MS - thesis

December 2020

Fluoride in the Icelandic horse

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60 ECTS thesis submitted in partial fulfilment of a *Magister Scientiarum* degree in Agricultural Sciences

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Clarification of contribution

I hereby declare that the writing of this thesis is my work under the supervision of my academic supervisor Charlotta Oddsdóttir and co-supervisor Sigríður Björnsdóttir.

The samples for the project came from four slaughterhouses in Iceland, where FEIF ID was accessible for each horse. The horses were tracked in Worldfengur, the studbook of Origin for the Icelandic horse.

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Brynja Valgeirsdóttir

Abstract

Fluoride in the environment distributes to water and vegetation from both natural and industrial sources. Vertebrates exposed to high levels of fluoride, accumulate fluoride in calcified tissues of the body, and are in danger of developing either acute or chronic symptoms of fluoride toxicity. The objective of this study was to provide background levels of fluoride concentration in the Icelandic horse and estimate whether a difference in fluoride accumulation is found in horses between regions and age. Mandibular fluoride concentrations were measured in 223 horses from four regions in Iceland (South, West, North and East) and divided by five age groups (foals, 1-4 y.o., 5-12 y.o., 13-20 y.o. and 21+ y.o.). The average mandibular fluoride concentration of the 223 horses was 244 ± 11.8 ppm, and 286 ± 12.6 ppm in horses over one year old, values beneath known tolerance levels. The highest mandibular fluoride concentrations for each age group were observed in horses from the West. A significant positive correlation was found between fluoride concentration and age of the horse, where the highest correlation coefficient was found in horses from the South. Furthermore, a significant difference was found between the regions, where horses from the West had significantly higher mandibular fluoride concentrations than horses from the South, but a significant difference was not found between horses from the West, North and East. Additionally, a correlation of fluoride concentration in horses from the West exclusively and increasing distance from the aluminium smelter in the region (Hvalfjörður) showed a significant negative regression, indicating that the greatest source of fluoride in the environment originates from the aluminium industry. The results are the first of their kind and can be used as a guideline of fluoride concentration in the Icelandic horse. Moreover, the results indicate that the Icelandic horses accumulate less fluoride on average than Icelandic sheep, which makes them less appropriate as an indicator species for environmental fluoride contamination.

Keywords: Fluoride, Icelandic horse, volcanic eruptions, aluminium smelter

Ágrip

Flúor í íslenska hestinum

Flúor í umhverfi á upptök sín frá bæði náttúrulegum uppsprettum og iðnaði, og dreifist þaðan til grunnvatns og gróðurs. Hryggdýr sem útsett eru fyrir verulegum styrk flúors frá umhverfi eiga á hættu að verða fyrir bráðri flúoreitrun, eða þróa með sér króníska flúoreitrun eftir uppsöfnun flúors í kalkríkum vefjum líkamans. Markmið þessarar rannsóknar var að afla upplýsinga um flúorstyrk í íslenska hestinum og fá þar af leiðandi bakgrunnsgildi sem hægt væri að styðjast við í framtíðinni. Einnig að meta hvort munur væri á flúoruppsöfnun í beinum hrossa milli landshluta, og aldurshópa. Styrkur flúors í kjálkasýnum 223 hrossa var mældur frá fjórum landshlutum á Íslandi (Suðurlandi, Vesturlandi, Norðurlandi og Austurlandi) og milli fimm aldurshópa (folalda, 1-4 vetra, 5-12 vetra, 13-20 vetra og 21 vetra og eldri). Meðalstyrkur flúors í þeim 223 sýnum sem safnað var, mældist 244 ± 11.8 ppm (milljónahlutar), og 286 ± 12.6 ppm þegar sýni úr folöldum voru tekin út, en þessi gildi eru vel undir þekktum viðmiðunarmörkum. Hæsti flúorstyrkur fyrir hvern aldurshóp var úr hrossum af Vesturlandi. Marktæk fylgni var á milli flúorstyrks og aldurs hrossa, þar sem hæsti fylgnistuðullinn var í hrossum af Suðurlandi. Enn fremur var marktækur munur á flúorstyrk milli landshluta, þar sem hross af Vesturlandi mældust með marktækt hærri flúorstyrk í kjálkum en hross af Suðurlandi, en marktækur munur fannst ekki á flúorstyrk milli hrossa af Vesturlandi, Norðurlandi og Austurlandi. Þegar fylgni flúrostyrks í hrossum af Vesturlandi eingöngu og fjarlægð þeirra frá álverinu í Hvalfirði var mæld, fannst marktæk neikvæð fylgni, sem gefur til kynna að drjúgur hluti flúors í íslensku umhverfi á rætur sínar að rekja til áliðnaðar. Niðurstöður rannsóknarinnar eru þær fyrstu sinnar tegundar og gefa innsýn í flúoruppsöfnun í beinum íslenska hestsins og mun á flúorstyrk í umhverfi milli landshluta. Einnig benda niðurstöðurnar til þess að íslenski hesturinn safni upp minna magni af flúor í bein heldur en íslenskt sauðfé og sé vegna þess óhentug vísitegund þegar kemur að flúormengun í umhverfi. Þessar niðurstöður er hægt að hafa að leiðarljósi í áframhaldandi rannsóknum, þar sem engar fyrirliggjandi upplýsingar eru tiltækar.

Lykilorð: Flúor, íslenski hesturinn, eldgos, álver

Acknowledgement

Financial support from the Environment Agency of Iceland is acknowledged, along with gratitude to Ester Inga Eyjólfsdóttir and Helga Dögg Flosadóttir at the Innovation Center of Iceland for fluoride analysis of the samples.

Special thanks go to Halla Eiríksdóttir and Guðjón Gunnarsson at Sláturfélag Suðurlands (SS) for their limitless help, willingness and obliging with sample collection. Other staff at SS is also thanked.

The staff at Kaupfélag Skagfirðinga (KS) get many thanks for obliging with sample collection, especially Edda Þórðardóttir, Birgir Hauksson and Robert Wika.

My supervisor, Charlotta Oddsdóttir, gets my sincere gratitude for her advice, guidance, time, and support.

I would like to thank my co-supervisor, Sigríður Björnsdóttir, for her advice and welcoming me to her home when needed.

Finally, I would like to thank my family and friends for their enormous support, help, patience and travelling company.

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1 Introduction

In this chapter, fluoride origination and distribution in the environment is reviewed, along with fluoride uptake, accumulation, and its effects on animals. Furthermore, fluoride guidelines and tolerance levels are presented. Understanding of the fundamental influence of fluoride on animals is important, as the thesis addresses fluoride concentrations in mandibles of the Icelandic horse.

1.1 Properties of fluorine

Fluorine is highly reactive and the most electronegative of all the elements. Therefore, it has a strong attraction for electrons and is not found in a free state in nature (Weinstein & Davison, 2004), but always in combination with other elements. Chemical and physical properties of fluorine compounds have a strong impact on fluorine uptake, metabolism, excretion and biological effects. The element itself is a pale yellow-green gas at room temperature (Ranjan & Ranjan, 2015) and fluorine is the thirteenth most abundant element in the Earth‘s crust (Edmunds & Smedley, 1996). Fluorine reacts with electropositive elements to form fluoride ions. The main ionic fluoride compounds that have been found responsible for fluoride toxicity in animals and humans are hydrogen fluoride, fluorosilicic acid, sodium fluoride and sodium silicofluoride (Ranjan & Ranjan, 2015).

Up to several hundred minerals contain fluoride, but the fluoride content varies between minerals from as little as 0.2% to 73%. The three primary fluoride-bearing minerals exploited for industry are fluorite (CaF_2), fluorapatite ($Ca_{10}F_2(PO_4)_6$) and cryolite (Na_3AlF_6), respectively (Weinstein & Davison, 2004). Furthermore, hydrogen fluoride is commonly used in the industry for many varieties. The greatest consumption of hydrogen fluoride is in the synthesis of fluorocarbons which are used as solvents, refrigerants and aerosols. Fluorosilicic acid is used in water fluoridation and in the aluminium industry for the synthesis of aluminium fluoride (Weinstein & Davison, 2004). The study is focused on fluoride (F^-), which is the toxic form.

1.2 Sources of fluoride

Fluoride sources for animal or human intake can be both of natural and industrial origin (Fig. 1) and include contaminated drinking water, forage and grasses, volcanic ash, phosphate fertilizers and fumes from various industries. Fluoride can be distributed or dispersed through the atmosphere, water, or blood, for example (Weinstein & Davison, 2004). Absorption is the process where fluoride is absorbed by another material, from the gastrointestinal tract to the blood vessels or from soil to plant roots, for example. On the other hand, adsorption is when molecules or ions deposit on the surface of objects from gas or liquid (McDonald et al., 2002).

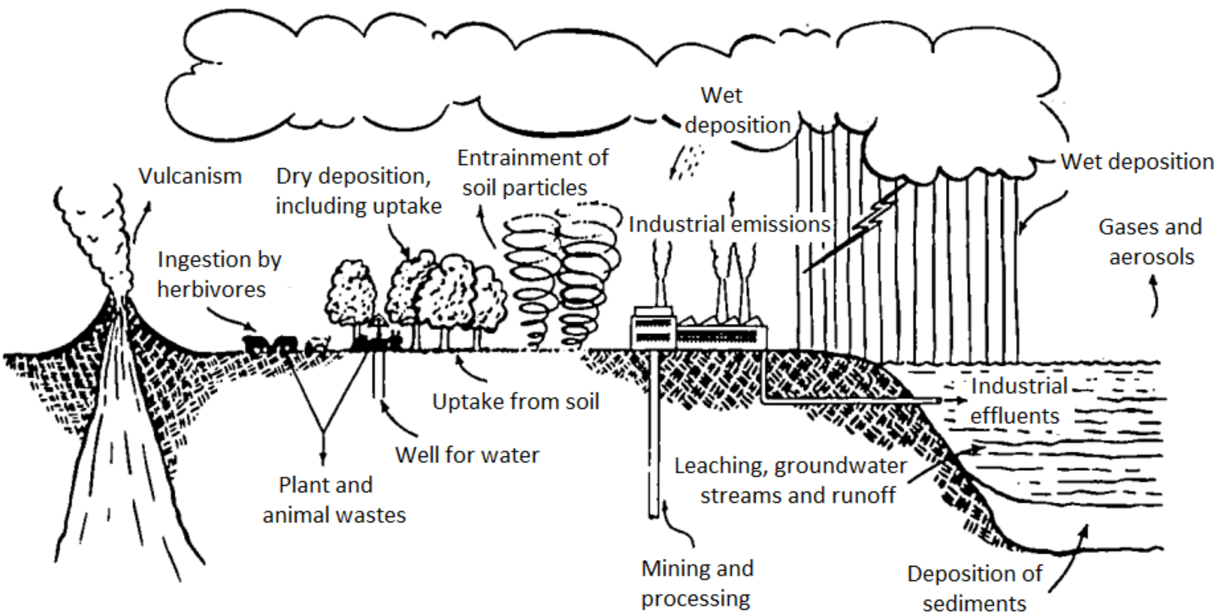

***Figure 1**. Fluoride cycle and transfer in the environment. Figure adapted from Weinstein & Davison (2004).*

1.2.1 Dispersion of fluoride

In the atmosphere, fluoride is either in particulate or gaseous (hydrogen fluoride) form. As a result of wind or turbulence, atmospheric fluoride can move over a large distance. The pattern of fluoride dispersion depends on the temperature of the plume, height of emission, wind speed and direction, topography, precipitation, and characteristics of vegetation. Additionally, pollutant concentration in plumes decreases with distance from the source because of wet and dry deposition. Wet deposition occurs when pollutants get removed from plumes by rain, mist, and snow, and is very effective. Dry deposition is when pollutants get attached to the surface of objects or are taken up into plant leaves through the stomata, for instance. In general, deposition varies with the form of fluoride (gaseous or particulate), nature of the plant canopy, wind speed, architecture of the particle and wetness (Weinstein & Davison, 2004). With the exception of sulphur hexafluoride, fluoride compounds do not remain in the troposphere for great periods of time, neither do they migrate to the stratosphere, where wind is faster. Sulphur hexafluoride, however, can remain in the atmosphere for 500 to a thousand years (WHO, 2002). Toxicity of fluoride is different between the gaseous and particulate form, and therefore it is important to know which form is examined for environmental monitoring, namely organic or inorganic form. When sampled closer to aluminium factories e.g., the gaseous form of fluoride is more widespread, but changes to particulate form with increasing distance from the source (Weinstein & Davison, 2004).

Fluoride transportation in water is influenced by water hardness, pH and the presence of ion-exchange substance such as clay. Generally, fluoride is transported in a compound with aluminium through the water cycle (WHO, 2002). Spring and well waters often contain higher concentrations of fluoride than surface water from streams and lakes, since they disperse over a larger area (Weinstein & Davison, 2004). Similarly, fluoride transformation and transport in soil is influenced by pH and the formation of aluminium and calcium compounds. At slightly higher pH values (5.5-6.5), adsorption is stronger to the solid phase of soil (WHO, 2002), which is combined of organic and secondary minerals (Ashman & Puri, 2002), and fluoride is not leached readily from soils (WHO, 2002).

