Male rats were exposed to 100 and 200 ppm NaF in their drinking water for 6 and 16 weeks. No effects were noted

on the development of the testes [31]. Finally, mice were administered 10 and 20 mg Naf/kg bw for 30 days [33]. While reductions in body weight were noted, no significant declines in testes weights were reported. The lack of effect of fluoride administration on testicular mass in this study is supported by similar results from the limited number of studies discussed above. It is important to note that a lack of change in this variable does not, in itself, rule out impacts at other points in the reproductive process as is discussed by Working [44].

Femur fluoride levels

That fluoride will accumulate in calciferous tissues has been confirmed many times in the literature [112-114]. The purpose of measuring fluoride burdens in the femurae of the Japanese quail in this study was not only to confirm that the method of administration was effective since fluoride in aqueous solutions is easily assimilated [72,115,116]. More importantly, the calculated bone fluoride levels allow for the interpretation of the effects discussed here within the larger context of levels encountered in the wild and those used in other studies.

Compared to bone fluoride levels measured in birds elsewhere, the dosing scheme here produced significant fluoride burdens. Culik [9] measured femoral fluoride levels of over 10 000 ppm in Antarctic gentoo penguins (*Papua papua*) known to ingest fluoride-rich prey, while black-crowned night herons (*Nycticorax nycticorax*) feeding near a source of industrial fluoride contamination were found to contain up to 11 000 ppm F in their femurae [37]. A survey of fluoride contamination of British birds revealed birds of prey with up to 5000 ppm F in the skeleton (values converted from dry weight to ash weight) [10]. Bone fluoride levels in red-billed gulls (*Larus novaehollandiae*) from New Zealand ranged from 1053 to 8050 ppm [5]. Clearly the range of bone fluoride levels induced in this study (3 000-9 000 ppm) are comparable with those measured in biota from naturally and anthropogenically contaminated areas.

Laboratory evaluations of screech-owl (*Otus asio*) reproduction where both sexes were treated with NaF in their food noted effects when bone F levels measured 2800-4000 ppm (values converted from dry weight to ash weight) [39]. While the number of young produced per clutch was significantly lower than the controls, there was no significant difference when expressed as a function of the number of fertile eggs produced. Interestingly, the number of fertile eggs produced, when expressed as a proportion of the number of eggs laid did not vary significantly among treatments. Male kestrels exposed to fluoride as a part of this research produced fewer fertile eggs, but the reduction was not statistically significant. In two reports male and female American kestrels were exposed to fluoride topically applied to the diet for 10 d [40], or fed cockerels (which had been fed fluoridated water for 10 d) as part of the diet [41] for 10 d. In another experiment American kestrel chicks were fed fluoride-contaminated food for 27 days with no apparent effects on growth and accumulated bone levels of approximately 18,000 ppm F [117]. The proportion of fertile eggs produced of those laid did not vary significantly among treatments, nor did the number of young produced. Although there were no effects on reproductive performance, bone fluoride levels were quite low in both studies (<1000 ppm) indicating that the exposure period may have been too short and did not adequately precede the period when the pairs would have been copulating. Numerous studies measured egg production and bone fluoride levels in hens fed fluoride. Merkley [21] added 100 ppm NaF to the drinking water of laying hens for 45 weeks and measured up to a mean of 9450 ppm in the tibiae. The treatment did not affect egg quality or production. Unfortunately, male reproductive performance was not evaluated.

The bone fluoride burdens measured in Japanese quail from this study are comparable with those documented in various species of birds inhabiting naturally and anthropogenically contaminated sites. Similarly, laboratory studies produced equal F levels in various osseous tissues of laying hens and raptors.

Because of the varied objectives of these experiments it is difficult to draw firm conclusions regarding levels of fluoride exposure and resulting impacts on reproductive success.

The results described above are somewhat consistent with those documented here and previously in the study with American kestrels. In both the screech owl study-of Pattee [39] and the kestrel reproductive assessment conducted as part of this thesis, birds were exposed to fluoride for a lengthy period of time and documented non-significant reductions in the proportion of eggs laid which were fertile. The screech- owls produced significantly fewer fertile eggs on a per clutch basis, unlike the kestrels. Since both species had approximately equal fluoride burdens, it is possible that the kestrels are relatively insensitive to the effects of fluoride. Using femoral fluoride burdens as an approximate yardstick, the Japanese quail used in this study, which accumulated twice the fluoride of either of the two previous species, still did not exhibit significant declines in egg fertility or semen quality. Since the fluoride in both experiments conducted as part of this thesis was adminstered in in a very bio-available form, the reproductive risk experienced by American kestrels and Japanese quail resulting from exposure to fluoride is not significant.

It is apparent from this study that chronic exposure to environmentally relevant levels of fluoride did not result in significant reductions in a number of semen parameters of Japanese quail. While a preceding assessment did not detect any effects of the median dose on extent or duration of fertility, the exposure period ended at approximately the time that the proportion of live sperm in ejaculates began to decrease in the subsequent study. Accordingly, it would be worthwhile to extend the length of the egg fertility experiment for an additional six to eight weeks to determine if the observed decline in the proportion of live sperm in the quail ejaculate would result in lower egg fertility.

REFERENCES CITED

1. **Kay, C.E., P.C. Tourangeau and C.C. Gordon.** 1975. Fluoride levels in indigenous animals and plants collected from uncontaminated ecosystems. *Fluoride* 8:125-133.

2. **Wright, D.A., A.W. Davidson and M.S. Johnson.** 1978. Fluoride accumulation by Long-tailed field mice (*Apodemus sylvaticus* L.) and Field voles (*Microtus agrestis* L.) from polluted environments. *Environ. Pollut.* 17:303-310.

3. **Newman, J.R. and J.J. Murphy.** 1979. Effects of industrial fluoride on Black-tailed deer (preliminary report). *Fluoride* 12:129-135.

4. **Adelung, D., K. Bößmann and D. Rößler.** 1985. The distribution of fluoride in some Antarctic seals. *Polar Biol.* 5:31-34.

5. **Stewart, D.J., T.R. Manley, D.A. White, D.L. Harrison and E.A. Stringer.** 1974. Natural fluorine levels in the Bluff area, New Zealand. *N. Z. J. Sci.* 17:105-113.

6. **Turner, J.C., S.R.B. Solly, J.C.M. Mol-Krijnen and V. Shanks.** 1978. Organochlorine, fluorine, and heavy metal levels in some birds from New Zealand estuaries. *N. Z. J. Sci.* 21:99-102.

7. **Seel, D.C.** 1982. Fluoride in the magpie. Report number 73. Institute of Terrestrial Ecology. Gwynedd, Great Britain.

8. **Seel, D.C. and A.G. Thompson.** 1984. Bone fluoride in predatory birds in the British Isles. *Environ. Pollut. (Ser. A)* 36:367-374.

9. **Culik, B.** 1987. Fluoride turnover in Adélie penguins (*Pygoscelis adeliae*) and other bird species. *Polar Biol.* 7:179-187.

10. **Seel, D.C., A.G. Thompson and R.E. Bryant** 1987. Bone fluoride in four species of predatory bird in the British Isles. In Coughtrey, P.J., M.H. Martin and M.H. Unsworth, eds., *Pollutant Transport and Fate in Ecosystems. Special Publication Number 6 of the British Ecological Society.* Blackwell Scientific Publications, Oxford, pp. 211-221.

11. **Karstad, L.** 1967. Fluorosis in deer (*Odocoileus virginianus*). *Bull. Wildl. Dis. Assoc.* 3:42-46.

12. **Shupe, J.L., A.E. Olson, H.B. Peterson and J.B. Low.** 1984. Fluoride

toxicosis in wild ungulates. *J. A. V. M. A.* 185:1295-1300.

13. **Suttie, J.W., R.J. Hamilton, A.C. Clay, M.L. Tobin and W.G. Moore.** 1985. Effects of fluoride ingestion on White-tailed deer (*Odocoileus virginianus*). *J. Wildlife Diseases* 21:283-288.

14. **Suttie, J.S., R. Dickie, A.B. Clay, P. Neilson, W.E. Mahan, D.P. Baumann and R.J. Hamilton.** 1987. Effects of fluoride emissions from a modern primary aluminum smelter on a local population of White-tailed deer (*Odocoileus virginianus*). *J. Wildl. Diseases* 23:135-143.

15. **Van Rensburg, S.W.J. and W.H. De Vos.** 1966. The influence of excess fluoride intake in the drinking water on reproductive efficiency in bovines. *Onderstepoort J. Vet. Res.* 33:185-194.

16. **Shupe, J.L. and A.E. Olson** 1983. Clinical and pathological aspects of fluoride toxicosis in animals. In Shupe, J.L. eds., *Fluorides: Effects on Vegetation, Animals and Humans.* Paragon Press Inc. Salt Lake City, pp. 319-338.

17. **Phillips, P.H., E.B. Hart and G. Bohstedt.** 1934. Chronic toxicosis in dairy cows due to the ingestion of fluorine. *Research Bulletin (Univ. Wisconsin Ag. Exp. Stn.)* 123:1-29.

18. **Mitchell, H.H. and M. Edman.** 1952. The fluorine problem in livestock feeding. *Nutrit. Abst. Rev.* 21:787-804.

19. **Udall, D.H. and K.P. Keller.** 1952. A report on fluorosis in cattle in the Columbia River Valley. *Cornell Vet.* 42:159-184.

20. **Shupe, J.L., M.L. Miner, D.A. Greenwood, L.E. Harris and G.E. Stoddard.** 1963. The effect of fluorine on dairy cattle II. Clinical and pathologic effects. *Am. J. Vet. Res.* 24:964-984.

21. **Merkley, J.W.** 1981. The effect of sodium fluoride on egg production, egg quality, and bone strength of caged layers. *Poult. Sci.* 60:771-776.

22. **Mehdi, A.W.R., K.A. Al-Soudi, N.A.J. Al-Jiboori and M.K. Al-Hiti.** 1983. Effect of high fluoride intake on chicken performance, ovulation, spermatogenesis and bone fluoride content. *Fluoride* 16:37-43.

23. **Van Toledo, B. and G.F. Combs Jr.** 1984. Fluorosis in the laying hen. *Poult. Sci.* 63:1543-1552.

24. **Hahn, P.H.B. and W. Guenter.** 1986. Effect of dietary fluoride and aluminum on laying hen performance and fluoride concentration in blood, soft tissue, bone and egg. *Poult. Sci.* 65:1343-1349.

25. **Schulz, J.A. and A.R. Lamb.** 1925. The effect of fluorine as sodium fluoride on the growth and reproduction of albino rats. *Science* 61:93-94.

26. **Messer, H.H., W.D. Armstrong and L. Singer.** 1972. Fertility impairment in mice on a low fluoride intake. *Science* 177:893-894.

27. **Messer, H.H., W.D. Armstrong and L. Singer.** 1973. Influence of fluoride intake on reproduction in mice. *J. Nutr.* 103:1319-1326.

28. **Kour, K. and J. Singh.** 1980. Histological finding of mice testes following fluoride ingestion. *Fluoride* 13:160-167.

29. **Chinoy, N.J. and E. Sequeira.** 1989. Effects of fluoride on the histoarchitecture of reproductive organs of the male mouse. *Reprod. Toxicol.* 3:261-267.

30. **Trautner, K. and J. Einwag.** 1989. Influence of milk and food on fluoride bioavailability from NaF and Na_2FPO_3 in man. *J. Dent. Res.* 68:72-77.

31. **Krasowska, A. and T. Wlotowski.** 1992. The effect of high fluoride intake on tissue trace elements and histology of testicular tubules in the rat. *Comp. Biochem. Physiol.* 103C:31-34.

32. **Chinoy, N.J., P.K. Pradeep and E. Sequeira.** 1992. Effect of fluoride ingestion on the physiology of reproductive organs of male rat. *J. Environ. Biol.* 13:55-61.

33. **Chinoy, N.J. and E. Sequeira.** 1989. Fluoride induced biochemical changes in reproductive organs of male mice. *Fluoride* 22:78-85.

34. **Chinoy, N.J., E. Sequeira and M.V. Narayana.** 1991. Effects of vitamin C and calcium on the reversibility of fluoride-induced alterations in spermatozoa of rabbits. *Fluoride* 24:29-39.

35. **Chinoy, N.J., M.V. Rao, M.V. Narayana and E. Neelakanta.** 1991. Microdose vasal injection of sodium fluoride in the rat. *Reprod. Toxicol.* 5:505-512.

36. **Andreasen, J.K. and R.K. Stroud.** 1987. Industrial halide wastes cause

acute mortality of snow geese in Oklahoma. *Environ. Tox. Chem.* 6:291-293.

37. **Henny, C.J. and P.M. Burke.** 1990. Fluoride accumulation and bone strength in wild Black-crowned Night-Herons. *Arch. Environ. Contam. Toxicol.* 19:132-137.

38. **Hoffman, D.J., O.H. Pattee and S.N. Wiemeyer.** 1985. Effects of fluoride on screech owl reproduction: teratological evaluation, growth, and blood chemistry in hatchlings. *Toxicol. Lett.* 26:19-24.

39. **Pattee, O.H., S.N. Wiemeyer and D.M. Swineford.** 1988. Effects of dietary fluoride on reproduction in Eastern Screech-Owls. *Arch. Environ. Contam. Toxicol.* 17:213-218.

40. **Bird, D.M. and C. Massari.** 1983. Effects of dietary sodium fluoride on bone fluoride levels and reproductive performance of captive American Kestrels. *Environ. Pollut. (Ser. A)* 31:67-76.

41. **Carriere, D., D.M. Bird and J.W. Stamm.** 1987. Influence of a diet of fluoride-fed cockerels on reproductive performance of captive American kestrels. *Environ. Pollut.* 46:151-159.

42. **Aitken, R.J., F.S. Best, D.W. Richardson, D. Djahanbakhch and M.M. Lees.** 1982. The correlates of fertilizing capacity in normal fertile men. *Fertil. Steril.* 38:68-76.

43. **Amann, R.P.** 1982. Use of animal models for detecting specific alterations in reproduction. *Fund. Appl. Toxicol.* 2:13-26.

44. **Working, P.K.** 1988. Male reproductive toxicology: comparison of the human to animal models. *Environ. Health Perspect.* 77:37-44.

45. **Overstreet, J.W. and W.F. Blazak.** 1983. The biology of human male reproduction: an overview. *Am. J. Ind. Med.* 4:5-15.

46. **Williams, J., B.C. Gladen, S.M. Schrader, T.W. Turner, J.L. Phelps and R.E. Chapin.** 1990. Semen analysis and fertility assessment in rabbits: statistical power and design considerations for toxicology studies. *Fund. Appl. Toxicol.* 15:651-665.

47. **Comhaire, F.H.** 1993. Methods to evaluate reproductive health of the human male. *Reprod. Toxicol.* 7:39-46.

48. **Eliasson, R.** 1975. Analysis of semen. In Behrman, S.J. and R.W. Kistner, eds., *Progress in Infertility.* Little Brown and Co. Boston, pp. 691-713.

49. **Cooper, D.M. and J.G. Rowell.** 1958. Relations between fertility, embryonic survival, and some semen characteristics in the chicken. *Poult. Sci.* 37:699-707.

50. **Meistrich, M.L. and R.C. Samuels.** 1986. Reduction in sperm levels after testicular irradiation in the mouse: a comparison with man. *Radiat. Res.* 102:138-147.

51. **Bird, D.M. and R.B. Buckland.** 1976. The onset and duration of fertility in the American kestrel. *Can. J. Zool.* 54:1595-1597.

52. **Bird, D.M. and P.C. Laguë.** 1977. Semen production of the American Kestrel. *Can. J. Zool.* 55:1351-1358.

53. **Bird, D.M.** 1985. Evaluation of the American kestrel (*Falco sparverius*) as a laboratory research animal. In J. Archibald, J. Ditchfield and H.C. Rowsell, eds., Eighth ICLAS/CALAS Symp. Vancouver, 1983. Gustav Fischer Verlag, New York pp.3-9.

54. **Wilson, W.O., U.K. Abbott and H. Abplanalp.** 1961. Evaluation of Coturnix (Japanese quail) as pilot animal for poultry. *Poult. Sci.* 40:651-657.

55. **Lin, M. and R.C. Jones.** 1990. Spatial arrangement of the stages of the cycle of the seminiferous epithelium in the Japanese quail, *Coturnix coturnix japonica. J. Reprod. Fert.* 90:361-367.