1.2.2 Water contamination

The contamination of groundwater happens when fluoride-bearing rocks, such as fluorspar, cryolite and apatite, break down and crumble when water percolates through the rocks. The level of fluoride pollution in groundwater is dependent on the nature of the rocks and the soil it runs through, the proportion of fluoride bearing minerals, pH and temperature of the soil and physical characteristics of the water. Furthermore, fluoride concentration in water is limited by fluoride solubility. It has been reported that when dissolved calcium is absent, water has a higher fluoride solubility, and vice versa (Edmunds & Smedley, 1996).

In geothermal areas, especially where the water circulates through granite rocks, compared to basaltic rocks, the fluoride content of the water is usually high. Fluorapatite and amphiboles are the primary sources of fluoride in geothermal waters. For a prolonged period of time, at temperatures higher than 200 °C, water-granite interaction results in a great content of fluoride in thermal water (Chandrasekhar et al., 2015). Geothermal water is occasionally used in agriculture and may therefore increase the proportion of toxic substances within plant tissues and consequently increase fluoride accumulation in grazing herbivores (Miller, Shupe & Vedina, 1999).

In a report from 2010 on chemical properties of Icelandic waterholes, fluoride was measured in waterholes all over Iceland. The waterholes contain fluoride on a scale of 0.015 mg/l in Glerá located in the North, to 4.04 mg/l in Flúðir located in the South region (Korneliusdóttir, 2010). The fluoride concentration in Flúðir exceeds the safe level of fluoride in drinking water, 1.5 mg/l (Regulation 536/2001), but otherwise, fluoride in Icelandic waterholes never exceed 1.0 mg/l (Korneliusdóttir, 2010) and should therefore be safe for consumption.

1.2.3 Vegetation

Plants are a significant source of fluoride in herbivores. Fluoride accumulates in plants as a result of absorption of fluoride from water and soil (Gritsan, Miller & Schumatkov, 1995). There are five vital factors that define fluoride concentration in plants; the amount of released fluoride, the distance from the emission source, the plant type, the soil type and weather conditions (National Research Council, 1955). Fluoride accumulates in plant leaves and varies from less than one to several thousand mg per kg between species. Accumulation in plant leaves usually involves highly soluble fluoride, such as hydrogen fluoride, so it readily dissolves in water within the cell wall and forms the fluoride ion. Following evaporation through the stomata, fluoride accumulates at the leaf margins and apex, as water loss is greatest in these areas. This unequal division of fluoride concentration in leaves is one of the reasons for its critical toxicity to some plant species (Weinstein & Davison, 2004). In soil, most of the fluoride cannot be assimilated easily by plants since soil fluoride is usually in compounds with other elements. Therefore, soil fluoride concentration is considered to have limited influence over fluoride concentration in vegetation versus fluoride uptake by stomata through soluble fluoride in the atmosphere

(Baunthiyal & Ranghar, 2014). However, when animals graze where soil is rich with soluble fluoride they are in danger of ingesting toxic doses of fluoride, especially if the pasture is highly grazed and the animals eat short plants close to the soil (Shupe & Olson, 1971). Fluoride content of organic soils is usually lower than that of fertilized soils since fluoride concentration of topsoil increases by the addition of fluoride containing fertilizers, pesticides, irrigation water or by emissions from industry (Ranjan & Ranjan, 2015).

1.2.3.1 Fluoride toxicity in plants

Fluoride is not essential for plants. Therefore, excessive fluoride uptake by plants can affect several metabolic processes and may result in chlorosis, leaf distortion, peripheral necrosis, and abnormal fruit development (Weinstein, 1977). It interferes with the overall response of plants including growth and productivity, photosynthesis, protein synthesis, seed germination and gene expression patterns (Yadu, Chandrakar & Keshavkant, 2016). First visible sign of plant fluorosis is when chloroplast activity is disrupted resulting in leaf tips and margins starting to wither. At excessive fluoride exposure cellular necrosis in leaves and shoots occurs and may result in death of the plant (Weinstein, 1977).

1.2.4 Industry

Swarup and Dwivedi (2002) reported that multiple types of industries release fluoride-rich effluents and fumes as waste products which pollute the environment. World Health Organization (WHO) has identified certain industries to be especially harmful in this matter, including the aluminium industry, superphosphate plants, steel production, ceramic factories and oil refineries. Fluoride released to the atmosphere from industry can be in various forms. Gaseous fluoride forms include hydrogen fluoride, hexafluorosilicic acid and silicon tetrafluoride. The particulate forms are aluminium fluoride, sodium aluminium fluoride and calcium fluoride, for example (WHO, 1984). The fluoride emissions released from industry are mostly airborne, as ash, dust, and fumes, which contaminate water, soil and vegetation. Hence, forage and grasses grown in industrial areas are generally contaminated. Areas in a considerable distance from the source of emission can also be contaminated (Radostits et al., 2007). Burns and Allcroft (1964) reported previously that fluoride pollution from industry in the United Kingdom had caused severe chronic fluorosis, such as dental fluorosis, bone lesions and lameness, in livestock grazing within a few kilometres of the source. Even though being fully supervised, industrial contamination might still cause chronic fluorosis in herbivores grazing near the source (Ranjan & Ranjan, 2015).

For decades, fluoride compounds such as sodium fluoride, cryolite and sodium fluorosilicates were used as pesticides in agriculture and have, through that time, caused fluoride toxicity and even death in animals. However, these compounds are rarely used in present times due to their harmful effects and are a limited source of fluorosis in animals today (Ranjan & Ranjan, 2015). Then again, the use of organofluoride compounds have been rising over the years. These compounds have a wide range of purposes and can serve as pharmaceuticals, agrochemicals, refrigerants, fire extinguishing agents and insulating materials. Due to the strength of the carbon-fluoride bond, these compounds are considered constant global contaminants and may be harming wildlife (Weinstein & Davison, 2004).

Rock phosphate fertilizers and mineral supplements can contribute to fluoride accumulation in livestock (Livesey & Payne, 2011). It has been estimated that in the intensive agricultural regions of the world, where use of phosphate fertilizers is widespread and continuous, fluoride content in the upper layers of agricultural soil increases significantly (Weinstein & Davison, 2004). There have been reports of fluorosis in cattle given access to commercial salt lick which contained 1400 mg/kg of fluoride. Four years after the introduction of the salt lick, 70% of the cattle had developed dental lesions including mottling, wear down of incisors and upper molars and increased fragility. Some cows showed lameness, especially in front limbs, and a few were seen walking on their knees. In the end, the salt lick was removed, and cattle born later showed normal teeth development (Schultheiss & Godley, 1995).

There are three active aluminium smelters in Iceland. The oldest, established in 1969, is located in Straumsvík, the capital area. Then, in 1998, an aluminium smelter was established in Hvalfjörður, West Iceland, followed by establishment of the third aluminium smelter in Reyðarfjörður, East Iceland, in 2007. Fluoride monitoring and surveillance has been carried out around these areas since the establishment of the smelters, where fluoride concentrations in atmosphere, precipitation, grass, leaves, pine needles, groundwater and sheep are measured (Stefánsdóttir, 2016; Pálsson, 1995, Yngvadóttir et al., 2017; Guðmundsdóttir et al., 2019).

1.2.5 Volcanoes

Volcanic ash has an especially high soluble fluoride concentration and can be deposited over a wide range of land after eruptions (Araya et al., 1993). Therefore, livestock exposed to volcanic ash, are in severe risk of developing symptoms of either acute or chronic fluorosis (Gregory & Neall, 1996). Fluorides found in volcanic ash contaminate glacier-melt waters, rainfall and surface water, forage, and grasses (Livesey & Payne, 2011). Fluoride concentration in grass and water can remain high for years after eruption in volcanic areas, even if volcanic activity has ended (Araya et al., 1993). Iceland is a highly active volcanic site and therefore thousands of sheep, cattle, horses, and other animals, both domestic and wild, have died through the years due to repeated volcanic eruptions (Pétursson, Pálsson & Georgsson, 1984).

In his review on volcanic eruptions in Iceland, Fridriksson (1983) quoted an Icelandic historian, Jón Steingrímsson, on the aftermath of Laki eruption in 1783:

> In horses, sheep and cattle the effects of the perilous fumes from the fires resulted either in death or various diseases: the horses lost all muscle, sometimes the hide decayed along the back. The hair of the tail and mane rotted and fell out if pulled. Joints became enlarged, especially above the hooves, the head puffed up with severe weakness in the jaws and the animals could not graze, swallow or chew. Sheep suffered even more. Partially all extremities were swollen with lumps, especially on the jaws where they tore out of the skin. Large bone lumps grew on the ribs, hipbones and legs. The legs bowed or became extremely fragile. Lungs, liver and heart were edemic and sometimes withered, the insides were decayed and soggy. The meat from these animals was foul smelling, bitter tasting and often poisonous, although people would try to make use of it. The cattle suffered similar hardships. Ossified lumps grew on the jaws and shoulder bones, and in some cases the leg bones would split. Hip joints and other joints moved out of place or became calcified. Part of the tail fell of and then the hooves would loosen in parts or drop off.

1.3 Absorption and metabolism of fluoride

1.3.1 Absorption

In animals, fluoride burden of the body is determined by the route of uptake, the diet, pH of the digestive system and the occurrence of calcified tissues in the body (Weinstein & Davison, 2004). Fluoride can be absorbed through different routes, including the gastrointestinal tract, respiratory tract and sometimes through skin and mucous membranes (Ranjan & Ranjan, 2015). Transport of fluoride through biological membranes occurs mainly through the non-ionic diffusion of hydrogen fluoride. Studies indicate that hydrogen fluoride is a highly permeant solute with a permeability coefficient similar to that of water. The hydrogen fluoride molecule seems to penetrate cell membranes faster than the dissociated fluoride ion, resulting in a more prominent intracellular intake (Whitford et al., 1994). Membrane permeability to hydrogen fluoride is five to seven orders of magnitude above that of fluoride (Gutknecht & Walter, 1981).

1.3.2 Gastrointestinal absorption

Up to 40% of ingested fluoride is absorbed from the stomach as hydrogen fluoride, as the acidic conditions in the stomach convert fluoride to hydrogen fluoride (Buzalaf, & Whitford, 2011). The higher the stomach acidity, the more fluoride is absorbed (Weinstein & Davison, 2004). Therefore, the peak plasma level is higher and occurs sooner when the contents are more acidic (Whitford & Pashley, 1984). High concentrations of cations that form insoluble complexes with fluoride (calcium, magnesium, aluminium) can decrease gastrointestinal fluoride absorption relative to the amount of fluoride ingested, causing hypocalcemia and inhibition of magnesium and manganese-dependent enzymes (Whitford, Pashley & Garman, 1997). When calcium and aluminium are absent and do not form insoluble compounds with fluoride, about 80-90% of the ingested amount of fluoride is absorbed from the gastrointestinal tract (Medical Research Council, 2002). Other factors that affect fluoride absorption are the species ingesting the fluoride, dietary components and age of the animal (Weinstein & Davison, 2004). Most of the fluoride that is not absorbed from the stomach will be absorbed from the proximal small intestine (Whitford, 1989). This is true for single-stomach animals such as horses and pigs. However, for ruminants a great amount is also absorbed in the rumen and abomasum (Parkins, 1971).

1.3.3 Respiratory absorption

Inhalation of fluoride has not been considered an important route of uptake for vertebrates, since the amount of inhaled fluoride is limited in relation to other routes (Weinstein & Davison, 2004). However, when gaseous fluoride forms, especially hydrogen fluoride, are released through industry or volcanic activity, fluoride may be absorbed in a great deal through the respiratory tract (WHO, 2002). Therefore, fluoride absorption from the respiratory tract could be a significant factor for animals living in areas close to fluoride emitting industries or recently erupted volcanoes (Radostits et al., 2007).

1.3.4 Dermal absorption

The Agency for Toxic Substances and Disease Registry reported that dermal exposure to fluoride is mainly through hydrofluoric acid. When it is applied straight to the skin, fluoride is absorbed through the epidermis and can cause extensive damage and later increased circulation of fluoride in the systemic circulation (ATSDR, 2003). In vertebrates, fluoride absorption through dermal routes does not appear to be significant for developing chronic fluorosis. Neither does it seem to be the primary route for fluoride absorption in invertebrates, such as silkworm larvae who are immensely sensitive to fluoride toxicity (Ranjan & Ranjan, 2015).