56. **Lin, M. and R.C. Jones.** 1993. Spermiogenesis and spermiation in the Japanese quail (*Coturnix coturnix japonica*). *J. Anat.* 183:525-535.

57. **Birkhead, T.R. and F. Fletcher.** 1994. Sperm storage and the release of sperm from the sperm storage tubules in Japanese quail *Coturnix japonica. Ibis* 136:101-105.

58. **Shrivastava, S.K., S.D. Ahuja, D. Chaudhury and A. Kundu.** 1994. Influence of rearing mixed and separate sexes of Japanese quail on semen quality and hatching performance. *Ind. J. Anim. Sci.* 64:953-955.

59. **Opel, H.** 1966. The timing of oviposition and ovulation in the quail (*Coturnix coturnix japonica*). *Br. Poult. Sci.* 7:29-38.

60. **Wentworth, B.C. and W.J. Mellen.** 1963. Egg production and fertility following various methods of insemination in Japanese quail (*Coturnix coturnix*

japonica). *J. Reprod. Fert.* 6:215-220.

61. **Sittman, K. and H. Abplanalp.** 1965. Duration and recovery of fertility in Japanese quail (*Coturnix coturnix japonica*). *Br. Poult. Sci.* 6:245-250.

62. **Taji, K. and K. Ikeda.** 1956. Studies on the artificial insemination of Japanese quail, *Coturnix coturnix japonica* T. et S. *Mem. Ehime Univ.* 6:1-5.

63. **Marks, H.L. and P.D. Lepore.** 1965. A procedure for artificial insemination of Japanese quail. *Poult. Sci.* 44:1001-1003.

64. **Biessmann, A.** 1982. Effects of PCBs on gonads, sex hormone balance and reproduction processes of Japanese quail *Coturnix coturnix japonica* after ingestion during sexual maturation. *Environ. Pollut. (Ser. A)* 27:15-30.

65. **Gish, C.D. and N.J. Chura.** 1970. Toxicity of DDT to Japanese Quail as influenced by body weight, breeding condition, and sex. *Toxicol. Appl. Pharmacol.* 17:740-751.

66. **Bryan, T.E., R.P. Gildersleeve and R.P. Wiard.** 1989. Exposure of Japanese quail embryos to o,p'-DDT has long-term effects on reproductive behaviors, hematology and feather morphology. *Teratology* 39:525-535.

67. **Hill, E.F. and C.S. Shaffner.** 1976. Sexual maturation and productivity of Japanese quail fed graded concentrations of mercuric chloride. *Poult. Sci.* 55:1449-1459.

68. **Hill, E.F. and J.H.J. Soares.** 1984. Subchronic mercury exposure in Coturnix and a method of hazard evaluation. *Environ. Toxicol. Chem.* 3:489-502.

69. **Dixon, R.J., G.G. Arzey and P.J. Nicholls.** 1992. Production, hatchability and fertility of eggs from breeding Japanese quail (*Coturnix coturnix japonica*) fed diets containing furazolidone. *Br. Poult. Sci.* 33:835-845.

70. **DeRosa, C.T., D.H. Taylor, P. Farrell and S.K. Seilkop.** 1976. Effects of sevin on the reproductive biology of the Coturnix. *Poult. Sci.* 55:2133-2141.

71. **Zavos, P.M., A.H. Cantor, R.W. Hemken, R.J. Grove, D.R. Varney and M.R. Siegel.** 1993. Reproductive performance of Japanese quail fed tall fescue seed infected with Acremonium coenophialum. *Theriogenology* 39:1257-1266.

72. **Fleming, W.J. and C.A. Schuler.** 1988. Influence of the method of fluoride administration on toxicity and fluoride concentrations in Japanese quail. *Environ. Tox. and Chem.* 7:841-845.

73. **Lake, P.E.** 1975. Gamete production and the fertile period with particular reference to domesticated birds. *Symp. Zool. Soc. Lond.* 35:225-244.

74. **Von Sinowatz, F., K. Wrobel and A. Friess.** 1976. Zur histopochemie der Uterovaginalregion bei der Wachtel (*Coturnix coturnix japonica*). *Acta Histochem.* 57:55-67.

75. **Al-Azawi, S.H., K.A. Al-Soudi and A.W.R. Mehdi.** 1986. Effect of fluoride intake on the structural-functional aspects of the reproductive system of the male domestic fowl. *J. Agric. Water Reso. Res.* 5:285-300.

76. **Schein, M.W., M. Diamond and C.S. Carter.** 1972. Sexual performance levels of male Japanese quail (*Coturnix coturnix japonica*). *Anim. Behav.* 20:61-67.

77. **Beach, F.A. and N.G. Inman.** 1965. Effects of castration and androgen replacement on mating in male quail. *Proc. Nat. Acad. Sci.* 54:1426-1431.

78. **Farris, H.E.** 1967. Classical conditioning of courting behaviour in the Japanese quail, *Coturnix coturnix japonica. J. Exp. Anal. Behav.* 10:213-217.

79. **Wilcox, F.H. and C.S. Shaffner.** 1958. The effect of different handling methods and added fructose on the fertilizing ability of chicken spermatozoa after storage. *Poult. Sci.* 37:1353-1357.

80. **Lin, M., R.C. Jones and A.W. Blackshaw.** 1990. The cycle of the seminiferous epithelium in the Japanese quail (*Coturnix coturnix japonica)* and estimation of its duration. *J. Reprod. Fert.* 88:481-490.

81. **D'agostino, R.B., A. Belanger and R.B. D'agostino Jr.** 1990. A suggestion for using powerful and informative tests of normality. *Am. Stat.* 44:316-321.

82. **SAS Institute Inc.** 1988. *SAS/STAT User's Guide.* SAS Institute Inc. Cary, N.C.

83. **Wilkinson, L.W., M. Hill, J.P. Welna and G.K. Birkenbeuel** 1992. *SYSTAT for Windows.* Systat Inc. Evanston, IL.

84. **Haseman, J.K. and L.L. Kupper.** 1979. Analysis of dichotomous response data from certain toxicological experiments. *Biometrics* 35:293

85. **Johnston, D.W.** 1956. The annual reproductive cycle of the California gull I. Criteria of age and and the testis cycle. *Condor* 58:134-162.

86. **Singer, L. and W.D. Armstrong.** 1968. Determination of fluoride in bone with the fluoride electrode. *Anal. Chem.* 40:613-614.

87. **Lepore, P.D. and H.L. Marks.** 1966. Intravaginal insemination of Japanese quail: factors influencing the basic technique. *Poult. Sci.* 45:888-891.

88. **Rattner, B.A., V.P. Eroschenko, G.A. Fox, D.M. Fry and J. Gorsline.** 1984. Avian endocrine responses to environmental pollutants. *J. Exper. Zool.* 232:683-689.

89. **Marks, H.L. and K.W. Washburn.** 1991. Body, abdominal fat, and testes weights, and line by sex interactions in Japanese quail divergently selected for plasma cholesterol response to adrenocorticotropin. *Poult. Sci.* 70:2395-2401.

90. **Ogasawara, F.X. and R. Huang.** 1963. A modified method of artificial insemination in the production of chicken-quail hybrids. *Poult. Sci.* 42:1386-1392.

91. **McFarquhar, A.M. and P.E. Lake.** 1964. Artificial insemination in quail and the production of chicken-quail hybrids. *J. Reprod. Fert.* 8:261-263.

92. **Merkley, J.W. and T.J. Sexton.** 1982. Reproductive performance of white Leghorns provided fluoride. *Poult. Sci.* 61:52-56.

93. **Nahorniak, N.A., P.E. Waibel, W.G. Olson, M.M. Walser and H.E. Dzuik.** 1983. Effect of dietary sodium fluoride on growth and bone development in growing turkeys. *Poult. Sci.* 62:2048-2055.

94. **Suttie, J.W., D.L. Kolstad and M.L. Sunde.** 1984. Fluoride tolerance of the young chick and turkey poult. *Poult. Sci.* 63:738-743.

95. **Guenter, W. and P.H.B. Hahn.** 1986. Fluorine toxicity and laying hen performance. *Poult. Sci.* 65:769-778.

96. **Birkhead, T.R. and A.P. Moller.** 1992. Number and size of sperm storage tubules and the duration of sperm storage in birds: a comparative study. *Biol. J. Linn. Soc.* 45:363-372.

97. **Domjan, M., P. Greene and N.C. North.** 1989. Contextual conditioning and the control of copulatory behaviour by species-specific sign stimuli in male Japanese quail. *J. Exp. Psychol. Anim. Behav. Processes* 15:147-153.

98. **Topham, J.C.** 1983. Chemically induced changes in sperm in animals and humans. Anonymous*Chemical Mutagens.* pp. 201-234.

99. **Amann, R.P.** 1986. Detection of alterations in testicular and epididymal function in laboratory animals. *Environ. Health Perspect.* 70:149-158.

100. **Overstreet, J.W., S.J. Samuels, P. Day, A.G. Hendrickx, S. Prahalada, T. Mast, D.F. Katz and C. Sakai.** 1988. Early indicators of male reproductive toxicity. *Risk Analysis* 8:21-26.

101. **Heywood, R. and R.W. James.** 1978. Assessment of testicular toxicity in laboratory animals. *Environ. Health Perspect.* 24:73-80.

102. **Buxton, J. and Orcutt, Jr.** 1975. Enzymes and electrolytes in the semen of Japanese quail. *Poult. Sci.* 54:1556-1566.

103. **Bedford, J.W.** 1983. Considerations in evaluating risk to male reproduction. In Christian, M.S., W.M. Galbraith, P. Voytek and M.A. Mehlman, eds., *Advances in Modern Toxicology, Assessment of Reproductive and Teratogenic Hazards.* Princeton Scientific Publishers, Princeton,N.J. pp. 41-78.

104. **Morton, D.** 1988. The use of rabbits in male reproductive toxicology. *Environ. Health Perspect.* 77:5-9.

105. **Amann, R.P.** 1981. A cririca review of methods for evaluation of spermatogenesis from seminal characteristics. *J. Androl.* 2:37-58.

106. **Bird, D.M., P.H. Tucker, G.A. Fox and P.C. Laguë.** 1983. Synergistic effects of Arochlor 1254 and Mirex on the semen characteristics of American kestrels. *Archiv. Environ. Contam. Toxicol.* 12:633-640.

107. **Clulow, J. and R.C. Jones.** 1982. Production, transport, maturation, storage and survival of spermatozoa in the male Japanese quail, *Coturnix coturnix*. *J. Reprod. Fert.* 64:259-266.

108. **Cheng, K.M., R.F. McIntyre and A.R. Hickman.** 1989. Proctodeal gland foam enhances competitive fertilization in domestic Japanese quail. *Auk* 106:286-291.

109. **Chong, A.P., C.A. Walters and S.A. Weinrieb.** 1983. The neglected laboratory test: the semen analysis. *J. Androl.* 4:280-282.

110. **Marks, H.L.** 1990. Abdominal fat and testes weights in diverse genetic lines of Japanese quail. *Poult. Sci.* 69:1627-1633.

111. **Eroschenko, V.P.** 1978. Alterations in the testes of the Japanese quail during and after the ingestion of the insecticide Kepone. *Toxicol. Appl.*

Pharmacol. 43:535-545.

112. **Roholm, K.** 1937. *Fluorine Intoxication: A Clinical-hygenic Study with a Review of the Literature and Some Experimental Investigations.* H.K. Lewis and Co. Ltd. London.

113. **National Academy of Sciences.** 1974. *The Effects of Fluorides in Animals.* National Research Council. Washington,D.C.

114. **Smith, F.A. and H.C. Hodge.** 1979. Airborne Fluorides and Man: Part 1. *C. R. C. Crit. Rev. Environ. Control* 8:293-371.

115. **Rao, G.S.** 1984. Dietary intake and bioavailability of fluoride. *Ann. Rev. Nutr.* 4:115-136.

116. **Smith, F.A.** 1993. Metabolism of Inorganic Fluoride. In Smith, F.A. eds., *Pharmacology of Fluorides. Part 1.* Springer-Verlag,

117. **Bird, D.M., D. Carriere and D. Lacombe.** 1992. The effect of dietary sodium fluoride on internal organs, breast muscle, and bones in captive American kestrels (*Falco sparverius*). *Arch. Environ. Contam. Toxicol.* 22:242-246.

118.**Tukey, J.W.** 1953. The problem of multiple comparisons. Unpublished. Dept. of Statistics, Princeton Univ.

Table 1. Fertility of eggs produced by Japanese quail when paired with a male for 30 minutes.

Length of egg-laying period	No. of females	No. producing eggs	No. of eggs (total)	No. fertile	Percent fertility[1]
Trial 1 (8 d)	20	19	101	47	46.5
Trial 2 (12 d)	20	19	139	59	42.4

[1] - fertility calculated excluding the first egg produced after pairing

Table 2. Proportion of fertile eggs produced by Japanese quail on the 9th day of five sequential trials after being paired with a treated male for 3.5 h. Each trial was 12-15 d in length and involved the same pairings.

Treatment	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
control	1/10[1]	0/7	3/10	4/9	1/8
250 ppm NaF	1/8	0/8	1/7	3/8	4/9

1 - 1 egg fertile of 10 laid

Table 3. Results of multiple comparisons tests of daily water consumption rates for male Japanese quail treated with 3 levels of fluoride or a control solution in their drinking water for 132 d.

Treatment	**Days sampled**	**Least squares mean (ml/bird/day)**	**Standard error**	**Tukey [118] comparisons**
control	14	23.18	0.90	a[1]
125 ppm NaF	22	20.20	0.72	ab
250 ppm NaF	21	19.26	0.74	b
500 ppm NaF	17	18.52	0.82	b

1- rows with the same letter are not significantly different at p=0.05

Table 4. Summary statistics of weekly semen collections from Japanese quail exposed to 3 levels of fluoride or a control solution for 132 d.

	control	125 ppm NaF	250 ppm NaF	500 ppm NaF
No. of sampling days	15	15	15	15
No. of birds/treatment	9	10	10	10
No. of samples	96	136	121	108
No. of samples missed (no semen or too small)	20	11	14	33

Table 5. Results of analysis of regression coefficients of semen volume of Japanese quail exposed to three levels of fluoride or a control solution added to their drinking water for 132 d . Means were calculated for the duration of the collection period.

	Control	**125 ppm NaF**	**250 ppm NaF**	**500 ppm NaF**
sample volume (µl) (mean ± 1 S.D.)	9.13 ± 2.17	10.29 ± 2.33	10.13 ± 2.25	9.47 ± 2.39
regression coeff. (treat. mean)[1]	0.188[a]	-0.145[b]	0.005[ab]	0.002[ab]

[1] columns with the same letter are not significantly different at p=0.05

Table 6. Results of analysis of regression coefficients of sperm motility of Japanese quail exposed to three levels of fluoride or a control solution added to their drinking water for 132 d. Means were calculated for the duration of the collection period.

	Control	**125 ppm NaF**	**250 ppm NaF**	**500 ppm NaF**
sample motility (mean ±1 S.D.)	3.76 ± 0.75	3.20 ± 1.01	2.89 ± 1.08	2.63 ± 1.12
regression coeff. (treat. mean)[1]	0.12[a]	-0.025[a]	-0.042[a]	0.015[a]

[1] columns with the same superscript are not significantly different at p=0.05

Table 7. Sperm live/dead ratios for Japanese quail treated with three levels of fluoride or a control solution in their drinking water for 132 d.

	control	125 ppm NaF	250 ppm NaF	500 ppm NaF
Overall mean ± 1 S.D.[1]	23.4±7.3[a]	19.1±8.3[b]	15.9±7.7[b]	14.3±7.7[b]
Live/dead ratio at wk 7[1]	23.33[a]	25.0[a]	19.8[a]	19.0[a]
Live/dead ratio at wk 15[1]	23.33[a]	14.1[b]	10.2[b]	10.1[b]

1 - columns with the same superscript are not significantly different at p=0.05

Table 8. Sperm concentrations for Japanese quail exposed to three levels of fluoride or a control solution in their drinking water measured on the first (pre-treatment) and last weeks of treatment.

	control	**125 ppm NaF**	**250 ppm NaF**	**500 ppm NaF**
week 1[1]	$1.25x10^6/\mu l$ [b]	$1.69x10^6/\mu l$ [a]	$1.82x10^6/\mu l$ [a]	$1.25x10^6/\mu l$ [b]
week 15[1]	$1.69x10^6/\mu l$ [a]	$1.66x10^6/\mu l$ [a]	$1.22x10^6/\mu l$ [b]	$1.38x10^6/\mu l$ [ab]

[1] within rows, values in columns with the same superscript are not significantly different at p=0.05

Table 9. Effect of three levels of fluoride or a control solution added to the drinking water of Japanese quail for 132 days on the total number of sperm per ejaculate.

	control	**125 ppm NaF**	**250 ppm NaF**	**500 ppm NaF**
treatment mean	$1.48x10^{7}$	$1.59x10^{7}$	$1.33x10^{7}$	$1.29x10^{7}$
regression coeff.[1]	0.05^{a}	-0.13^{b}	-0.0062^{b}	0.007^{b}
intercept[1]	$1.03x10^{5a}$	$1.69x10^{5b}$	$1.37x10^{5a}$	$1.20x10^{5a}$

[1] within rows, values in columns with the same superscript are not significantly different at p=o.05

Table 10. Femur fluoride levels measured in male Japanese quail after being treated with three levels of fluoride or a control solution in the drinking water for 132 d (ppm, ash weight).

treatment	**n**	**mean (±S.D.)[2]**	**minimum value**	**maximum value**
control	10	1529 (±392)[a]	970	2167
125 ppm NaF	9[1]	4602 (±1082)[b]	3047	6135
250 ppm NaF	9[1]	5774 (±810)[c]	4905	7280
500 ppm NaF	10	7733 (±1083)[d]	6234	9174

1- one quail escaped prior to euthanasia

2-values with the same superscripts are not significantly different at p=0.05

Table 11. Testicular masses of Japanese quail exposed to three levels of fluoride or a control solution for 132 d (weights in grams).

	left testis mass (mean ± S.D.)	**right testis mass** (mean ± S.D.)	**comb. mass**[1]	**GSI**[1,2] (comb.mass/bw*100)
control	2.77 ± 0.53	2.48 ± 0.54	5.25[a]	2.73[a]
125 ppm NaF	2.59 ± 0.46	2.42 ± 0.49	5.01[a]	2.66[a]
250 ppm NaF	3.05 ± 0.51	2.84 ± 0.41	5.58[a]	2.96[a]
500 ppm NaF	2.81 ± 0.52	2.49 ± 0.39	5.30[a]	2.88[a]

1 - within columns, values with the same superscript are not significantly different at p=0.05

2 - GSI= combined testicular mass/body mass * 100

Fig. 1. Weight change of twenty 7-week old male Japanese quail fed *ad libitum*. Bars represent mean ± 1 S.D.

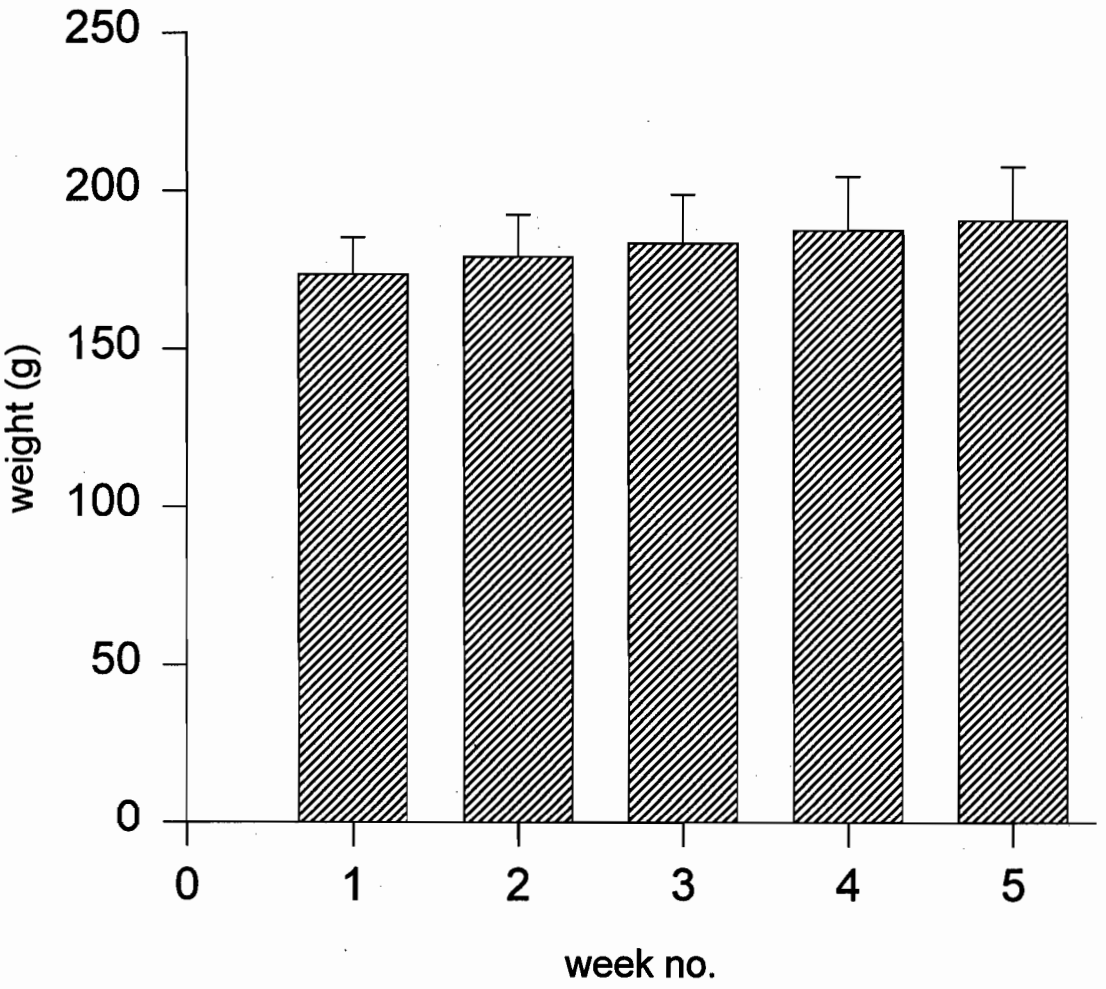

250
200
150
100
50
0
weight (g)
0
1
2
3
4
5
week no.

Fig. 2. Daily rate of disappearance of food for 20 male Japanese quail expressed in terms of 10 birds (values include spillage and consumption) measured over a 17 d period.

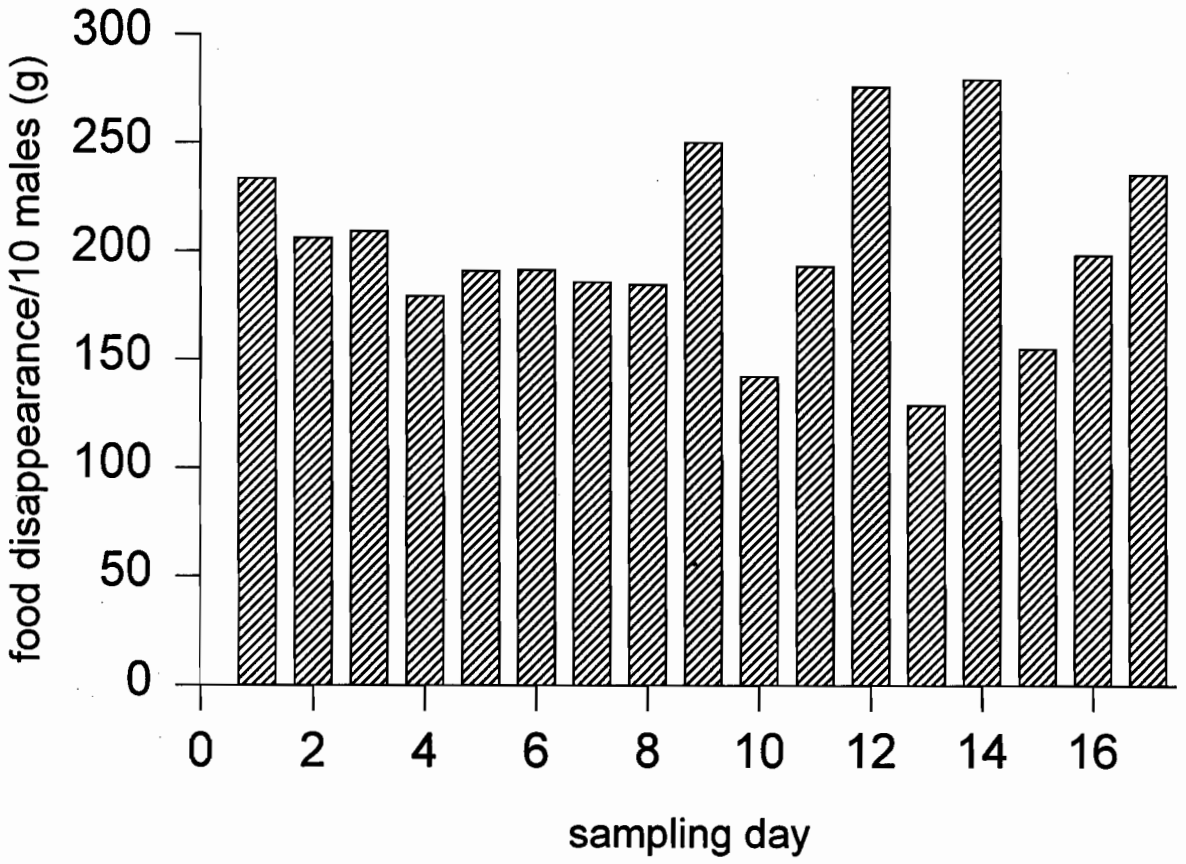

food disappearance/10 males (g)
300
250
200
150
100
50
0
0
2
4
6
8
10
12
14
16
sampling day

Fig. 3. Daily water consumption of 20 male Japanese quail expressed in terms of 10 birds measured 9 times over a 5 week period.

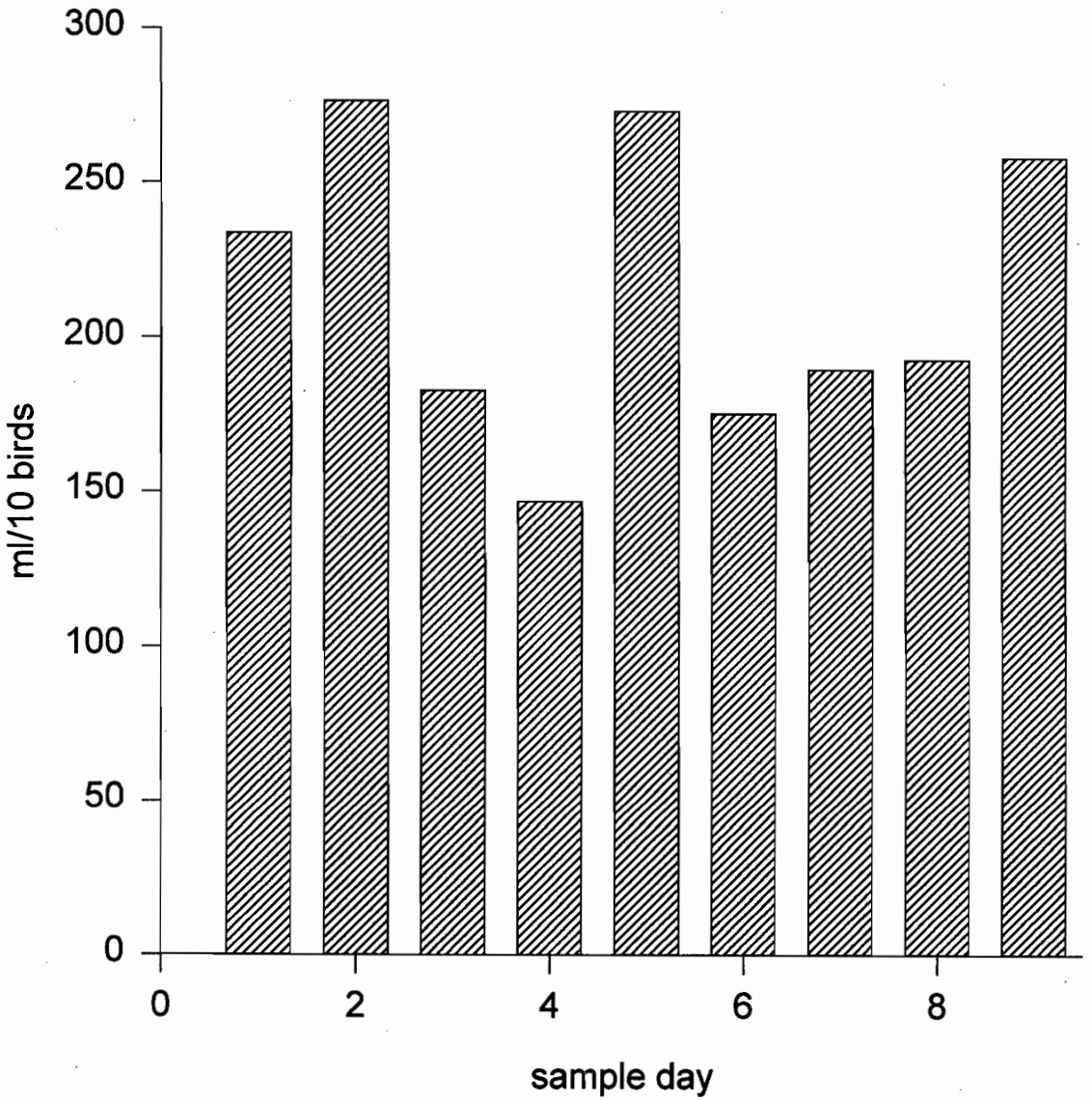
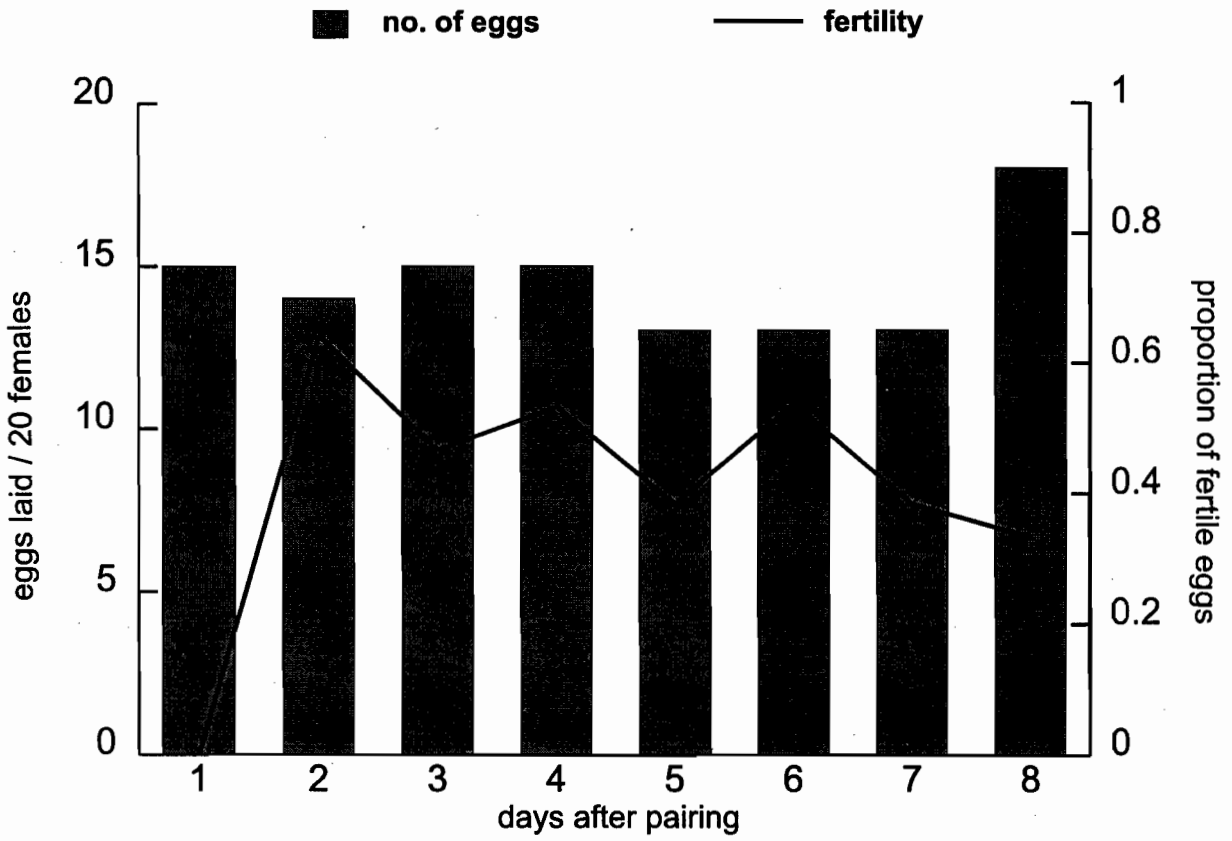