1.3.5 Distribution of fluoride after absorption

Fluoride distributes readily throughout the body when absorbed into the blood. Plasma fluoride levels increase measurably within the first few minutes after ingestion and reach a peak concentration within 20-60 minutes. In general, the numerical value of the fasting plasma concentration of adults whose main source of fluoride is through diet, is approximately equal to the concentration in drinking water (Guy, Taves & Brey, 1976). However, variations in the expected values of plasma are due to the amount of fluoride ingested, solubility of the fluoride compound, rate of absorption and individual differences in the rates of removal of fluoride by the kidneys and skeleton (Whitford, 1994). Rapid decline in plasma concentration occurs as the absorption rate declines and the renal and skeletal clearance of fluoride happens (Whitford, 1989). In plasma, fluoride distributes across cell membranes of almost all soft tissue with a steady-state-ratio, except to brain and fat, which have a lower ratio, and kidneys which have a higher ratio since fluoride is concentrated in the renal area. (Ranjan & Ranjan, 2015).

Fluoride concentration in plasma is not a good biomarker for fluoride pollution since there are several physiological variables that affect the levels, including the fluoride concentration in the exchangeable pool of bone, the rates of bone accretion and resorption, and the renal clearance of fluoride. Urinary fluoride levels are comparable to plasma concentrations, but they are more variable since it affects urinary flow rate and the extent of tubular reabsorption (Whitford, 1994).

1.3.6 Accumulation of fluoride in calcified tissues

Since fluoride has a high affinity for calcium, around 50% of the absorbed fluoride becomes associated with calcified tissues within 24 hours, whether it is the shell of a crab or an equine bone, while all of the remainder is excreted in urine (Weinstein & Davison, 2004). The clearance of fluoride from plasma by the skeleton continues at a high rate, that even exceeds the rate of calcium clearance from the blood to the skeleton (Costeas, Woodard & Laughlin, 1971).

Bone is composed of water (45%), inorganic salts such as calcium and phosphorus (35%) and osteoid (20%), which consists of collagen and non-collagenous glycoproteins and proteoglycans. In bone and tooth enamel, the main inorganic constituent is hydroxyapatite ($Ca_5(PO_4)_3(OH)$), which forms crystals that are parallel to collagen fibers. A hydration shell surrounds the crystals (Swarup & Dwivedi 2002). Whitford (1989) suggested that fluoride uptake by bone occurs in stages. During the first stage fluoride migrates into the hydration shells of the bone crystals. The hydration shells are connected to the extracellular fluid and since fluoride in this pool is rapidly exchangeable it can migrate either way depending on the concentration in the hydration shells and the extracellular fluid. Fluoride is then either associated or incorporated into the hydroxyapatite and substituted for the hydroxyl group, forming fluorapatite. Fluorapatite has a lower solubility, greater crystallinity and forms a more stable crystal than hydroxyapatite. Furthermore, fluorapatite crystals run perpendicular to the collagen fibre opposite to hydroxyapatite crystals as mentioned before. Therefore, the skeletal response to fluoride toxicity is to increase bone formation and proliferation of osteoblasts (Grynpas, 1990; Farley, Wergedal & Baylink, 1983). Approximately 99% of the body burden of fluoride is associated with calcified tissues. The fluoride concentration in bone is not homogeneous. For example, in long bones the concentration is highest in the periosteal region (Weatherell et al., 1977). The concentration declines sharply within a few millimetres of the periosteal surface and increases slightly in the endosteal region. Furthermore, fluoride concentration is higher at the end of long bones than in the middle (Ranjan & Ranjan, 2015). A cancellous bone has higher fluoride concentration than a bone that is compact.

When livestock is exposed to great levels of fluoride during the formative state of the teeth (Shupe, Miner & Greenwood, 1963), from birth and up to the fourth year (Lowder & Mueller, 1998), the dentine and enamel are affected, resulting in ameloblastic and odontoblastic damage. The abnormal tooth matrix can therefore not incorporate required minerals as a normal matrix would. Enamel mineralization is thus poor and faulty, and dental lesions appear (Shupe, Miner & Greenwood, 1963). When the tooth is fully developed the enamel is unable to undergo repair or remodelling, therefore making the dental lesions by fluoride permanent (Kierdorf, Kierdorf & Sedlacek, 1999). In contrast, if animals are exposed to high levels of fluoride after their permanent enamel has fully developed, if they migrate to a fluoride endemic area for example, dental fluorosis would not be expected in the animals (Kierdorf et al., 1996).

Fluoride concentrations in bones are cumulative throughout the life of the animal, unlike that of teeth, as described above. Therefore, fluoride content in bones of young animals is usually low, and increases with rising age of animals, when exposed to continuous levels of fluoride. However, the metabolic activity of the skeletal system is more rapid in younger animals, and thus affects the degree of fluoride accumulation in the skeleton. High levels of fluoride could therefore be more prominent in bones of animals exposed to elevated levels of fluoride and a young age (Shupe, Miner & Greenwood, 1963; Kierdorf et al., 1995).

1.3.7 Clearance of fluoride

Fluoride is excreted mostly by urine (Fig. 2). Urinary fluoride clearance increases with urine pH due to a decrease in the concentration of hydrogen fluoride (ATSDR, 2003). Fluoride is freely filtered through the glomerular capillaries and then undergoes a variable degree of tubular re-absorption. Renal clearance of fluoride is usually low compared to the other halogens (Whitford, 1994).

The mechanism of tubular re-absorption appears to be the diffusion of hydrogen fluoride, similar to the gastric absorption of fluoride and migration across cell membranes. Furthermore, factors such as the composition of diet, metabolic or respiratory disorders, and the altitude of residence that affect urinary pH, have been reported to affect the metabolic balance and tissue concentration of fluoride (Whitford, 1989).

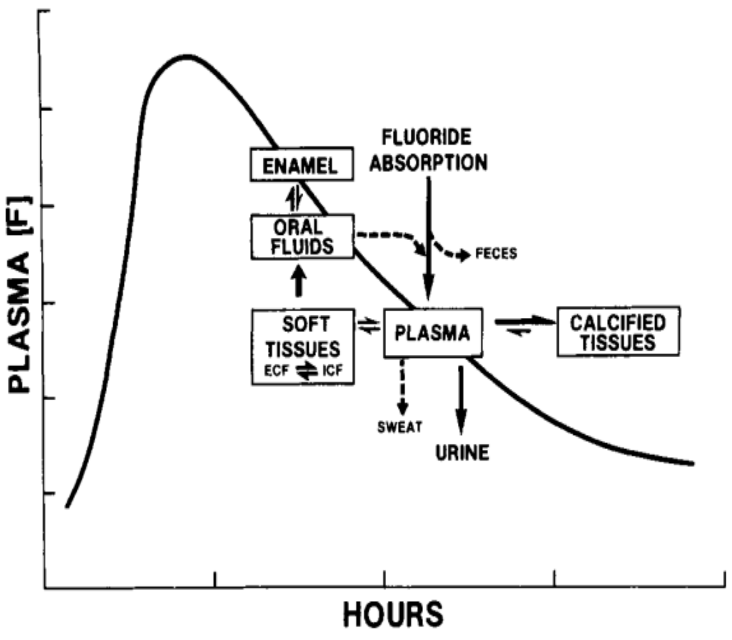

Figure 2. *A typical fluoride concentration curve in plasma after the ingestion of small amount of fluoride and when the major metabolisms happen. Figure adapted from Whitford (1994).*

1.4 Health effects of fluoride toxicity

1.4.1 Acute toxicity

Acute fluorosis is relatively rare, and it requires extensive exposure to highly soluble inorganic fluoride compounds. It can appear after a major environmental incident, industrial accident or when livestock consumes superphosphate fertilizer (Livesey & Payne, 2011). Acute fluorosis causes multiple organ failure, toxic shock, and death. Degeneration of heart muscle, bone marrow, parathyroid, central nervous system, thyroid, abdomen, kidney, adrenal and liver has been reported as a consequence of acute fluorosis (Wheeler & Fell, 1983). Exposure to hydrofluoric acid can cause acute fluorosis via respiratory or dermal exposure, where hypocalcaemia and death can occur within a few minutes. Acute oral exposure to soluble fluorides, such as in volcanic ash, can cause death within 12 hours (Gregory & Neall, 1996).

Cases of acute toxicity have been described in Icelandic livestock after volcanic eruptions as mentioned on the aftermath of Laki eruptions in 1783-1784 (Fridriksson, 1983) and further by Georgsson and Pétursson (1972) following the Hekla eruption in 1970. Sheep grazing outdoors were volcanic ash fell showed loss of appetite, general prostration, drowsiness, blood-stained diarrhoea and sometimes cough and dyspnea. Around 3% of adult sheep and 8-9% of lambs in that area died. Necropsy from the deceased sheep showed blood-filled lungs, toxicity in liver and kidneys and dilution in the heart muscles. It was estimated that in the first days after the eruption, grazing sheep could have ingested up to 100 mg/kg of fluoride (Georgsson & Pétursson, 1972).

In an experiment conducted on Icelandic lambs, diarrhoea, general prostration and loss of appetite were observed after 20 weeks of ingesting sodium fluoride in 10 mg/kg and 15 mg/kg doses, five days each week. These symptoms were absent in lambs which received 5 mg/kg F doses. Fluoride levels in plasma rose in context to the fluoride doses during the experiment, but no pathological changes were seen in the soft tissue, teeth or bone after necropsy. It was estimated that fluoride plasma levels above 860 ng/ml were consistently associated with fluoride toxicity, and levels above 500 ng/ml were considered potentially harmful (Kristinsson et al., 1997).

1.4.2 Chronic toxicity

Fluoride is a highly cumulative toxin and when organisms are exposed to fluoride over a long period it can induce fluorosis. If exposure is moderate, clinical signs of chronic toxicity might not appear for several weeks or months (Suttle, 2010). Dental lesions appear to be the earliest visible sign of chronic fluorosis in mammals, followed by symptoms of skeletal deformities. When exposure to toxic doses of fluoride is continuous, toxic effects on soft tissues and other organ systems might become visible (Perumal et al., 2013). The risk of fluorosis depends on the level and duration of exposure, species, age, nutritional status, physiological factors and grazing management (Weinstein & Davison, 2004).

1.4.3 Dental fluorosis

Either acute or chronic exposure to sodium fluoride, or other soluble fluoride, affects formation of the enamel and triggers dental fluorosis that manifests as mottled, discoloured and porous enamel (Yan et al., 2007). It is suggested that these effects are associated with precipitation of hydroxyapatite by fluoride ions, altering enamel mineralization, similar to that of bone. Several authors have also concluded that the clinical signs of dental fluorosis are associated with the action of fluoride on secretory functions of ameloblasts and epithelial cells responsible for enamel development, inducing endoplasmic reticulum stress in the ameloblasts, resulting in a reduction of protein synthesis, secretion and protein concentration in the enamel (Matsuo et al., 1996; Sharma, Tsuchiya, & Bartlett, 2008; Kubota et al., 2005).

Mild tooth lesions can be subtle and are particularly hard to see. More serious lesions are easier to see since the enamel might be stained. Enamel hypoplasia can cause enormous wear to the tooth. It becomes brittle and early tooth loss is therefore possible. Severely affected molars have uneven wear and develop spurs, spikes and step mouth. These symptoms can cause difficulty in eating, weight loss and reduced productivity (Ranjan & Ranjan, 2015). Extreme dental wear can lead to reduced milk production and impaired reproduction and can therefore affect agricultural profit (Weinstein & Davison, 2004).

A system to score dental lesions by severity was developed by Kierdorf et al. (1996) to estimate dental fluorosis in populations of wild red deer (*Cervus elaphus)* in Europe (Table

1). Dental lesions are scored in six levels (0-5), in premolars and molars of deer mandible. Kierdorf's system has been used to estimate fluoride accumulation in Icelandic sheep, in relation to fluoride pollution (Yngvadóttir et al., 2017). Furthermore, Livesey and Payne (2011) described a scoring system using photographs of cattle teeth (Fig. 3).

***Table 1**. Scoring system for teeth lesions in red deer. Table adapted from Kierdorf et al. (1996).*

Score	Tooth characteristics
0	Normal (white and translucent) appearance of enamel. Due to its hardness, the enamel forms distinct ridges on the occlusal surface. Physiological, i.e. age-related attrition.
1	Occurrence of enamel opacities and posteruptively of (yellow to brownish) enamel discoloration. Changes mainly confined to cuspal regions and sometimes present in the form of horizontal bands.
2	The whole tooth crown is affected by opacity and discoloration. A reduction in height or disappearance of the enamel ridges is often discernible.
3	Complete loss of enamel ridges leads to a flattening of the occlusal surface. Occurrence of enamel surface lesions affecting up to 5% of the tooth crown.
4	Pathologically increased wear leading to an abnormal tooth shape. Between 5% and 25% of the tooth crown exhibit enamel surface lesions.
5	Dysfunctional tooth shape due to grossly increased wear. Some teeth may be fractured or are completely lost as a result of periodontal breakdown. More than 25% of the tooth crown affected by enamel surface lesion.

Vikøren and Stuve (1996) studied fluoride related dental lesions in cervids living near aluminium smelters in Norway. Dental lesions such as staining of the enamel, generalized mottling and excessive wear of the tooth were observed in context to high fluoride concentrations in mandibles, and were most common in cervids habiting closest to the aluminium smelters, with fluoride levels above 2000 ppm (Vikøren & Stuve, 1996). Furthermore, dental lesions were examined in cattle residing at Cornwall Island, Canada, where an an aluminium smelter is located on the south bank of St. Lawrence River, U.S.A. Brown discolouration, mottling, excessive wear of the tooth and bulging of the gingiva were symptoms observed in a large proportion of the group. Delays in development of the incisor permanent teeth were noted as well. Mandible fluoride concentrations ranged from 319 ppm in a 4-month-old calf, to 4733 ppm in a 3-year-old cow (Krook & Maylin, 1979).

Reports of dental fluorosis have generally been associated with volcanic eruptions in Iceland. The earliest report dates back to 1694, (probably due to volcanic eruptions in Hekla and Katla, 1693), where dental fluorosis was described in young sheep, cattle and horses (Georgsson, Pétursson & Pálsson, 1981). Following the Hekla eruption in 1970, young sheep in South and North Iceland were examined for dental fluorosis. Teeth erupting few months post fluoride exposure, were usually damaged, showing excessive wear and discolouration (Georgsson & Pétursson, 1972). Likewise, dental fluorosis was observed in young horses from both the North and South regions, three years subsequent to the 1970 Hekla eruption. Dental lesions were exclusively found in horses which were either one or two years old at the time of the eruption (Georgsson, Pétursson & Pálsson, 1981).

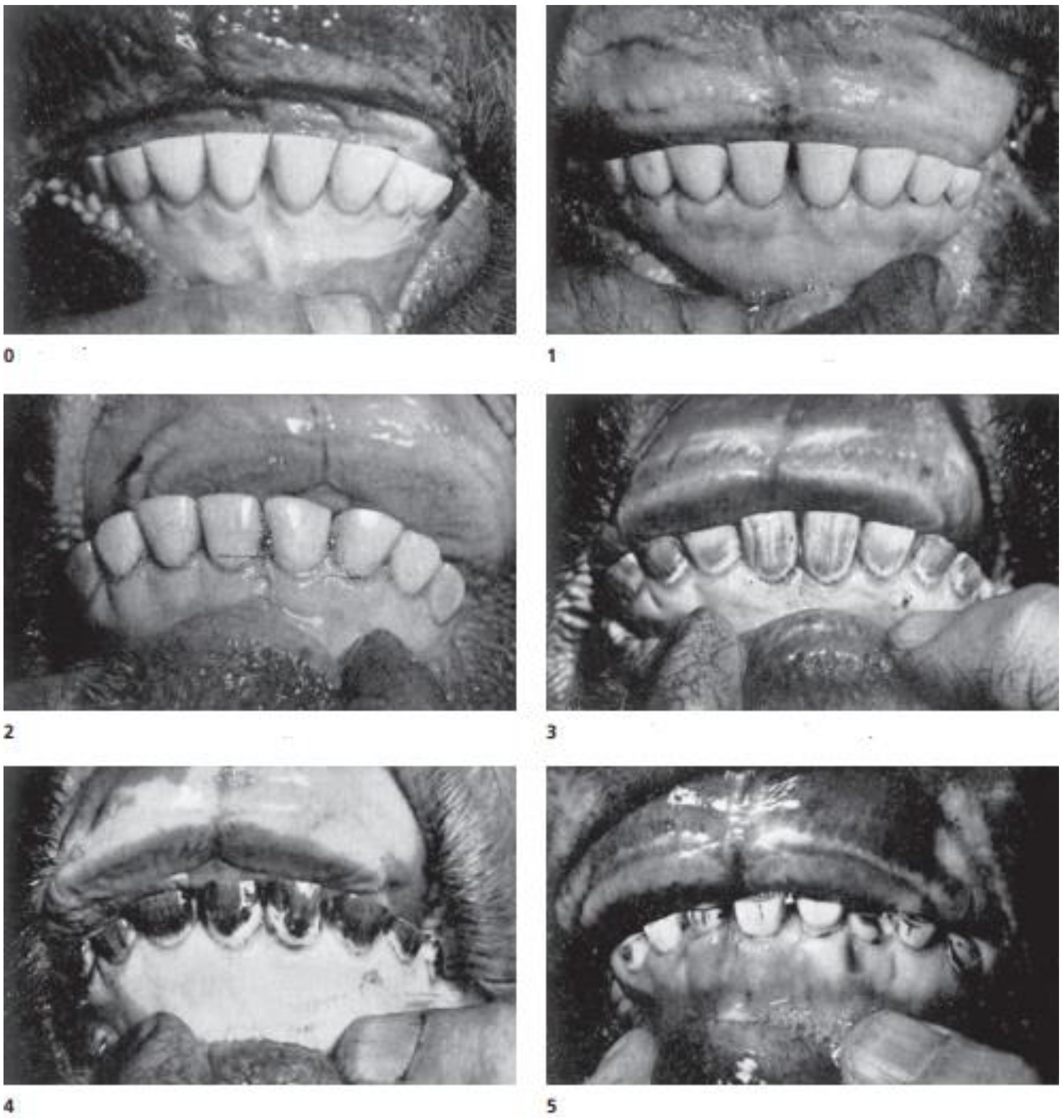

***Figure 3**. Scoring system of cattle incisor lesions related to fluoride toxicity. Level 0 normal teeth. Level 1 slight occurrence of enamel opacities. Level 2 tooth crown is affected, slight stain. Level 3 flattening of tooth crown and severe stain. Level 4 abnormal tooth shape and definite stain. Level 5 excessive wear, some teeth may be fractured. Figure adapted from Livesey & Payne (2011).*

1.4.3.1 Possible benefits of fluoride in teeth

It is important to note that fluoride has been added to drinking water for caries control in people in several countries for more than half a century (Dean et al., 1956). Fluoride promotes the remineralization of white spot lesions, which are the early stages of dental damage. When the spot has been repaired by fluoride it has been shown to be more resistant to additional damage, than spots that have not been repaired by fluoride (Koulourides et al., 1980). However, in spite of the dental health effects that artificially fluoridated water has demonstrated, over the years there have been numerous harm-arising claims from people ingesting fluoridated water. These claims include allergic reactions, cancer and genetic disorders (Browne, Whelton & O'Mullane, 2005).

1.4.4 Skeletal fluorosis

When vertebrates are exposed to fluoride over a long period it alters the composition and crystalline state of bone mineral as mentioned before (Weinstein & Davison, 2004). Early stages of skeletal fluorosis start with pain in joints and bones, muscle weakness and chronic fatigue. Abnormal growth of bone and calcification of ligaments result in enlarged joints, including the spine vertebrae (Choubisa & Choubisa, 2016). Therefore, animals can suffer from stiffness, crippling and intermittent lameness, where forelimbs are usually more affected than hind limbs (Ranjan & Ranjan, 2015). In skeletal fluorosis bone lesions are variable and depend upon duration of fluoride exposure, age and sex of the individual, type of bone affected and hormonal response. The bone lesions include osteosclerosis (abnormal hardening of bone and an elevation in bone density), osteophytosis (presence of bony outgrowth), osteoporosis (decrease in bone tissue density), osteomalacia (softening of bone due to decreased content of calcium ions) and periosteal hyperostosis (increase in the size of the cartilage-capped protuberance at the end of the bone; Ranjan & Ranjan, 2015). Ultimately skeletal fluorosis can cause severe pain, which reduces food intake and productivity, causes weight loss and might lead to death (Livesey & Payne, 2011).

Bone is a dynamic tissue which is formed and resorbed constantly in response to altered serum calcium levels, changes in mechanical loading and in response to multiple endocrine and paracrine factors (Walkley et al., 2007). Shupe et al. (1992) proposed that bone formed during high fluoride exposure became mottled. The collagen, polysaccharide, lipid and protein of the osteoid develops abnormally and inhibits bone mineralization in undeveloped bones (Shupe et al., 1992). This is in context to a previous study by Shupe (1963) and explains why juveniles are more sensitive to high amounts of fluoride exposure than adults. A completely developed bone accumulates fluoride but shows no histological changes, until the fluoride content reaches a critical level. Then, the bone is resorbed by precocious osteoclastic activity. Consequently, the bone marrow cavity becomes enlarged and the remaining cortex is converted to porotic matter. Later, as a reinforcing mechanism, periosteal osteoblastic activity enlarges the bone and therefore permits continued function of mechanically inadequate bone, which in the end causes the fluoride related bone deformities (Shupe et al., 1992).

Skeletal fluorosis has been observed in animals around the world. In Dungapur district, a fluoride endemic area in India, Choubisa (2010) examined fourteen domestic horses and nine donkeys (*Equus africanus asinus*) where mean fluoride concentration in drinking water ranged from 1.4 to 3.3 ppm. Eleven horses and all the donkeys suffered from exostoses in bones, stiffness, and lameness. Furthermore, these animals were afflicted with colic and intermittent diarrhoea, dental lesions, and males showed decreased reproductive activity. However, fluoride concentrations in bones was not measured (Choubisa, 2010). Additionally, at a farm in Pagosa Springs, U.S.A., several horses began showing signs of fluoride toxicity after artificial fluoridation of water was introduced in 1985. Symptoms such as exostosis in the rib and back bones were seen. Dental lesions were also observed. Bone fluoride concentration was measured in cannon bone of three horses and one control horse, which had not been exposed to fluoride (Krook & Justus, 2006). In Australia, Australian eastern grey kangaroos (*Macropus giganteus*) residing near aluminium smelter in Portland, Victoria, showed clinical signs of lameness. To determine the cause of these symptoms, bone histopathology, microradiography, quantitative ultrasonography, radiography and multi-element analysis of bone ash samples was carried out. Dental lesions such as abnormal teeth wear and incisor enamel hypoplasia were observed, as well as various skeletal lesions in distal tibia and fibula, femur, tarsal bones, metatarsus IV and proximal coccygeal vertebrae. Elevated fluoride levels in bones were detected (Clarke, 2003).

Following volcanic eruptions in Iceland, the emphasis has been on observing dental fluorosis in animals, and information of skeletal fluorosis are therefore scarce. However, when Georgsson, Pétursson and Pálsson (1981) estimated fluorosis in sheep after the 1970 Hekla eruption, radiographic examination showed slight thickening of periosteum in leg bones, in a small portion of the group. Fluoride concentrations in these animals was six to eightfold what was considered a normal value (Georgsson, Pétursson & Pálsson, 1981). Skeletal fluorosis has never been reported in horses in Iceland.

In 2011, three adult horses that were suspected to suffer from fluorosis, were subject to postmortem examination. The horses had been living at a farm in Hvalfjörður, West Iceland, where an aluminium smelter is located. Clinical signs included stiff gait and lameness as well as thickening of the neck. Radiography of the legs did, however, not reveal any signs of skeletal fluorosis, while laminitis was confirmed. Dental fluorosis was excluded by macroscopic examination. The average concentration of fluoride in the mandible of the horses was 886 ppm (Sigurðardóttir & Björnsdóttir, 2011a; Sigurðardóttir & Björnsdóttir, 2011b). Similar figures for mandible fluoride concentration were reported in 12 horses from the same farm in 2016, 811 ppm at average (Sigurðarson & Kristinsson, 2016).

1.4.4.1 Possible benefits of fluoride in bone

Fluoride exerts a biphasic action at the level of osteoblasts: on bone mineral and on bone structure and function. When the fluoride concentrations are low, skeletal uptake is limited and the effects can be beneficial. In isolated preparations of osteoblast-like cells in culture, fluoride has been shown to cause cellular proliferation, enhanced collagen synthesis and increased alkaline phosphatase activity (Farley, Wergedal & Baylink, 1983). Turner et al. (1989) showed that rats treated with fluoride increased trabecular and cortical bone mass in therapeutic doses but reduced trabecular bone mass at toxic doses. The increase in the volume of bone at low fluoride doses resulted from stimulated periosteal bone formation and apposition rates (Turner et al., 1989). Furthermore, fluoride was found to exert a dose-dependent effect on remodelling of bone (Chavassieux et al., 1991).

1.4.5 Soft tissue toxicity

Other, non-skeletal symptoms can also appear as a result of chronic fluorosis as mentioned before. Excessive thirst, nasal irritation, asthma and irregular reproductive cycles with repeated abortions and stillbirths are also seen (Choubisa & Choubisa, 2016; Choubisa et al., 2012). Fluoride alters mechanisms at a cellular and molecular level (Zuo et al., 2018). Metabolic, structural and functional damage caused by chronic fluorosis has been reported in many tissues as a result of fluoride interaction with enzymes. Fluoride can act as an enzyme inhibitor at millimolar level, yet occasionally stimulates enzyme activity at micromolar level. The toxic mechanisms depend on the type of enzyme in question (Adamek, Pawłowska-Góral & Bober, 2005; Mendoza-Schulz et al., 2009). Few studies have associated fluoride exposure to increased oxygen generation resulting in organ- and tissue-specific illnesses such as diabetes, heart, liver and neurodegenerative diseases (Suzuki et al., 2014; Mailoux & Harper, 2011). The liver, heart and kidney are the most susceptible soft tissues to fluoride toxicity. When exposed to continuous doses of fluoride, all three organs exhibit histopathological and functional changes (Ranjan & Ranjan, 2015).