300
250
200
150
100
50
0
ml/10 birds
0
2
4
6
8
sample day

Fig. 4. Number of eggs laid and the proportion fertile for 20 Japanese quail after being paired once for 30 min with a male and returned to their pens for 8 d (Trial 1) or returned to their pens for 12 d (Trial 2).


no. of eggs
fertility
eggs laid / 20 females
proportion of fertile eggs
days after pairing
0
5
10
15
20
0
0.2
0.4
0.6
0.8
1
1
2
3
4
5
6
7
8

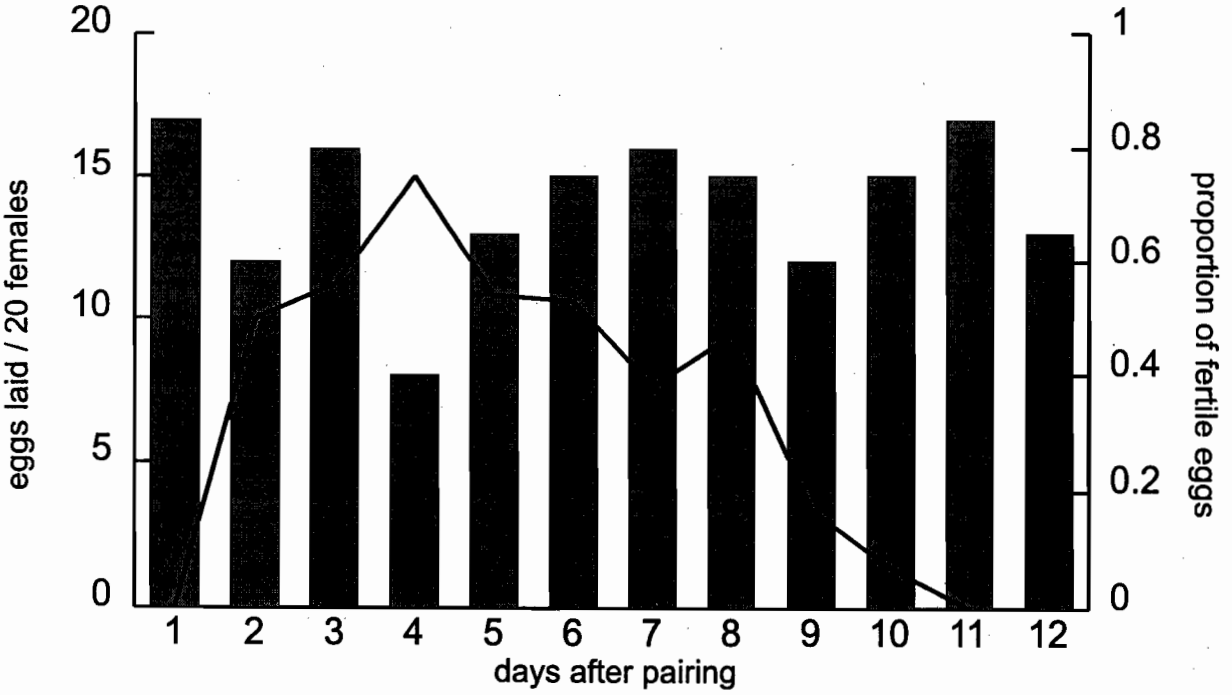

eggs laid / 20 females
proportion of fertile eggs
days after pairing
0
5
10
15
20
0
0.2
0.4
0.6
0.8
1
1
2
3
4
5
6
7
8
9
10
11
12

Fig. 5. Average individual weight change of 20 male Japanese quail exposed to 250 ppm NaF (hatched bars) or a control solution (clear bars) in their drinking water over an 8 week period.

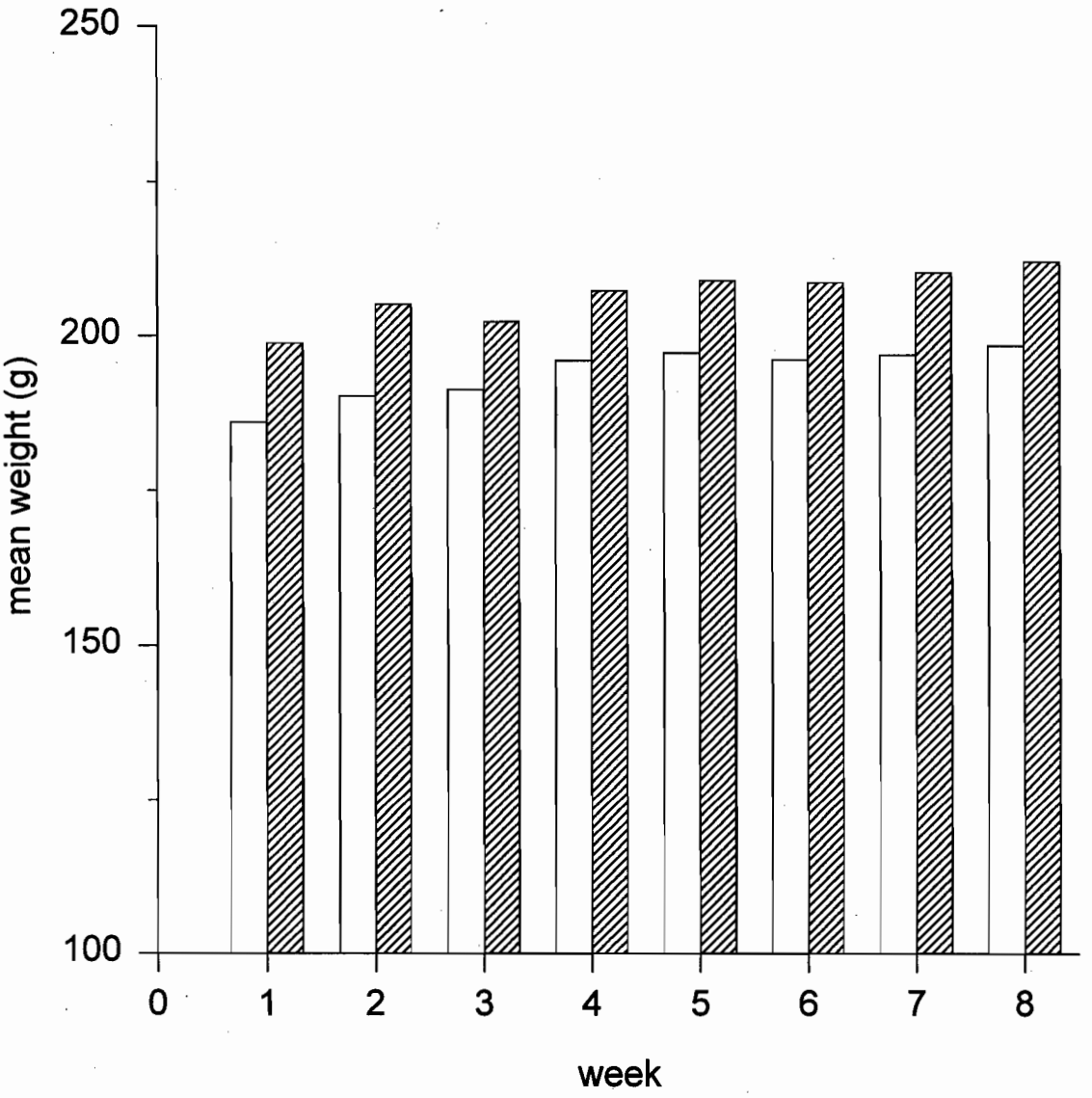

250
200
150
100
mean weight (g)
0
1
2
3
4
5
6
7
8
week

Fig. 6. Water consumption of 20 male Japanese quail exposed to 250 ppm NaF (hatched bars) or a control solution (clear bars) in their drinking water measured over an 8 week period. Expressed in terms of 10 birds.

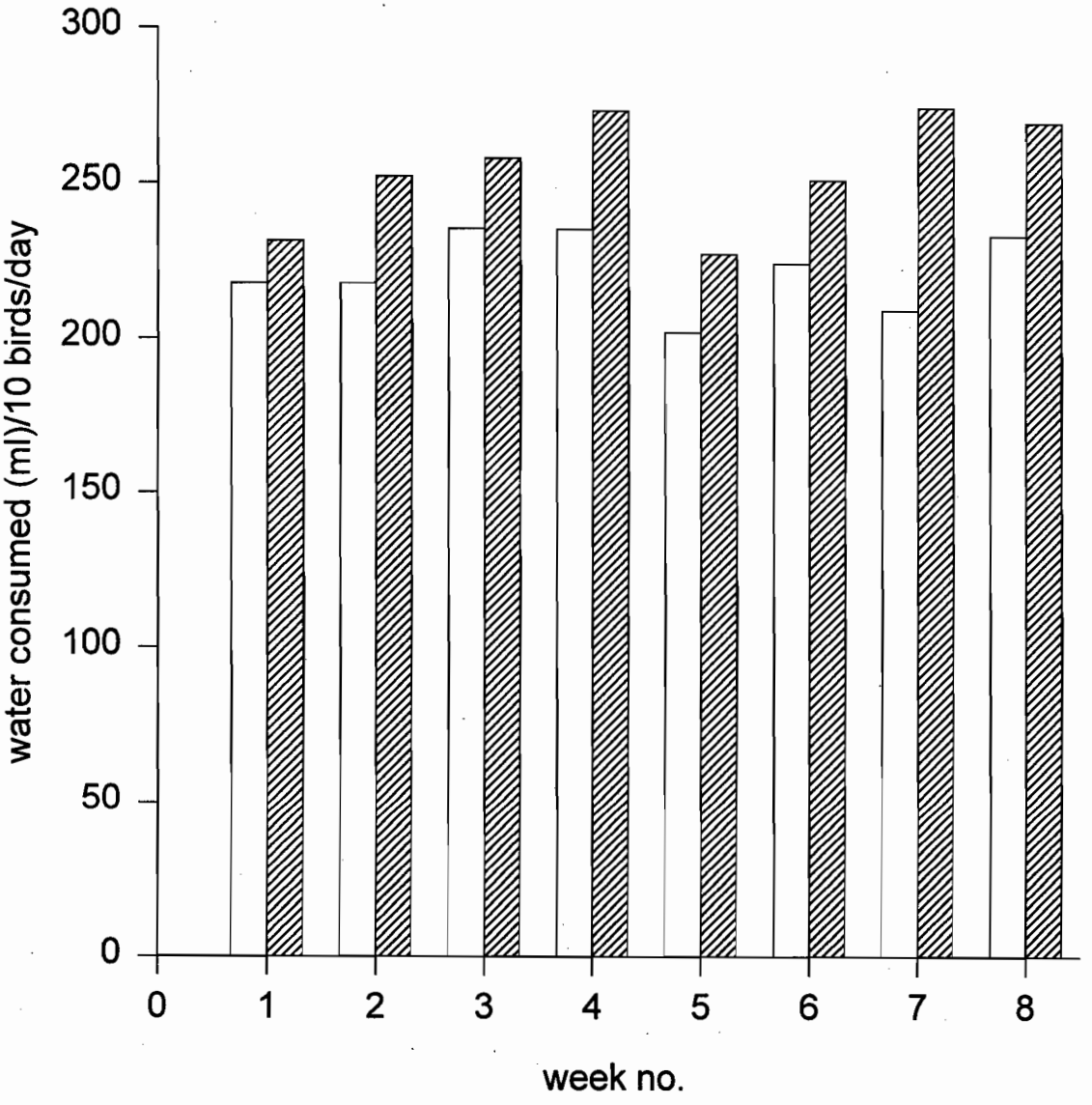

300
250
200
150
100
50
0
water consumed (ml)/10 birds/day
0
1
2
3
4
5
6
7
8
week no.

Fig. 7. Individual daily water consumption rates, expressed as a function of body weight, of 20 male Japanese quail treated with NaF (hatched bars) or a control solution (clear bars) in their drinking water over an 8 week period.

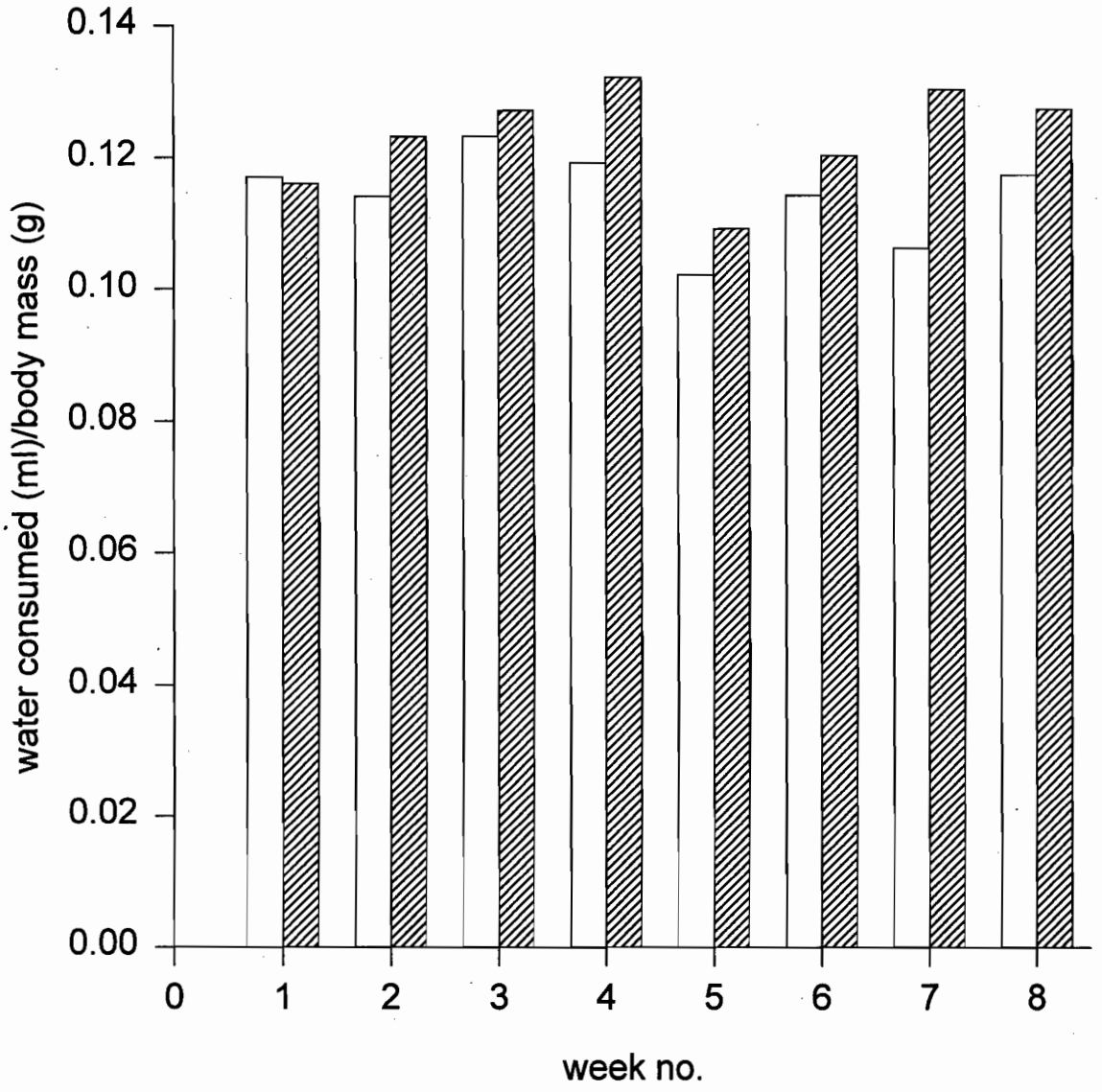

0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
water consumed (ml)/body mass (g)
0
1
2
3
4
5
6
7
8
week no.

Fig. 8. Mean number of copulations performed by individual male Japanese quail, during a one hour observation period, exposed to 250 ppm NaF (hatched bars) or a control solution (clear bars) in their drinking water.