Furthermore, excessive fluoride concentrations in the body have been shown to penetrate the blood-brain barrier and may therefore be significantly harmful for the mammalian nervous system (Zuo et al., 2018). A study by Shivarajashankara et al. (2002) revealed that high amounts of fluoride lead to brain-specific metabolic disorders in rats due to increased lipid peroxidation and inhibition of important natural enzymes.

Even though the symptoms described above have not all been reported in herbivores, the studies demonstrate the ability of fluoride to interact with multiple cellular processes and the importance that all these mechanisms function in the right manner. Therefore, it is vital to highlight the possible effects of fluoride toxicity in all circumstances.

1.5 Fluoride tolerance and guidelines for animals

Tolerance for fluoride exposure varies between species, individual resistance, diet and environmental factors. For instance, it appears that ruminants are more susceptible to fluoride accumulation than monogastric herbivores, such as horses or swine (Ranjan & Ranjan, 2015). It has been suggested to be related to the different pH levels in the stomach of ruminants and monogastric animals (McDonald et al., 2002), or a negative calcium balance in cattle (Ranjan & Ranjan, 2015).

1.5.1 Water quality standards

Since fluoride is added to drinking water in several countries for caries control (Dean et al., 1956), the optimal fluoride concentration in drinking water has been determined to be in the range between 0.7-1.2 mg/l. However, it has been recommended that the maximum fluoride level in water does not exceed 1.5 mg/l when organisms are exposed for a long period of time (WHO, 1984; WHO, 2002). According to Regulation (536/2001) on drinking water in Iceland, by the Ministry for the Environment and Natural Resources, the maximum level of fluoride in drinking water is not to exceed 1.5 mg/l, in accordance to that of the World Health Organization.

1.5.2 Air quality standards

Air quality standards are designed to protect the most sensitive organisms and will therefore automatically protect the less sensitive receptors. Gaseous atmospheric levels of fluoride should not exceed 0.82 $\mu g/m^3$ on a monthly average, protecting sensitive receptors, such as plants (Weinstein & Davison, 2004). Maximum fluoride levels in smelter buffer zones in Iceland are set to 0.3 $\mu g/m^3$ of hydrogen fluoride, as an average for the period between April and September each year (Regulation on air quality no. 787/1999).

1.5.3 Forage quality standards

The tolerance level of fluoride content in forage or feed for the most sensitive herbivores is 40 mg/kg dry weight over the period of twelve months. For more tolerant animals the tolerance level is modified to 30-35 mg/kg dry weight (Weinstein & Davison, 2004). In

Iceland, the maximum tolerance level of fluoride in forage for herbivores is set to 30 µg F/g according to the Icelandic Regulation (340/2001) on fodder supervision.

1.5.4 Tolerance level in animals

Guideline limits for horses do not exist. Therefore, a standard has been adapted based on a study by Vikøren and Stuve (1996) and their research on Norwegian red deer. Red deer are in risk of developing dental lesions when fluoride concentration in mandibles are between 1000-2000 µg/g (ppm). When the concentration exceeds 2000 ppm F it causes dental fluorosis and skeletal lesions are likely to develop. This standard has been adapted as a maximum level of fluoride concentration in bones of other herbivores (Yngvadóttir et al., 2017; Vikøren & Stuve, 1996).

1.6 Objective

The main objective of the present study is to provide background levels of fluoride concentration in general. Further, to estimate whether fluoride accumulation in bone tissue is dependent on age and geographical regions in Iceland, where specific sources of fluoride would be expected.

2 Materials and methods

2.1 Sampling method

Mandibles from 223 cadavers of Icelandic horses were sampled at slaughterhouses over the period of two years to conduct a chemical analysis of fluoride content. The samples were obtained from four regions of Iceland (South, West, North and East) with varying potential sources of fluoride, both natural and industrial. The samples were collected from four Icelandic slaughterhouses (Table 2, Figure 4). Two are located in North Iceland, Sauðárkrókur (KS) and Blönduós (SAH), and two in South Iceland, at Selfoss (SS) and Höfn (HH). The aim was to collect samples from the four regions, as evenly distributed between them as possible. Additionally, samples within each region were divided into five age groups; foals, juveniles aged 1-4 years, 5-12-year-old horses, 13-20-year-olds and lastly, horses aged 21-years and older.

All horses in Iceland are tracked by the International Federation of Icelandic Horse Associations (FEIF) and registered in the studbook *Worldfengur*. The FEIF provides them with an identification number, which determines their gender, age and origin. This studbook makes tracking of each horse possible. If there were any doubts concerning origin or where they resided for the greatest part of their life, the owner, or previous owner, was contacted.

Table 2. *Dates and locations of mandible sampling, as well as number of samples per date.*

Date (dd/mm/yy)	Slaughterhouse	No. Samples
23/10/17	SAH ehf. Blönduós	21
22/11/17	SS svf. Selfoss	26
05/12/17	Norðlenska ehf. Höfn	8
22/02/18	SS svf. Selfoss	19
17/05/18	SS svf. Selfoss	23
31/05/18	SS svf. Selfoss	3
21/09/18	SS svf. Selfoss	17
26/11/18	KS svf. Sauðárkrókur	38
10/12/18	KS svf. Sauðárkrókur	42
06/02/19	SS svf. Selfoss	8
11/02/19	KS svf. Sauðárkrókur	18
Total		223

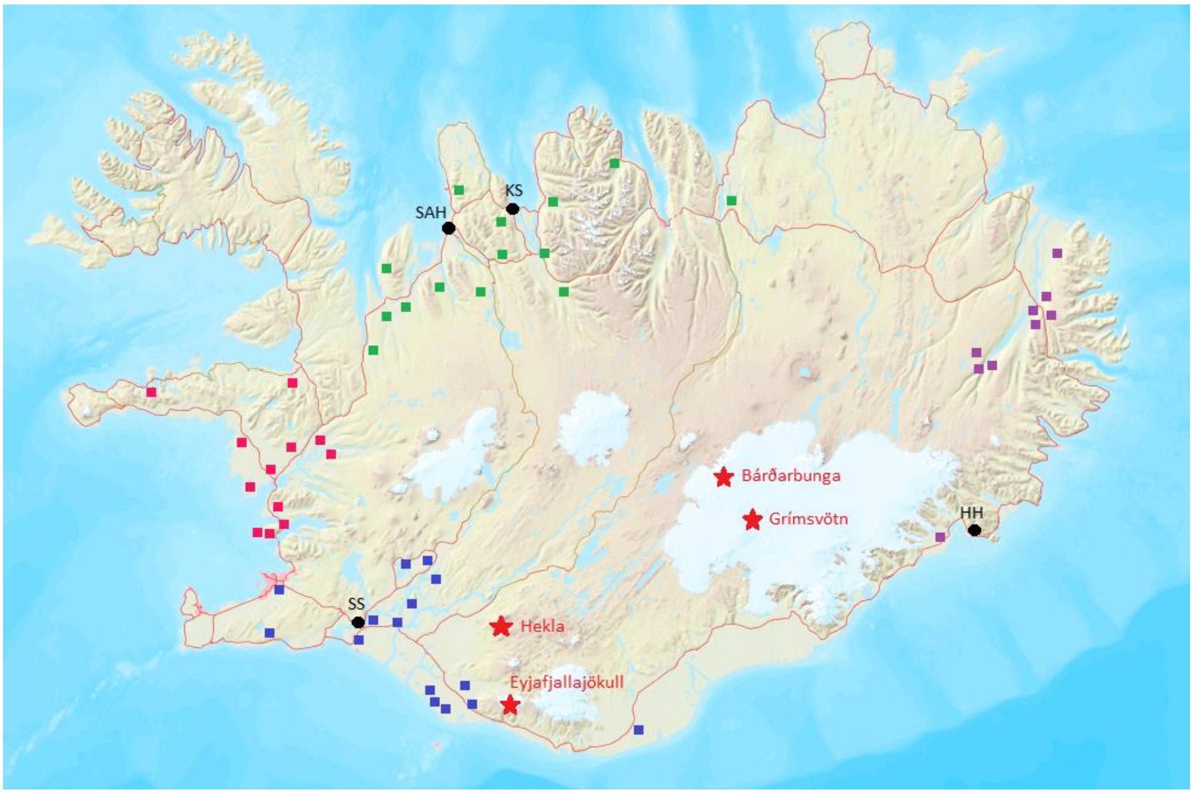

Figure 4. *Origin of horses sampled and number of samples from each region. Blue boxes represent horses from the South (52), pink boxes represent horses from the West (71), green boxes represent horses from the North (63) and purple boxes represent horses from the East (37). Each location box could represent more than one sample. The black circles stand for the slaughterhouse locations, SS in Selfoss, SAH in Blönduós, KS in Sauðárkrókur and HH in Höfn. The red stars represent the volcanoes that have erupted in Iceland since 1991. Figure adapted from Landmælingar Íslands, retrieved and modified May 14, 2020, from http://kortasja.lmi.is/.*

2.2 Mandible preparation

Heads of the slaughtered horses were collected in the slaughterhouse. The front part of the lower jaw was skinned, then sawn apart (Fig. 5) in the interdental space with a *Black&Decker* oscillating multi-tool. Each jaw sample was marked with the sampling date and given a serial number and then kept frozen in plastic storage pockets. Specific slices fit for chemical analysis were later cut out of the frozen mandibles with a *Hitachi* reciprocating saw (Fig. 5).

2.3 Chemical analysis

Fluoride analysis was carried out on the slices at the Innovation Center of Iceland (*Nýsköpunarmiðstöð Íslands*). Each bone sample was incinerated at 600°C and teeth, if any, were removed. The bone tissue was then pulverized and fully dissolved in acid (HNO_3). The solution was mixed with TISAB buffer and fluoride then quantitatively analyzed with a fluoride specific electrode. Therefore, a linear scale could be measured corresponding to the bone samples. A linear calibration curve was obtained using dilutions of Merck fluoride standard (1000 ppm, no. 1.19814.0500) in a background matched matrix. The bone sample quantification was calculated using that calibration. Blank and in-house reference material were measured once per group of 10 samples, and a repetition was made for one sample of each ten. The blank samples were used for extracting detection limit and measurement error. Fluoride concentration was given as a mass ratio of dry matter (parts per million, ppm).

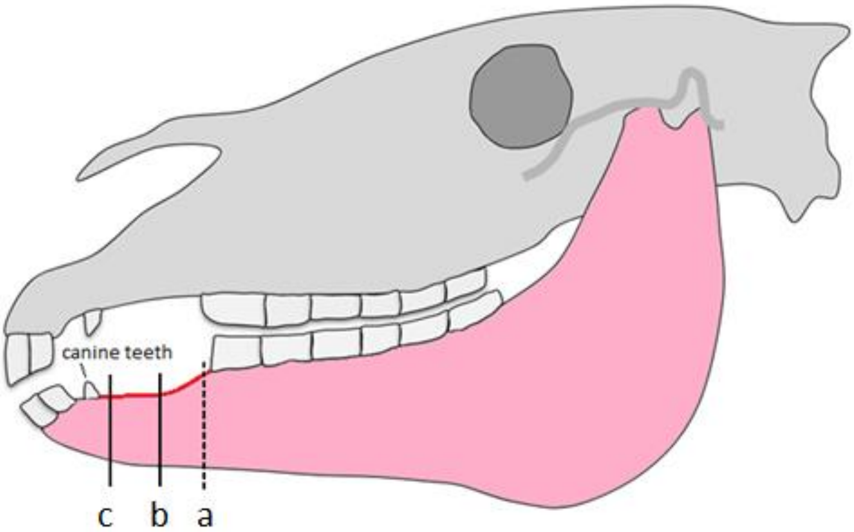

Figure 5. *The horse mandible is represented with the colour pink and the red line indicates the interdental space. The broken line a) represents the cut separating the sample from the jaw. Lines b) and c) stand for the cuts forming the two cm slice. Figure adapted from JayciParke, retrieved and modified May 14, 2020, from https://en.wikipedia.org/wiki/Equine_malocclusion*

2.4 Statistical analysis

Statistical analysis was made using *JMP 14* software. A two-way analysis of variance (*ANOVA*) was used to compare the fluoride values between the five different age groups, the four geographical regions and between three groups of horses from the West with various distance from the aluminium smelter in Hvalfjörður, followed by Tukey Test to estimate the difference between each group at a 95% confidence level. Correlation test between age and fluoride concentration of each sample was constructed using a bivariate correlation with a linear fit. Additionally, correlation test of fluoride concentration and age was carried out for horses from each region. Furthermore, correlation test of fluoride concentration in horses from the West and their location as a distance from the aluminium smelter in Hvalfjörður was carried out as well. The mean fluoride concentration of all samples and mean for each region and age group was calculated using *Excel* software.

3 Results

3.1 Distribution of samples

Distribution of sample numbers between regions and age groups is shown in Figure 6. Fewer samples were obtained in some regions than others, with some age groups less represented as well. No samples were collected for the 1-4-year-old (y.o.) group in the East, and samples from this age group were fewest from all the regions, especially the North. Additionally, the 13-20 y.o. group was small in the South compared to the other regions, as well as few samples from foals in the East.

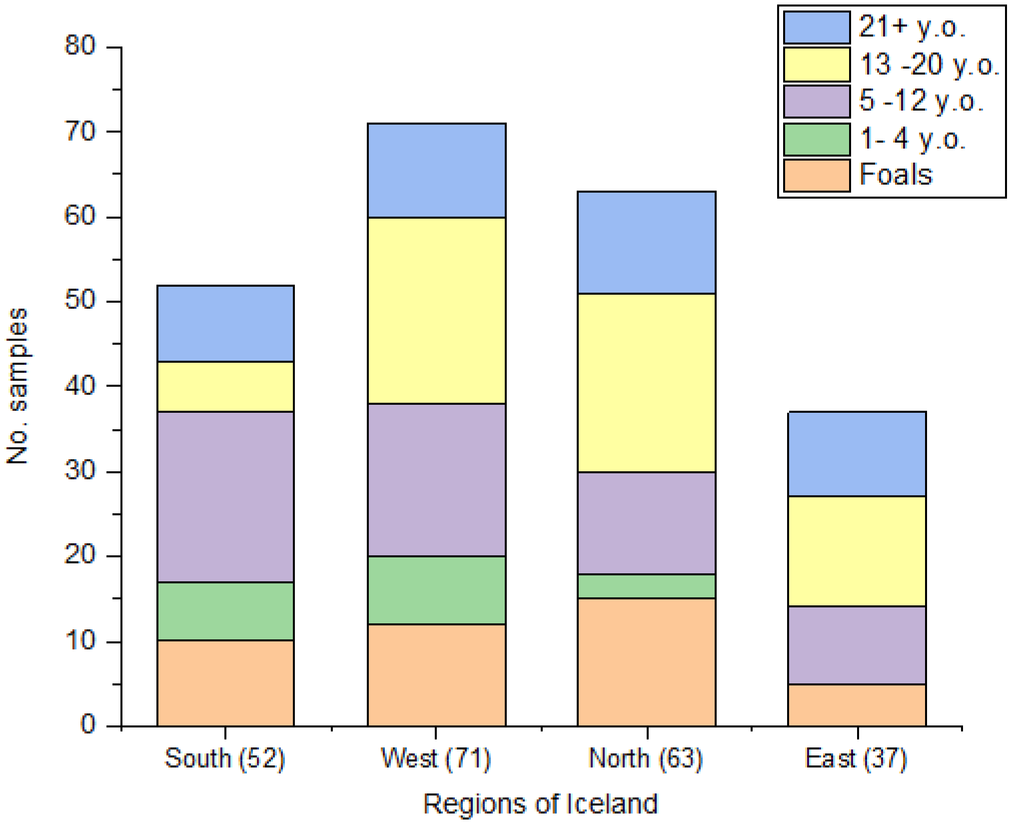

Figure 6. *Distribution of samples defined by age groups for each region.*

3.2 Fluoride analysis

Mandibular bone fluoride values in the 223 samples ranged from the lowest value of 71 ppm F in a two-year-old juvenile, to the highest value of 1583 ppm F in a 25-year-old mare. For foals, fluoride concentrations ranged from 25 ppm F to 224 ppm F. The horses and foals with both the lowest and highest values were all from the West, which indicates the widest range of fluoride concentration in that area. Three horses had fluoride values higher than 1000 ppm F, the level where Norwegian deer were shown to be at risk of developing dental lesions (Vikøren & Stuve, 1996). This included the previously mentioned 25-year-old mare from the West and two other horses from the same region, a 20-year-old gelding with fluoride concentration of 1192 ppm, and a 14-year-old mare with 1082 ppm F.

Average fluoride concentration for all the samples collected was 244 ± 11.8 ppm (Table 3). When results are broken down to each region the highest average values were reached in samples from the West, and the lowest values were found in the South. This is also true for average fluoride concentration between regions in all horses over one year old. For foals exclusively, the highest average concentration is in the West as well. However, the lowest average concentration for foals is in the North.

Table 3. *Highest fluoride concentration of all regions (ppm F), along with average fluoride concentration and standard deviation of all samples, samples excluding foals, and only foals divided by regions. Number of samples for each group are in brackets (no.)*

	South (no.)	**West (no.)**	**North (no.)**	**East (no.)**	**All regions (no.)**
Highest concentration	377	1583	563	464	1583
Average concentration all ages	182 ± 12 (52)	295 ± 30 (71)	234 ± 17 (63)	248 ± 16 (37)	244 ± 11 (223)
Average concentration excluding foals	226 ± 12 (42)	336 ± 34 (59)	294 ± 15 (48)	270 ± 9 (32)	286 ± 12 (181)
Average concentration of foals	47 ± 2,2 (10)	97 ± 14 (12)	43 ± 3,4 (15)	49 ± 2,1 (5)	60 ± 5,5 (42)

When fluoride concentration is broken down to the five age groups, unrelated to region distribution (Table 4), the lowest average concentration is in the foals, followed by the juveniles (1-4 y.o.). The highest average values are in the oldest age group (21+ y.o.).

Table 4. *Highest fluoride concentration of all age groups (ppm F), along with average fluoride concentration and standard deviation of all age groups. Number of samples for each group are in the brackets (no.)*

	Foals (no.)	1-4 y.o. (no.)	5-12 y.o. (no.)	13-20 y.o. (no)	21+ y.o. (no)
Highest concentration	224	304	812	1192	1583
Average concentration	60 ± 5 (42)	164 ± 16 (18)	247 ± 15 (59)	334 ± 23 (62)	357 ± 32 (42)

3.3 Fluoride concentration by age

A correlation test was carried out for fluoride concentration and the age of each horse (Fig. 7). The results demonstrate a significant positive regression between increasing fluoride concentration and rising age of the horses ($P < 0.0001$, $r^2 = 0.268$).

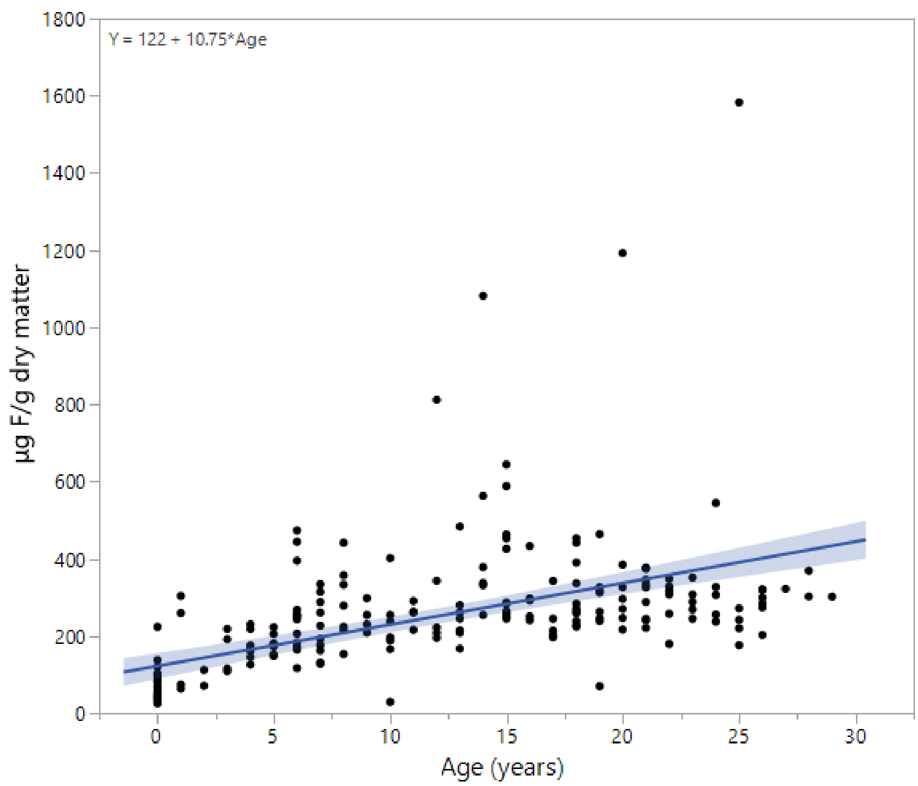

Figure 7. *Regression of fluoride concentration and age of each horses. The regression slope is presented in the top left corner.*

As figure 7 shows, four samples have fluoride concentrations higher than 800 ppm and influence the regression slope for the whole group. Therefore, a correlation test was further carried out for fluoride concentration and age of each horse, excluding the four samples

(Fig 8). A significant positive regression was found between increasing fluoride concentration and rising age of each horse ($P < 0.0001$, $r^2 = 0.413$), with a slightly higher correlation coefficient when the four samples were excluded.

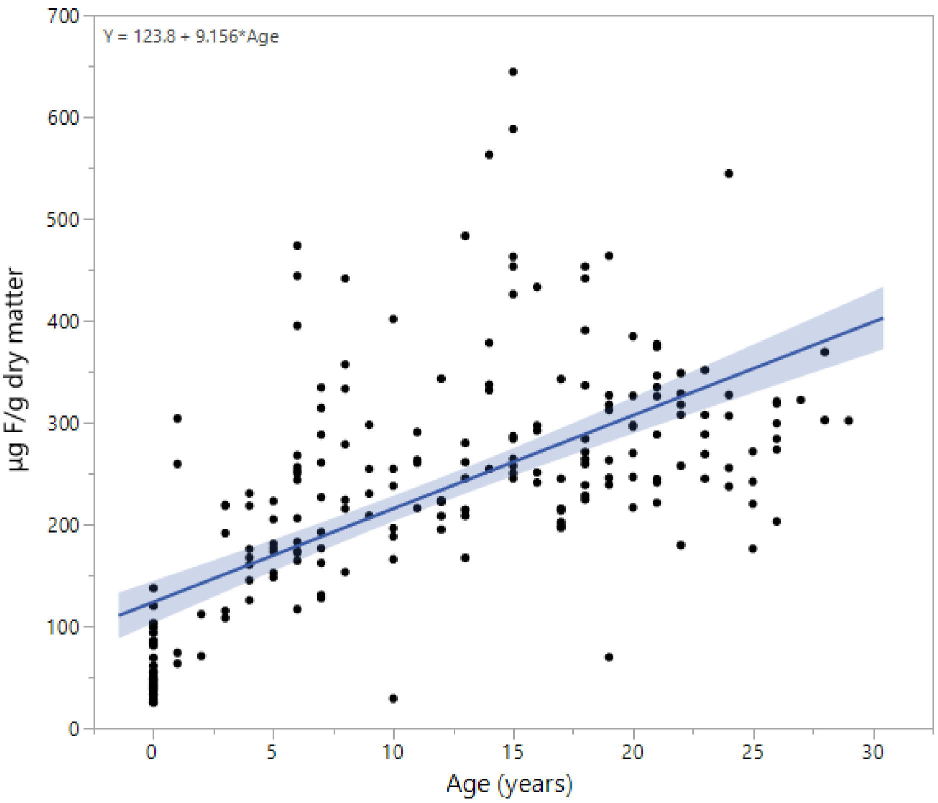

***Figure 8**. Regression of fluoride concentration and age of each horse excluding the four highest samples. The regression slope is presented in the top left corner.*

Furthermore, a correlation test for age and fluoride concentration was carried out for each region (Fig. 9). In all regions, a significant positive regression was found between age and fluoride concentration ($P < 0.0001$). The highest correlation coefficient was found in the South ($r^2 = 0.675$), followed by the East ($r^2 = 0.449$) and the North ($r^2 = 0.404$). The lowest coefficient was found in the West ($r^2 = 0.201$). As figure 11 shows, the four highest values observed in figure 7, are all from the West. Therefore, a correlation test was further carried out for fluoride concentration and the age of each horse, excluding the four highest values (Fig. 10). Evidently, the coefficient did only change for the West when the four values were removed ($r^2 = 0.288$).