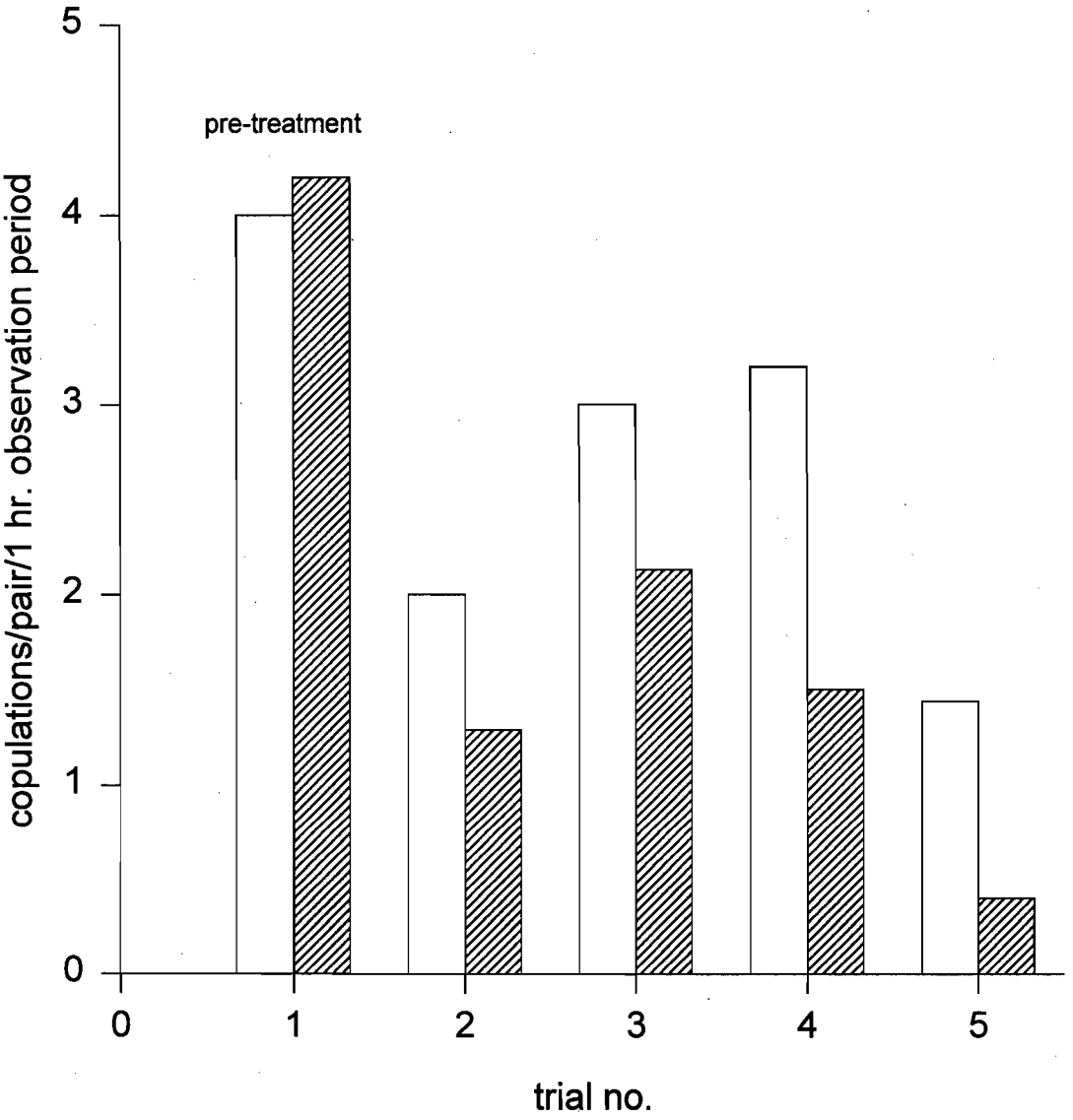

pre-treatment
copulations/pair/1 hr. observation period
trial no.
0
1
2
3
4
5

Fig. 9. Mean number of neck grabs performed by individual male Japanese quail, during a one hour observation period, exposed to 250 ppm NaF (hatched bars) or a control solution (clear bars) in their drinking water.

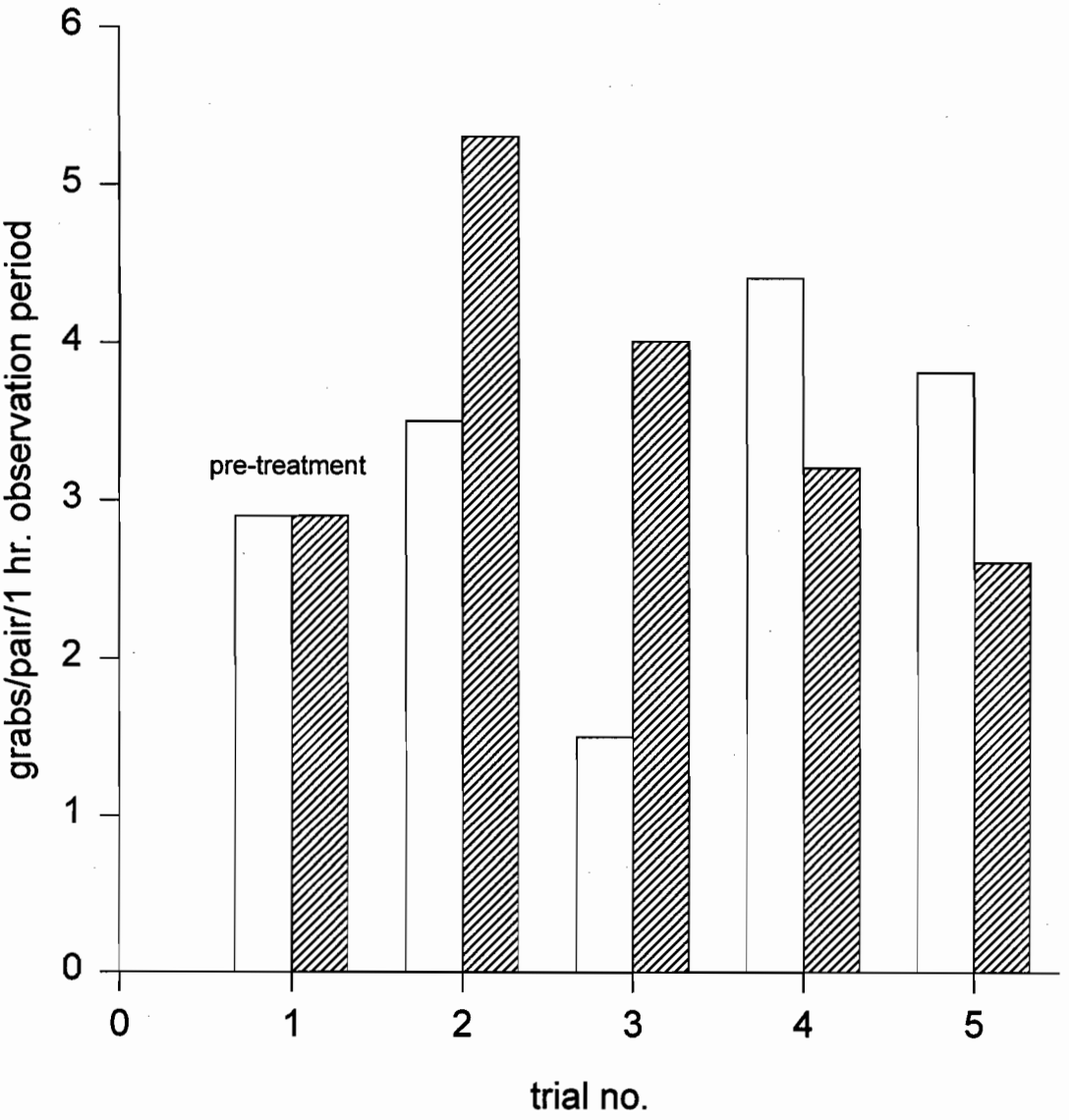

6
5
4
3
2
1
0
grabs/pair/1 hr. observation period
pre-treatment
0
1
2
3
4
5
trial no.

Fig. 10. Proportion of fertile eggs produced by Japanese quail paired with males exposed to 250 ppm NaF (hatched bars) or a control solution (clear bars) in their drinking water. In each trial birds were paired for 3 h and eggs collected for the following 12-15 d. The first egg produced following pairing is included in the analysis.

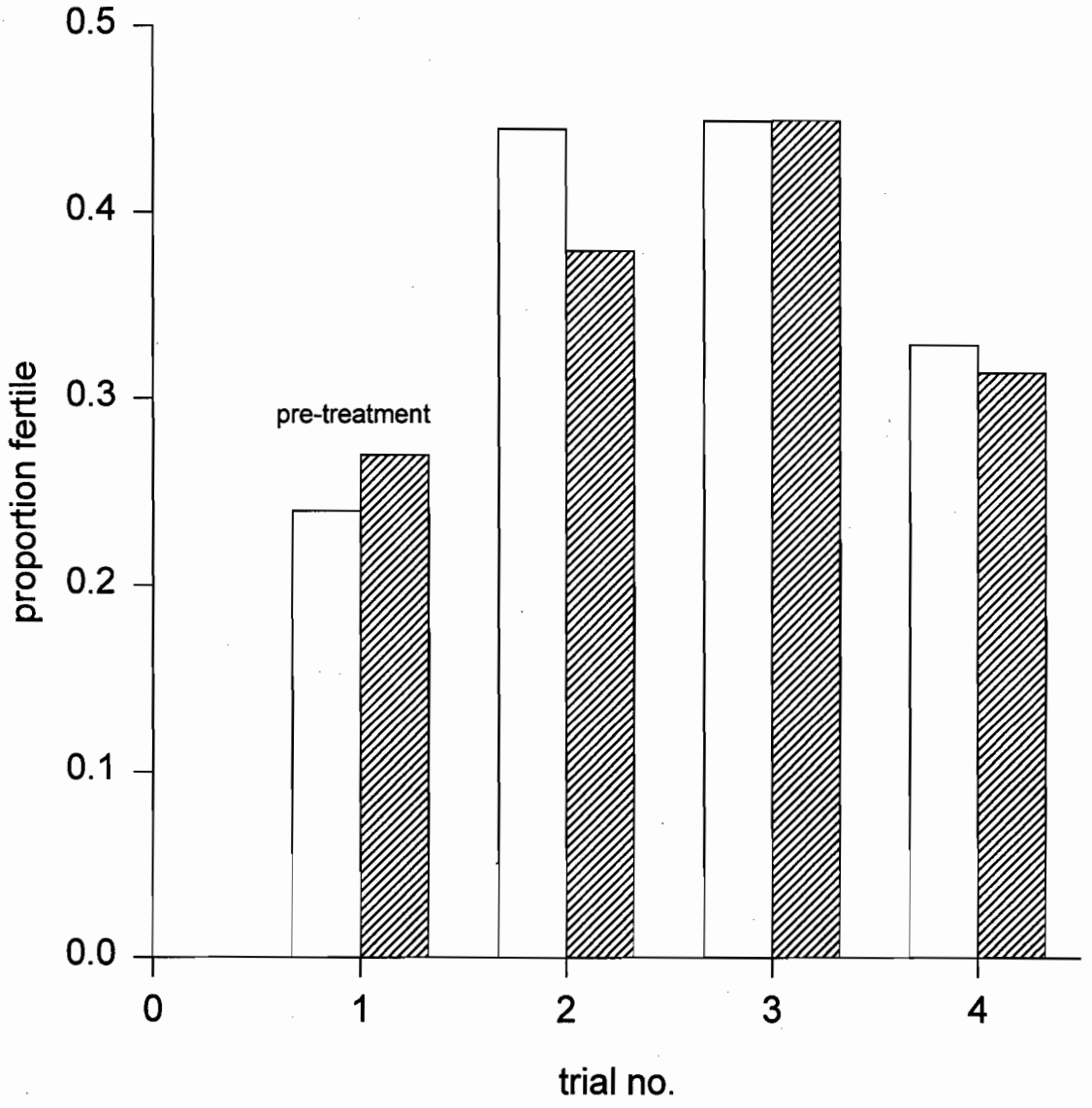

0.5
0.4
0.3
0.2
0.1
0.0
proportion fertile
pre-treatment
0
1
2
3
4
trial no.

Fig. 11. Proportion of fertile eggs produced by Japanese quail paired with males exposed to 250 ppm NaF(hatched bars) or a control solution (clear bars) in their drinking water. In each trial birds were paired for 3 h and eggs collected for the following 12-15 d. The first egg produced following pairing is excluded from the analysis.

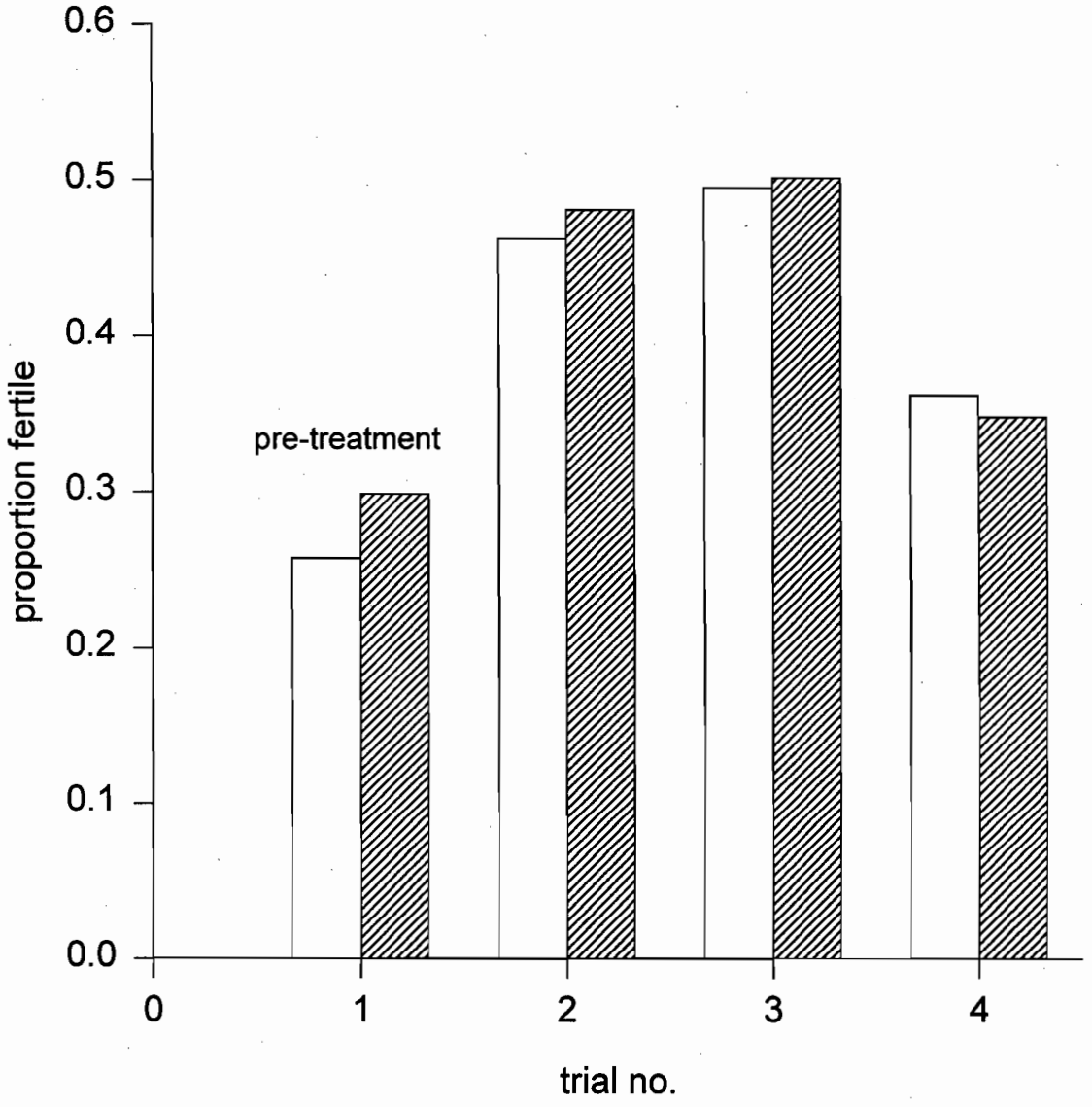

0.6
0.5
0.4
0.3
0.2
0.1
0.0
proportion fertile
pre-treatment
0
1
2
3
4
trial no.

Fig. 12. Weight change over a four month period of male Japanese quail exposed to 125, 250, 500 ppm NaF or a control solution added to their drinking water. The control males were added to the study on May 20.

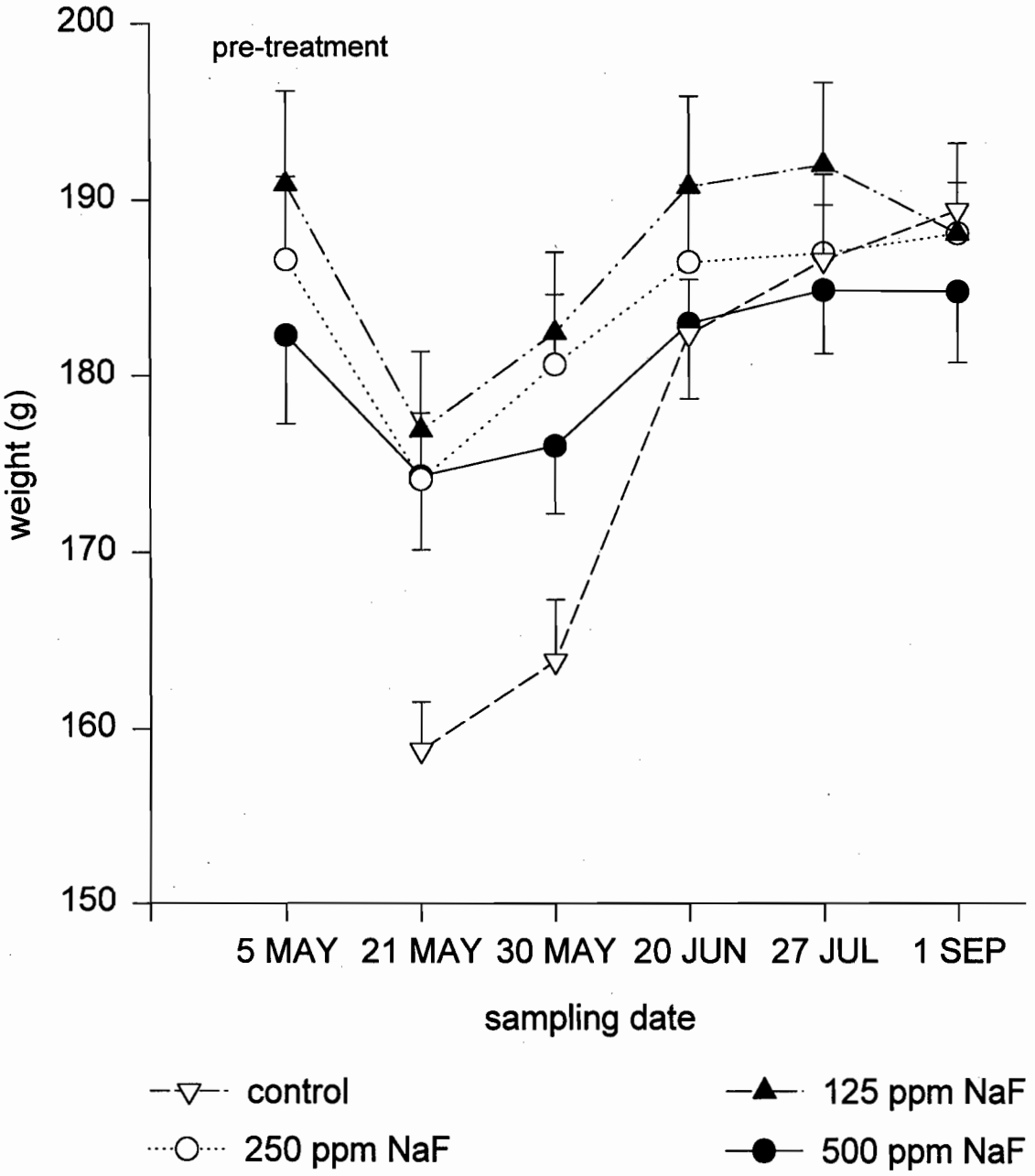

200
190
180
170
160
150
pre-treatment
weight (g)
5 MAY
21 MAY
30 MAY
20 JUN
27 JUL
1 SEP
sampling date
control
125 ppm NaF
250 ppm NaF
500 ppm NaF

Fig. 13. Estimated water consumption of male Japanese quail exposed to three levels of fluoride or a control solution in their drinking water, sampled over a two month period.

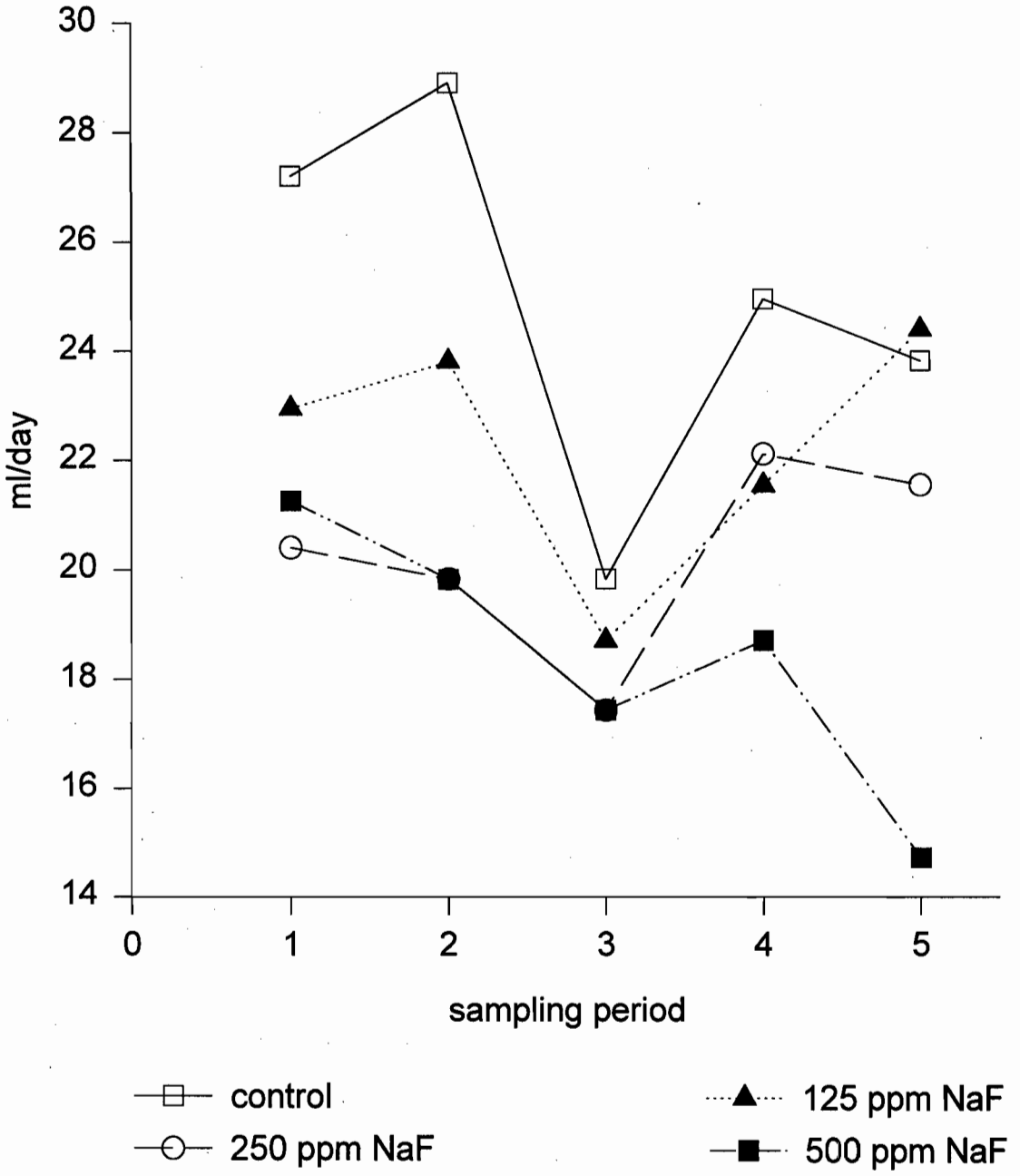
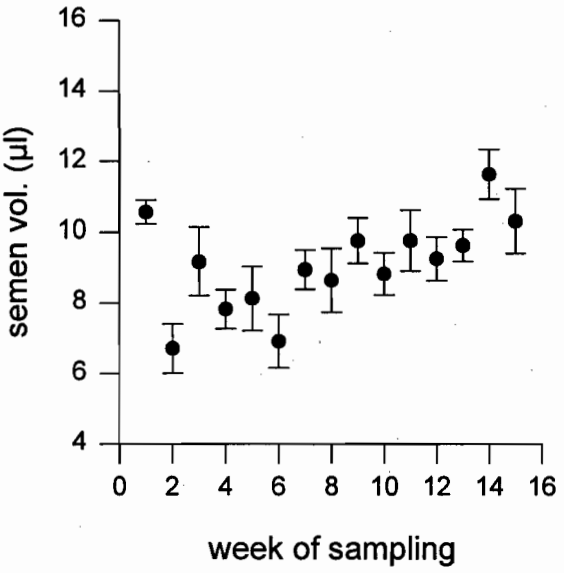
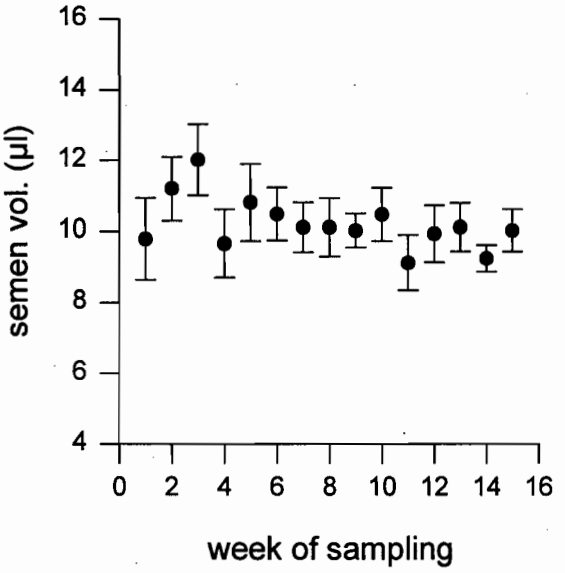

30
28
26
24
22
20
18
16
14
ml/day
0
1
2
3
4
5
sampling period
control
125 ppm NaF
250 ppm NaF
500 ppm NaF

Fig. 14. Changes in semen volume collected weekly from Japanese quail exposed to three levels of fluoride or a control solution in their drinking water. Each point represents the mean of up to 10 males ±1 S.E.

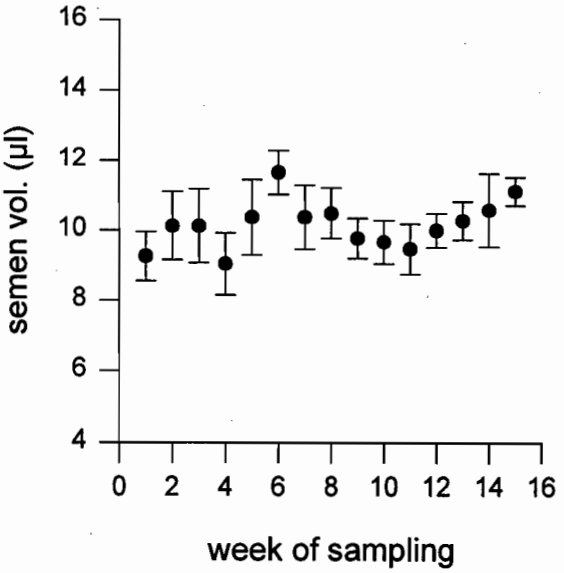
Control



125 ppm NaF



250 ppm NaF



500 ppm NaF

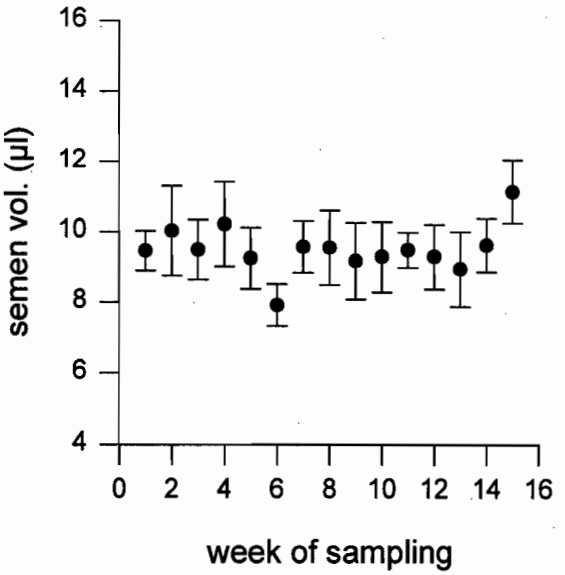
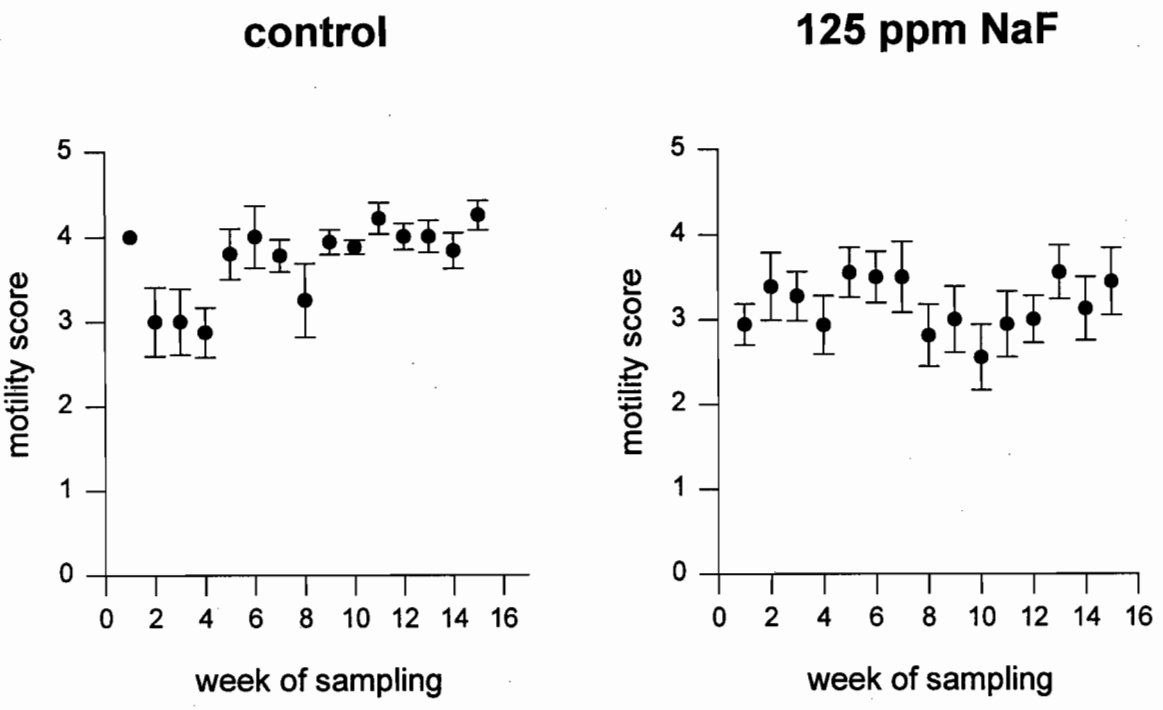


Fig. 15. Changes in sperm motility of semen samples collected weekly from Japanese quail exposed to three levels of fluoride or a control solution in their drinking water. Each point represents the mean of up to 10 males ±1 S.E.


control
125 ppm NaF
motility score
week of sampling
0
1
2
3
4
5
0
2
4
6
8
10
12
14
16