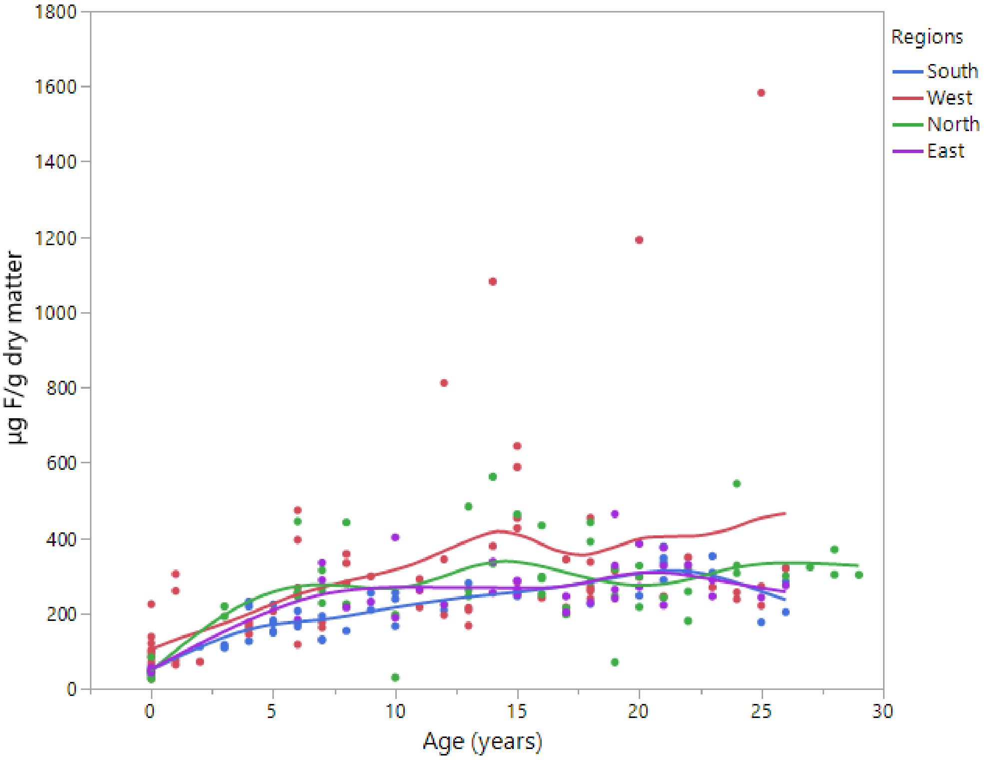

Figure 9. *Regression of fluoride concentration and age of each horse divided by the four regions.*

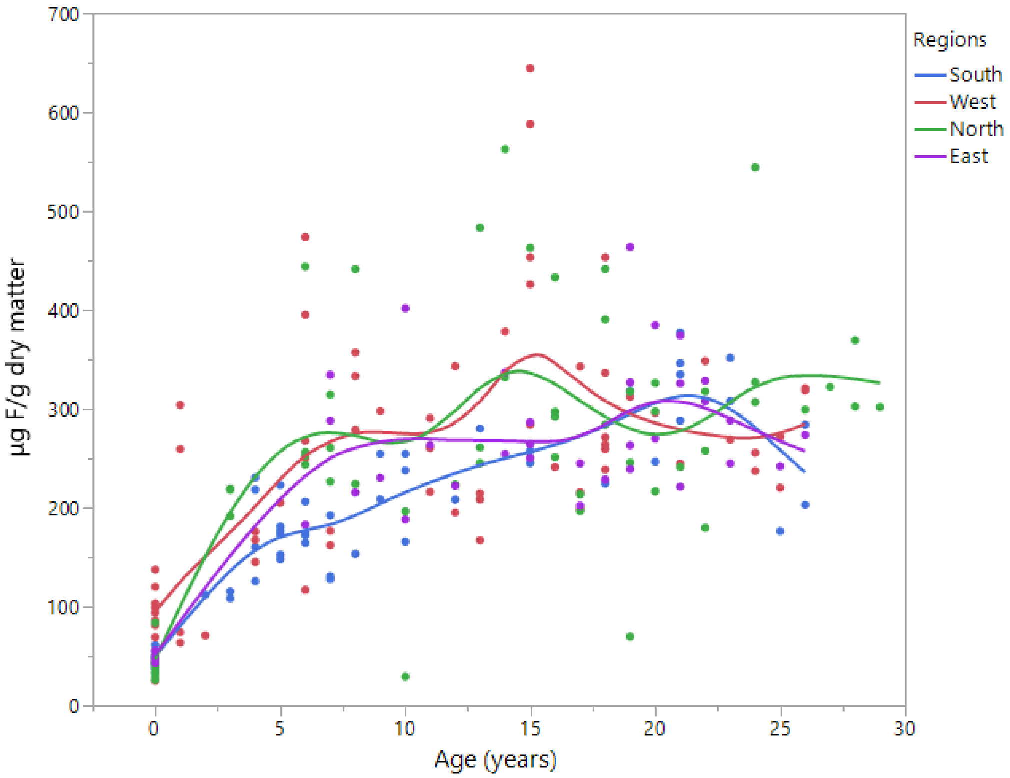

Figure 10. *Regression of fluoride concentration and age of each horse divided by the four regions excluding the four highest samples.*

3.4 Fluoride concentration by regions

A One-way analysis was carried out on the samples to determine the difference of fluoride concentration in horses between regions. For foals exclusively, the One-way analysis revealed a significant difference between the regions ($P < 0.0001$). All pair Tukey HSD determined that foals from the West had significantly higher fluoride concentration than foals from the three other regions ($P < 0.0001$; Fig. 11b). A statistical difference was not found between foals from the South, North and East. For horses aged one year and older, the One-way analysis revealed similar results, a significant difference ($P = 0.0095$; Fig. 11a). All pair Tukey HSD however determined that fluoride concentration in the West was solely significantly different from fluoride concentration in the South ($P = 0.0049$), and not statistically different between the other regions.

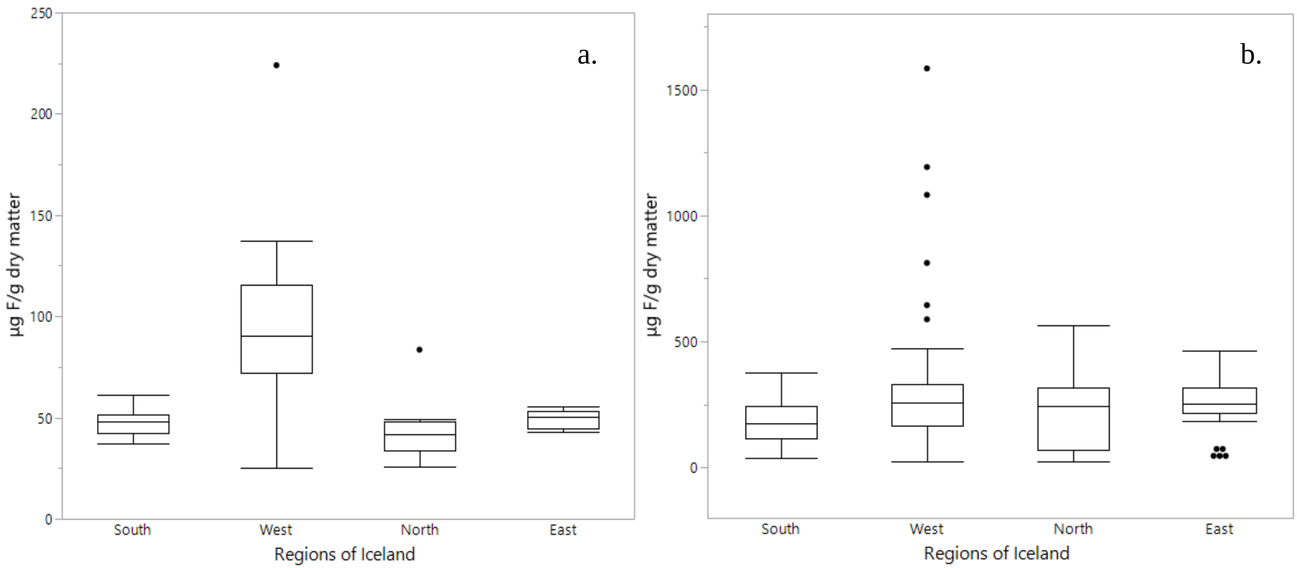

Figure 11. *Fluoride concentration between regions explained by boxplots in a) only foals and b) all age groups excluding foals. Dots are outliers, whiskers are highest and lowest values (excluding outliers), the box represents upper and lower quartile, and the middle line is the median. Note that the scale of the two figures is not the same, as the foals have lower values.*

3.4.1 Fluoride concentration in the West region

As was established in chapter 3.3, the four horses with the highest fluoride concentration were all from the West. Therefore, an attempt was made to determine whether the high values could be connected to the aluminium industry located in the region, in Hvalfjörður.

A correlation test was carried out for fluoride concentration and the origin of each horse from the West, as a distance from the aluminium smelter measured in kilometres (Fig. 12a). Foals were excluded from the correlation test. A significant negative regression between fluoride concentration and increasing distance from the aluminium smelter was demonstrated ($P = 0.001$, $r^2 = 0.17$). Furthermore, a correlation test was carried out on samples from the West excluding the four highest values. The results similarly demonstrate a significant negative regression between fluoride concentration and increasing distance from the aluminium smelter, but weaker ($P = 0.042$, $r^2 = 0.14$; Fig. 12b).

Furthermore, adult horses from the West were divided to groups according to their distance from the aluminium smelter; group one represents horses located within 30 km from the smelter (n = 20); group two represents horses located between 31 and 60 km from the smelter (n = 31); group three represents horses located 61 km from the smelter and further (n = 8). When fluoride concentration in the three groups was compared using One-way analysis, a significant difference between the groups was demonstrated ($P = 0.0011$). All pair Tukey HSD determined that fluoride concentration in group one was significantly higher than in group two ($P = 0.0017$) and group three ($P = 0.0162$). A statistical difference was not found between group two and three. When the three groups were compared excluding the four highest values, a significant difference was observed, but weak ($P = 0.021$).

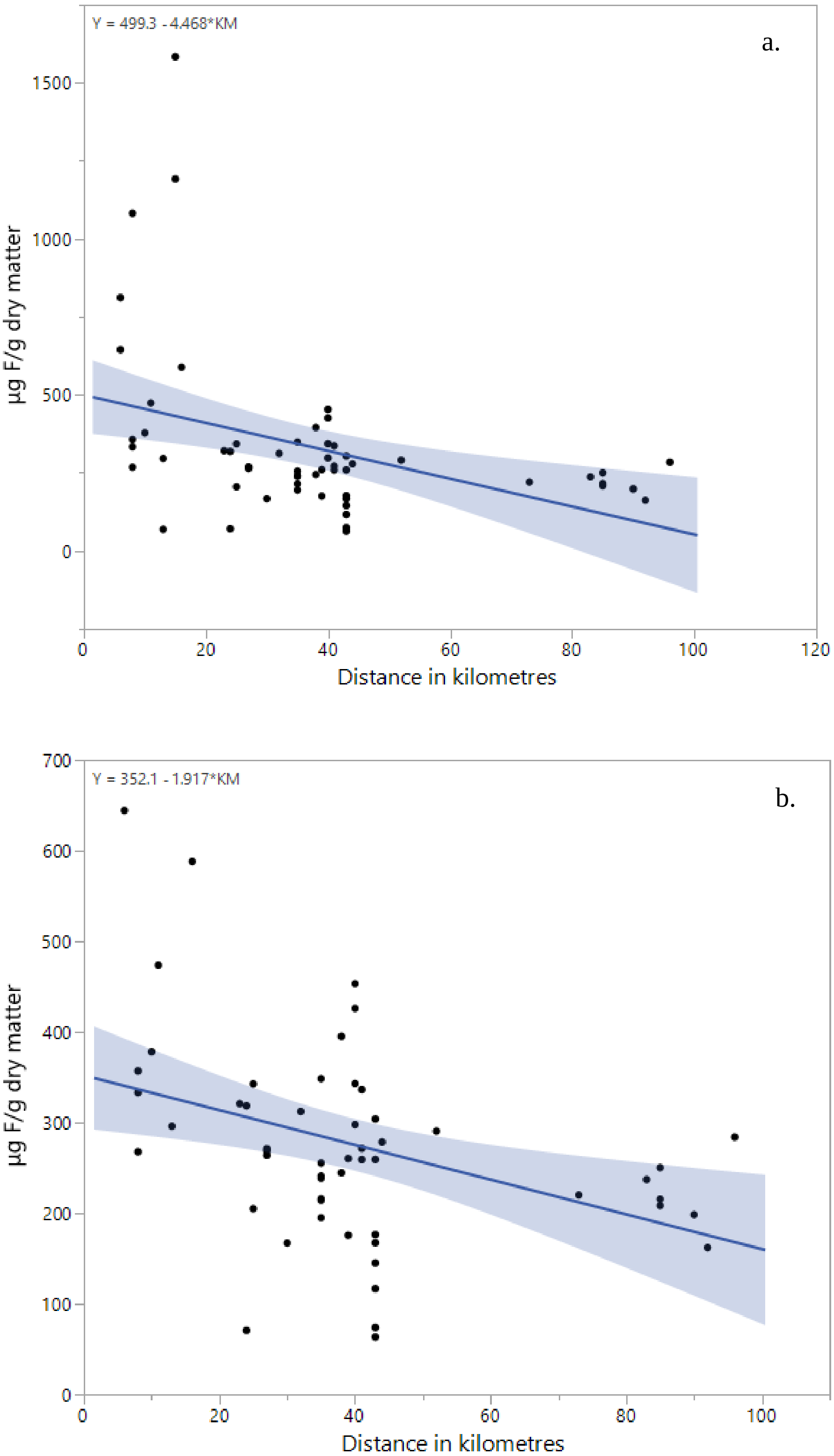

Figure 12. *Regression of fluoride concentration and location of each horse in the West as a distance from the aluminium smelter in Hvalfjörður a) the whole group aged one year and older and b) the whole group aged one year and older excluding the four highest samples. The regression slope is presented in the top left corner. Note that the scale of the two figures is different due to the missing outliers on the latter one.*

4 Discussion

The results demonstrated in the study are the first of their kind, where fluoride concentrations in the Icelandic horse are studied in a range of horses regarding age and origin in Iceland. Therefore, an important objective in the study was to obtain the samples as evenly distributed by age and regions as possible. Since horse slaughterhouses in Iceland are few and far between, acquiring samples in some age groups from the East was difficult, and it was impossible to get mandible samples from the Westfjords, a region not included in the study because of this issue. However, horses in Iceland are unequally divided by regions and when looking into horse numbers of each region, independent of age by the Statistics of Iceland (*Hagstofa Íslands*), the greatest numbers are in the South (28,086) and North regions (23,718). Fewer horses are in the West (9582), East (2698) and Westfjords (770). Given these numbers, the study represents a sufficient percentage of horses from all regions, except in the South, and Westfjords, as Figure 6 shows. Although every effort was made to equalize the age distribution in samples from each region, the specified time was insufficient to follow it through.