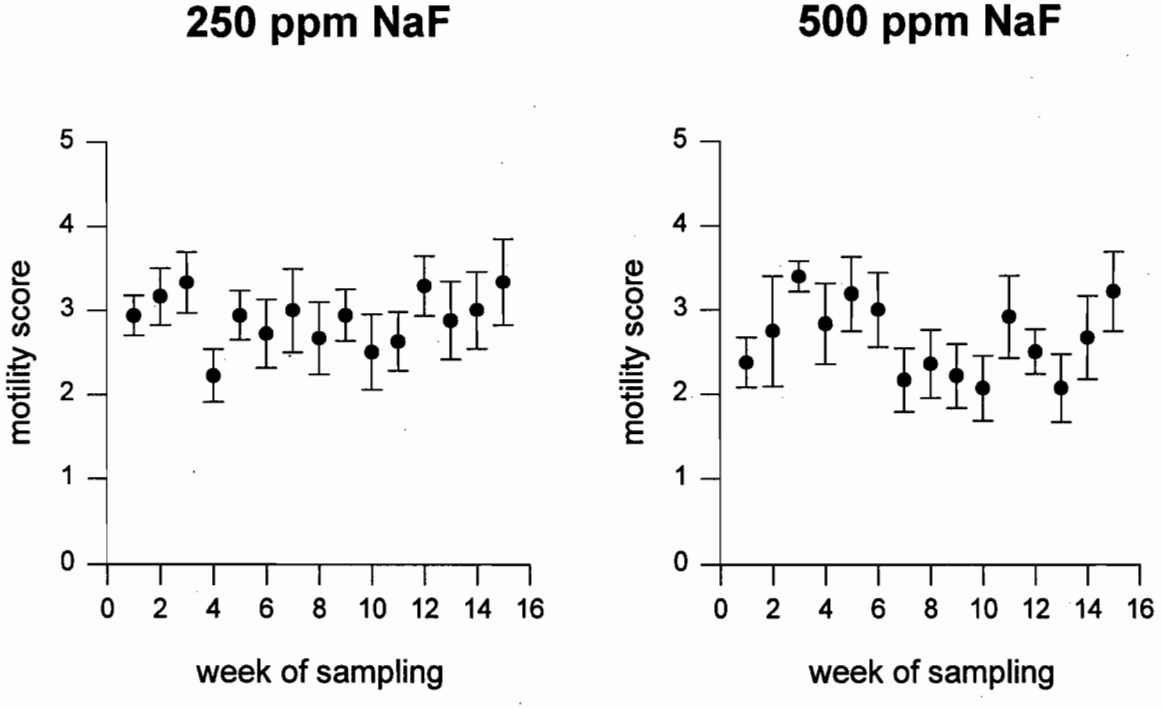
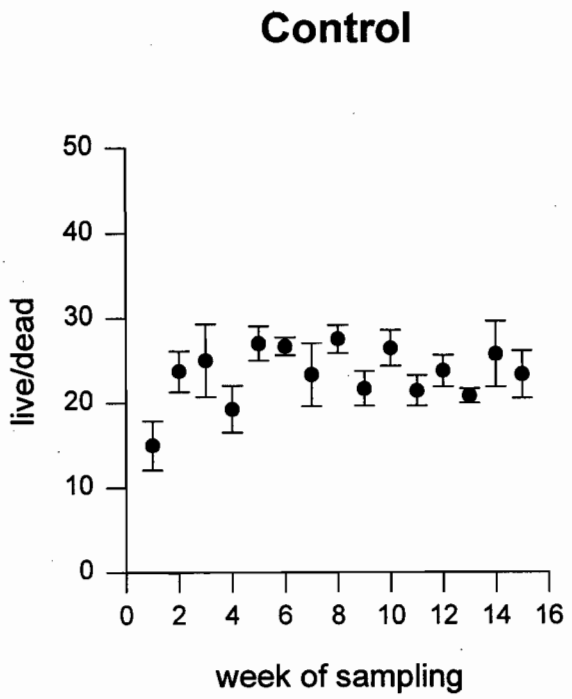
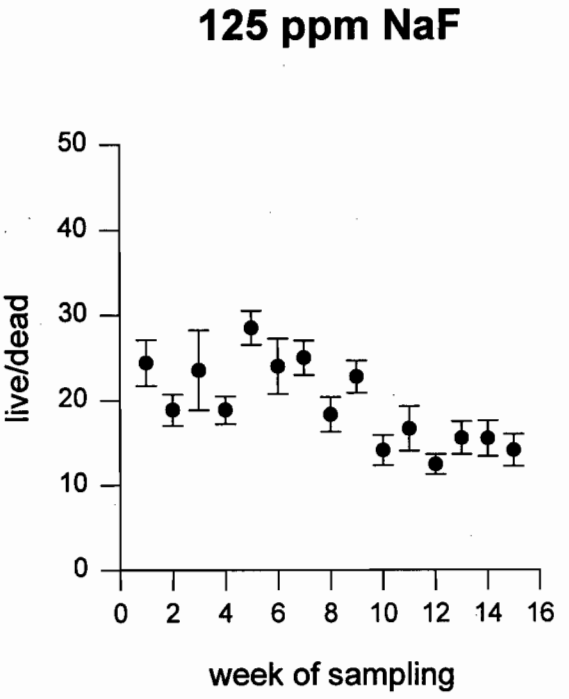
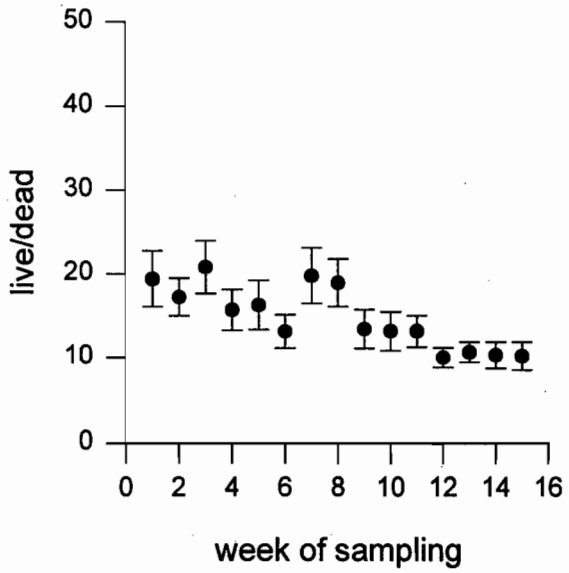

250 ppm NaF
500 ppm NaF
motility score
week of sampling
0
1
2
3
4
5
0
2
4
6
8
10
12
14
16

Fig. 16. Changes in sperm live/dead ratios of semen samples collected weekly from Japanese quail exposed to three levels of fluoride or a control solution in their drinking water. Each point represents the mean of up to 10 males ± 1 S.E.


Control
live/dead
week of sampling
0
10
20
30
40
50
0 2 4 6 8 10 12 14 16


125 ppm NaF
live/dead
week of sampling
0
10
20
30
40
50
0 2 4 6 8 10 12 14 16


250 ppm NaF
live/dead
week of sampling
0
10
20
30
40
50
0 2 4 6 8 10 12 14 16

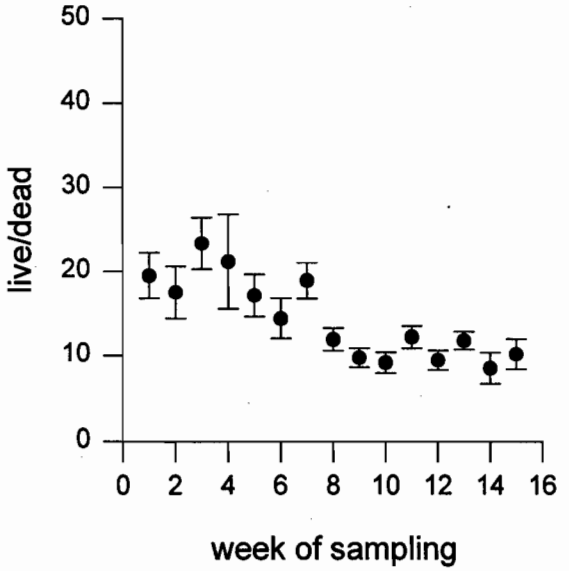
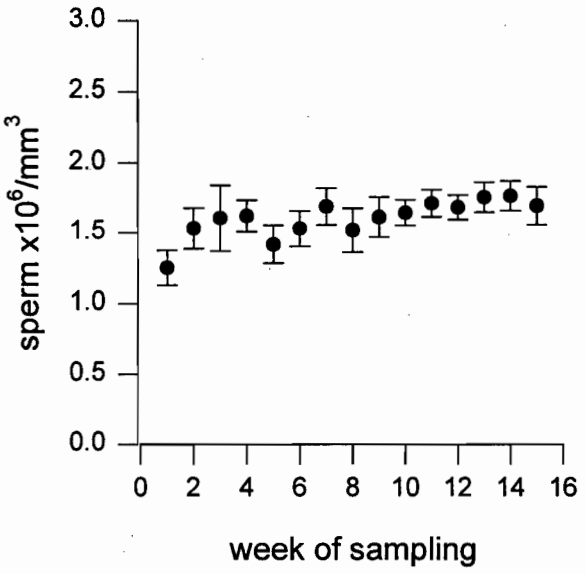
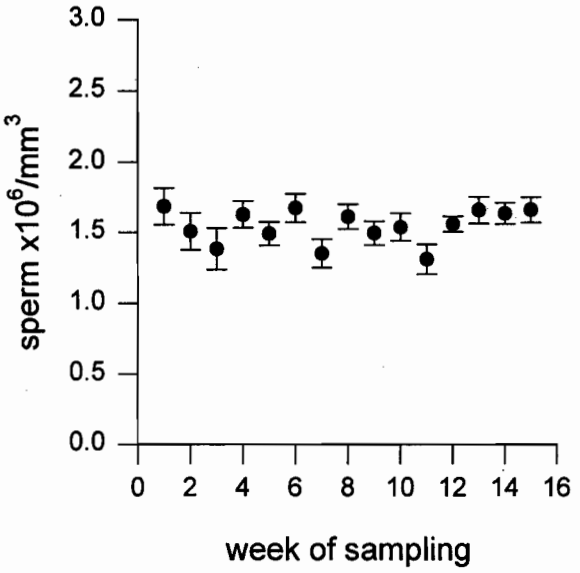

500 ppm NaF
live/dead
week of sampling
0
10
20
30
40
50
0 2 4 6 8 10 12 14 16

Fig. 17. Changes in sperm concentration of semen samples collected weekly from Japanese quail exposed to three levels of fluoride or a control solution in their drinking water. Each point represents the mean of up to 10 males ± 1 S.E.

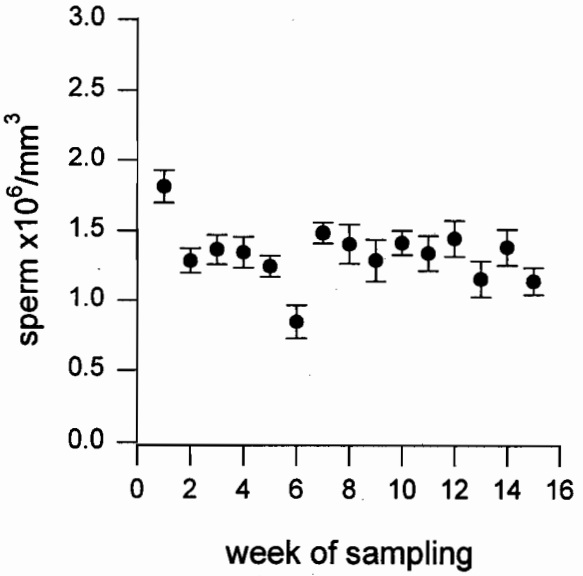
Control



125 ppm NaF



250 ppm NaF



500 ppm NaF

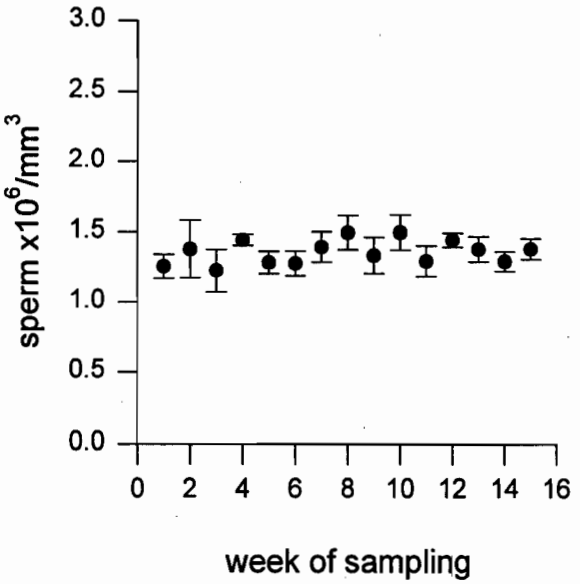
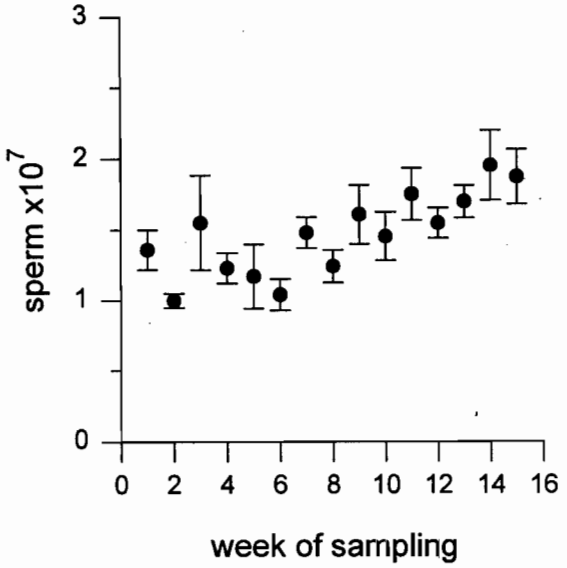
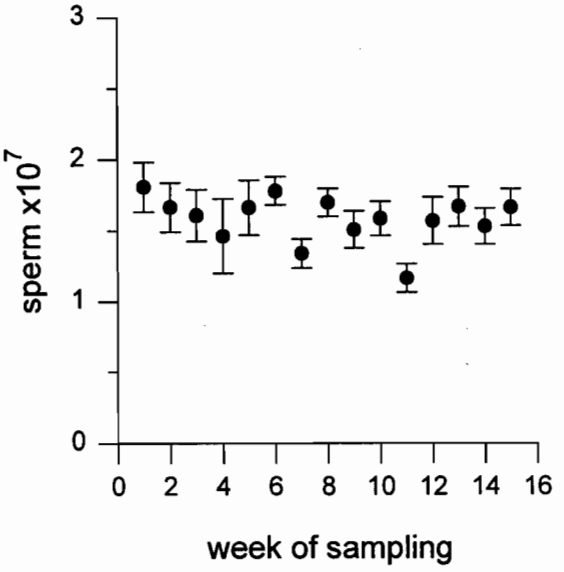


Fig.18. Changes in sperm number per ejaculate of semen samples collected weekly from Japanese quail exposed to three levels of fluoride or a control solution in their drinking water. Each point represents the mean of up to 10 males ±1S.E.

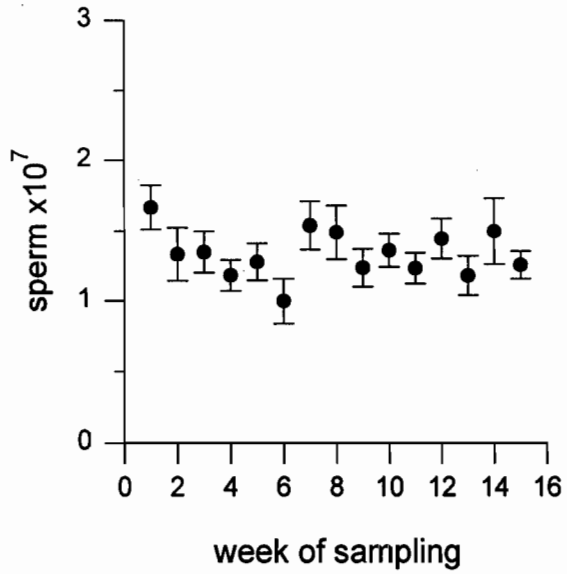
control



125 ppm NaF



250 ppm NaF



500 ppm NaF

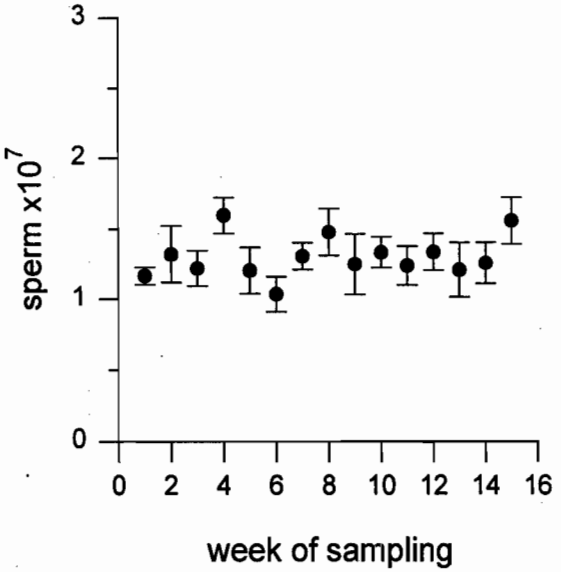


Fig. 19. Regression of combined testes mass on body mass of 38 Japanese quail exposed to three levels of fluoride or a control solution in their drinking water.

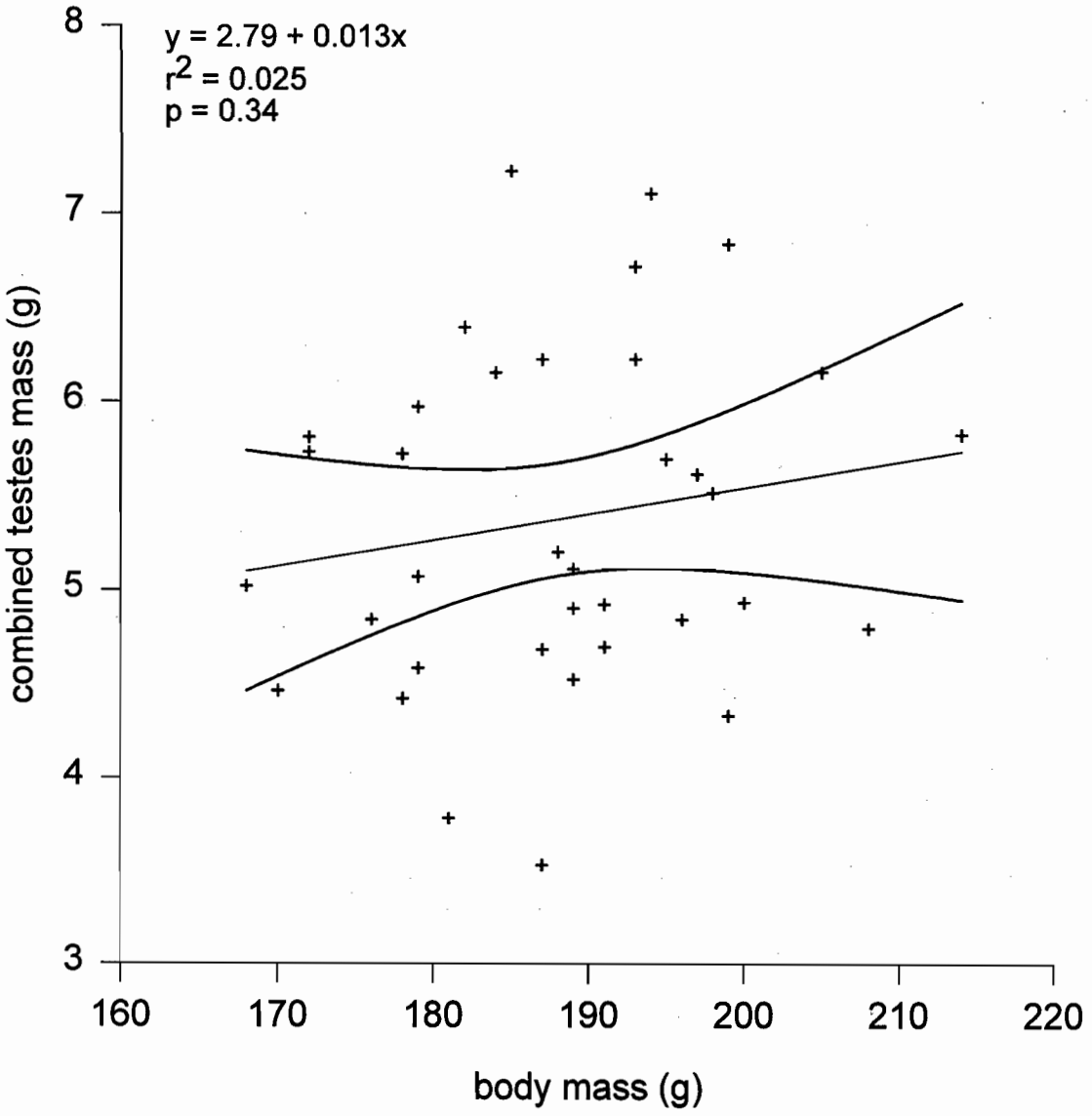
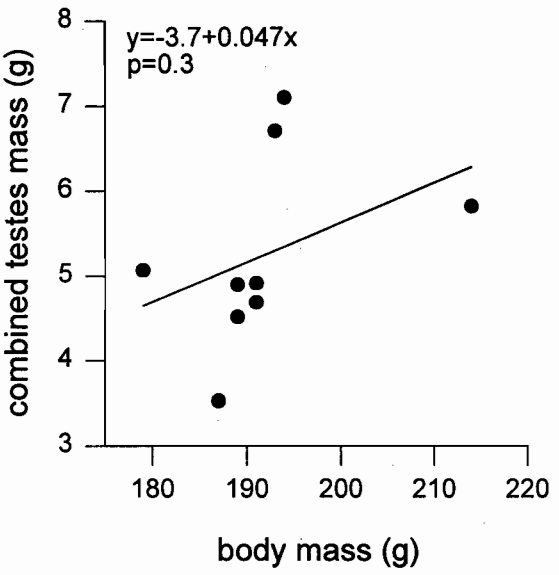
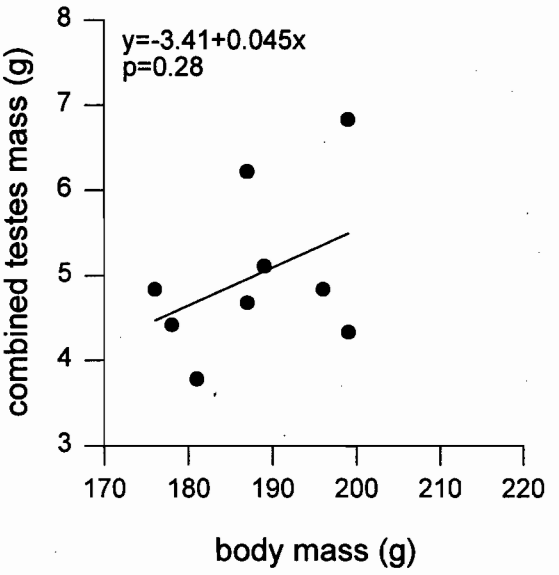

y = 2.79 + 0.013x
r2 = 0.025
p = 0.34
combined testes mass (g)
body mass (g)
8
7
6
5
4
3
160
170
180
190
200
210
220

Fig. 20. Regression of combined testes mass on body mass by treatment for Japanese quail exposed to three levels of fluoride or a control solution in the drinking water.

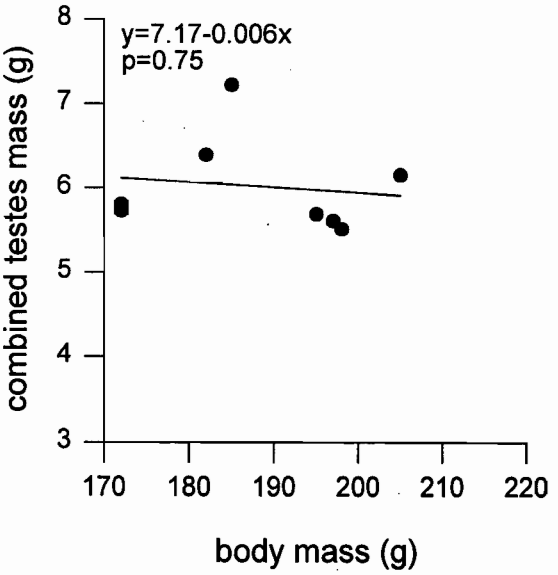
Control



125 ppm NaF



250 ppm NaF



500 ppm NaF

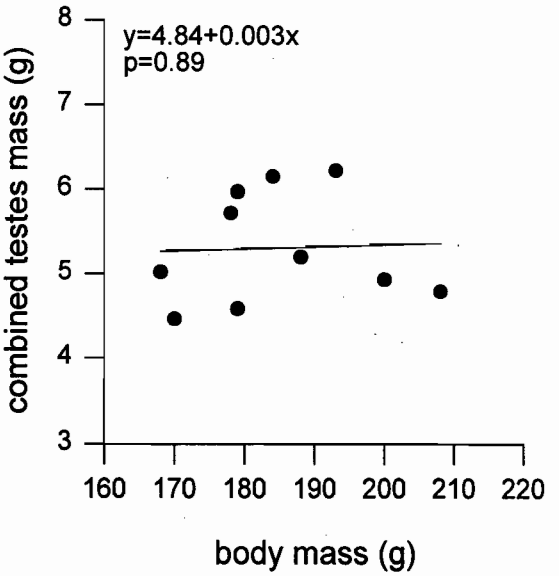
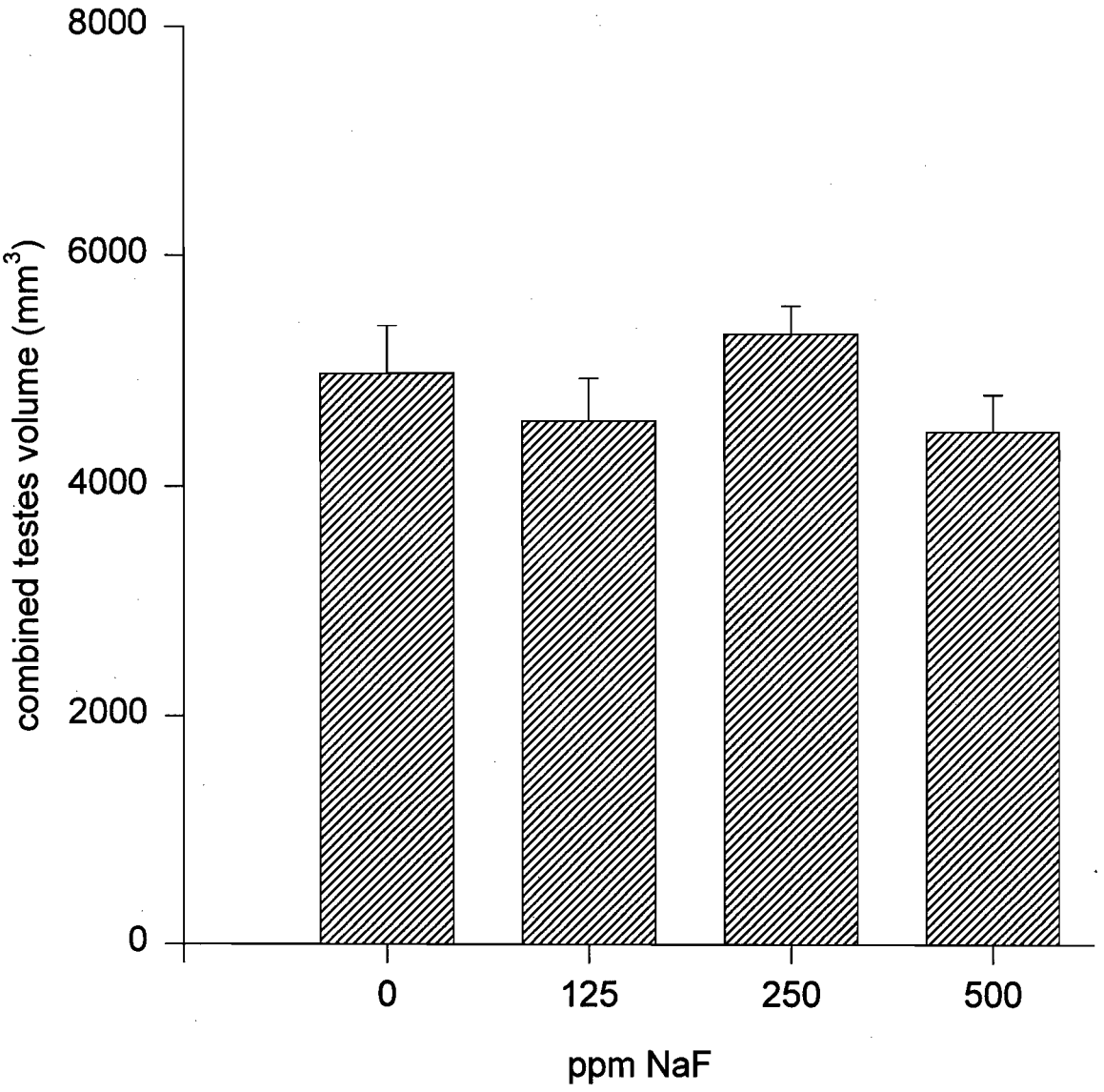


Fig. 21. Mean testes volumes of Japanese quail exposed to three levels of fluoride or a control solution in their drinking water. Each column represents the treatment mean ± 1 S.E. Treatments were not significantly different (p=0.27).


8000
6000
4000
2000
0
combined testes volume (mm3)
0
125
250
500
ppm NaF

CONCLUSIONS

The American kestrels and Japanese quail used in this study were treated with a significant daily exposure of fluoride over an extended period of time. In addition, the treatment, as sodium fluoride, was administered in a highly bio-available form. Sodium fluoride is known to be more soluble than other fluoride compounds. In addition, the compound was administered in water, increasing the ease which it would cross the gut wall. Fluoride complexed in bone matrix would not be as easily assimilated as that administered in this study. To assess potential impacts on male reproductive performance various aspects of the reproductive biology of these species were examined. Emphasis was placed upon the ability to produce viable sperm, and ultimately, the production of fertile eggs, a critical measure for any free-living species.

Despite the significant fluoride exposure which resulted (as measured by bone fluoride levels) from the experimental procedures, no significant impacts on any of the parameters measured was documented as a result of exposure to fluoride. Based upon these results it would appear unlikely that a male American kestrel would be exposed to levels of environmental fluoride which would affect its reproductive performance. Rather, effects on body condition and mobility could arise resulting from the development of lesions associated with chronic fluoride exposure. While interspecies extrapolations must be made with extreme caution, based on the results obtained for the Japanese quail in addition to the kestrel, it is unlikely that the male of any avian species would experience a reduction in reproductive success without first suffering from acute fluoride toxicity.

While there was no statistically significant reduction in the number of fertile eggs produced per clutch, during the second trial of the kestrel component of the study this value declined in a dose-dependent fashion from a high of 3.9 in the

control group to 2.25 in the 6.6 mg NaF/d group. This decrease may be ecologically relevant and must be considered when evaluating the risk experienced by free-living birds based on this research. The mechanism by which this reduction in fertility occurred may be related to the significant increase in the proportion of dead sperm observed in the Japanese quail component of this research. Those species which produce relatively dilute semen (including the kestrel) may be more susceptible to to these impacts than the quail which produce very concentrated semen.

While avian species appear to be more resistant to the effects of fluoride than mammals, the male's ability to produce many more sperm than are actually required to fertilize an embryo may serve to protect them, to some extent, from exogenous compounds which are known to compromise semen quality. It is the apparently robust nature of the male reproductive system to the effects of chronic fluoride ingestion which contributed, at least in part, to the negative results obtained here.

STATEMENT OF ORIGINALITY

This thesis includes the following original contributions to the scientific literature:

- determination of the effects of fluoride administration on testosterone levels in an avian species.
- evaluating the contribution of the male to the reproductive success of the breeding pair after exposure to fluoride.
- use of the oral gavage method to administer a known amount of fluoride to an animal over an extended period of time.
- correlating testosterone levels in males exposed to fluoride and their subsequent reproductive performance.
- use of Japanese quail semen to evaluate the impacts of fluoride on semen quality.
- evaluation of the effects of chronic exposure to fluoride on individual semen quality over time.
- use of the avian female as a bioassay of sperm quality after exposing the male to fluoride.
- documentation of changes in semen characteristics of aging Japanese quail.
- determination of the effects of fluoride administration on male Japanese quail reproductive performance.
- measurement of the effects of chronic exposure to fluoride on testicular mass and volume of an avian species.