Compared to previous studies on fluoride concentrations in both horses and other species, the average values in the study are low (244 ppm; 286 ppm excluding foals), considerably lower than results from the few studies done on a small number of horses previously; 345 ppm (Pálsson, 1995) and 503 ppm (Sigurðarson & Kristinsson, 2016). All values were under 2000 ppm, the level associated with increased risk of pathological changes to teeth due to environmental fluoride (Vikøren & Stuve, 1996). The highest fluoride concentration (1583 ppm) was greater than the highest values previously measured in Icelandic horse bones; 926 ppm F (Sigurðardóttir & Björnsdóttir, 2011b), and 1070 ppm F (Sigurðarson & Kristinsson, 2016). Unusually low values were measured in two adult horses from the North, 29 ppm F in a 10-year-old gelding and 70 ppm F in a 19-year-old gelding. These values correspond to the values of foals in the study, born in the summer and slaughtered in the winter (Appendix II). The highest fluoride concentration found in foals was 224 ppm, in a foal born in the West region, which corresponds to the average fluoride concentration in the whole sample group. Moreover, the highest values in horses from each of the five age groups are in horses from the West, indicating a higher environmental fluoride burden in this region.

Although little has been known until now regarding fluoride accumulation in horses, considerable information has been gathered on sheep, as monitoring has been conducted in relation to aluminium smelters intermittently from 1969 (Pálsson, 1995). Average fluoride concentration in adult sheep, from various locations around Iceland, was 1150 ppm, before aluminium production had started in Iceland, with fluoride concentration in lambs being 180-200 ppm (Pálsson, 1995). These values are substantially higher than values in horses from the present study, even though aluminium production did not exist in Iceland at the time. These values are indicative of the environmental levels of fluoride at that time, indicating that without an increased environmental fluoride burden, sheep accumulate fluoride more readily than horses, most likely due to less fluoride being absorbed in the gastrointestinal tract of horses. When fluoride monitoring around the aluminium smelter in Straumsvík (South-West Iceland) started, concentrations in lamb mandibles ranged from 650 ppm to 2140 ppm, and from 3500 ppm up to 9000 ppm F in adult sheep from that area. No signs of bone deformities were visible in sheep with the highest fluoride concentrations. However, dental fluorosis and molar deformities were evident in the sheep grazing close to the smelter. Control samples were taken from sheep in Lundarreykjadalur in West Iceland, with the average of 1000-1500 ppm in jawbone ash (Pálsson, 1995). In Reyðarfjörður, East Iceland, environmental monitoring since the establishment of an aluminium smelter has shown an increase in average fluoride concentration in both lambs and adult sheep (Jóhannsdóttir, Flosadóttir & Þórðarson, 2014). Despite greatly increased concentrations of fluoride and an average in adult sheep over 2000 ppm, no animals showed signs of fluorosis. In some adult sheep dental cavities were observed, which were not explained by fluoride concentration (Guðmundsdóttir et al., 2019).

Studies have been conducted on fluoride concentration in other species. Shupe et al. (1992) studied fluoride concentrations in 200 cattle from the U.S.A., which had either been exposed to fluoride over a prolonged period or been treated with sodium fluoride for few years. The cattle were divided into three groups by the strength of fluoride treatment dose or strength of fluoride in forage they ingested. The average bone fluoride concentration in cattle from the high exposure group was 4296 ppm (n = 87); 2431 ppm in the intermediate group (n = 87) and 1004 in the low exposure group (n = 26). The highest concentration was 12,500 ppm F in an animal from the high exposure group. Dental fluorosis was observed in all the groups, most severe in the high exposure group, but minimal in the low exposure group. More than half of the cattle in the high exposure group showed bone lesions such as osteosclerosis, periosteal hyperostosis and exostoses (Shupe et al., 1992). Death et al. (2015) examined fluoride concentrations in six marsupial species residing near an aluminium smelter in Portland, Australia, and a control group from a low-fluoride area. The average fluoride concentration in all species from the high-fluoride area was 3200 ppm and 196 ppm in the low-fluoride area. Occurrence of dental fluorosis was positively correlated with increasing bone fluoride levels in all species from the high-fluoride area (Death et al., 2015). Similarly, Hufschmid et al. (2015) reported that Eastern grey kangaroo (*M. giganteus*), which resided near the same aluminium smelter in Portland, suffered from exostoses in long bones when fluoride concentration in bones exceeded 3000 ppm. Vikøren and Stuve (1996) found that fluoride levels in mandibles of roe deer (*Capreolus capreolus*) and red deer (*C. elaphus*) exceeding 8500 ppm F were correlated with gross osteofluorotic lesions, whereas Newman and Murphy (1979) reported that black-tailed deer (*Odocoileus hemionus*) showed thickening in bones and chalky white and roughened periosteal surfaces, with metatarsal fluoride concentrations exceeding only 2000 ppm F (Vikøren & Stuve, 1996).

As previously stated, only four horses in the present study showed values which would be considered abnormally high compared to the sample group. All four horses were from the West region, living relatively close to the aluminium smelter in Hvalfjörður. It is possible that these four horses were exposed to temporary spike levels of fluoride emissions from the aluminium smelter when they were still growing, since uptake of fluoride in bones is the greatest during the growth phase. Icelandic horses have been shown to increase their height until they reach five years of age (Strand et al., 2007). The twenty-five-year old mare with 1583 ppm F and the 20-year-old gelding with 1192 ppm F where potentially exposed to the same spike during their youth. Similarly, it could be hypothesized that the 14-year-old mare with 1082 ppm F and the 12-year-old gelding were exposed to another fluoride emission spike during their youth. Additionally, two horses from the West had quite high levels compared to the whole sample group, a 15-year-old gelding with 644 ppm F and another 15-year-old gelding with 588, which were probably exposed to the same spike. Even though the results indicate, that the closer to the aluminium smelter the horses in the West had lived, the higher their fluoride concentration was, the correlation was weak. Therefore it is more likely that the horses with the highest fluoride concentrations were exposed to high levels of fluoride for a short amount of time during their growth phase, as a result of isolated incidents of increased emission from the aluminium smelter production, on top of the continuous levels of fluoride exposure from the aluminium smelter during their lives. Furthermore, even though the highest values from the four horses are relatively high compared to the sample group presented in the study, the values are fairly low compared to high values observed in other species, as mentioned above (Vikøren & Stuve, 1996; Shupe et al., 1992; Death et al., 2015; Hufschmid et al., 2015).

The results of the present study therefore are in agreement with previous observations that ruminants, such as sheep and cattle, accumulate fluoride more readily in their bones when exposed to excessive amount of fluoride than monogastric animals, such as horses (Ranjan & Ranjan, 2015). Fundamental differences in the digestive system lead to differences in the metabolism of substances between them, and the different accumulation between the species could possibly be explained by the acidity of the digestive system of ruminants (McDonald et al., 2002), as lower stomach pH (high acidity) correlates with increased fluoride absorption (Weinstein & Davison, 2004). Corresponding to the two Australian journals above, Hume (1981) described a parallel between the ruminant and marsupial digestive systems, although the pH is lower in marsupials, therefore indicating that marsupial species are prone to fluoride accumulation as well. Studies in rats have shown that low stomach pH increased fluoride absorption, but that the smallest part of the absorption occurs in the stomach (Messer & Ophaug, 1993; Messer & Ophaug, 1991). It is possible that fluoride absorption takes place in the ruminant fore stomachs, thus removing considerable quantities of fluoride before the cud reaches the abomasum, an organ comparable to the equine stomach. Furthermore, the digesting period of cud in the gastrointestinal tract of ruminants is longer than digestion of feed in the equine stomach (McDonald et al., 2002). This could explain why the average fluoride concentration ruminants tends to be higher than in horses.

We have no reason other than to believe that once a certain level of fluoride accumulation is reached in calcified tissues, the pathological processes are the same in these different species. Therefore, it is likely that chronic pathological changes begin to manifest at similar fluoride concentration in all the species. However, no examination on either bone or teeth was executed on samples in this study, therefore information on chronic toxicity of the horses are absent. The sole available data on tooth and bone examination in the Icelandic horse is from the Sigurðardóttir and Björnsdóttir studies (2011a;2011b), where no tooth or bone deformities were diagnosed in the bones of horses from Hvalfjörður, consistent with the maximum values of 926 ppm F (Sigurðardóttir & Björnsdóttir, 2011b). Therefore, it could be estimated that none of the horses from the present study suffered from fluoride toxicity, even when the values exceeded 1000 ppm F.

Previous research on fluoride accumulation in animals demonstrate the fluoride concentration rises with increasing age of the animal (Kierdorf et al., 1995; Weatherell, Robinson & Hallsworth, 1972; Weinstein & Davison, 2004). This is in coordination with the results of this study. The correlation coefficient between age and fluoride concentration of the samples can be used to estimate the value of age as an explanation of fluoride concentration in the horse. In the South, where the R square is relatively high, age could be the sole explaining factor of fluoride accumulation in the horse mandibles. Similarly, R square is quite high in the North and East, and it could therefore be assumed that fluoride concentrations in horses from those regions are age related. In the West, the R square is relatively low, before the outliers are removed. After removal of the highest samples, the correlation coefficient rises for horses in the West region as well. As figure 10 shows (where outliers are excluded), the curves for each region flattens again with increasing age of the horses and the difference between the regions decreases. On that note, compared to sheep, the horse life span is longer on average than that of sheep. This difference gives an interesting opportunity of observing the development of fluoride accumulation in horse bones for a long time. Nonetheless, horses still accumulate relatively little fluoride over their lifetime in comparison to sheep, and it may even seem that the accumulation rate decreases in the more senior horses. Since horses from the South have both the lowest average fluoride concentration and the highest correlation coefficient, the values from the South are representative for what can be considered as a normal exposure to fluoride from the environment, especially considering the values in foals and youngsters. However, it can be assumed that values from the West would be representative for when the fluoride burden in the environment arises from aluminium smelters, considering both high values in foals from the West and the values measured above 600 ppm F in horses living close to the smelter in Hvalfjörður.

The study substantiated noticeable differences in mandibular fluoride content between the four regions included in the study. Kierdorf et al. (1995) found that higher rates of bone fluoride accumulation in youngsters are regarded as indicative of intense fluoride burden in the surroundings (Kierdorf et al., 1995). These results support the proposal that fluoride accumulation in horses from the West region is affected by external factors, given the results from the foals and the effect of outliers in the region. However, the results also suggest that the South is the overall region that has values remarkably different from the other regions, even though the difference is that they are significantly low.

Multiple environmental factors can affect fluoride accumulation in the Icelandic horse. Gunnarsdottir et al., (2016) reported that geothermal water could blend with groundwater and increase the amount of specific chemicals such as fluoride and arsenic (Gunnarsdottir et al., 2016). Although some samples from the West were collected near an active low-temperature geothermal area, and could influence fluoride concentration in horses from that area to some extent, active low-temperature geothermal areas are also found in the other regions, as well as high-temperature geothermal areas in the North, and the South (Appendix I), which had the lowest average fluoride concentration.

Furthermore, fluoride in the bedrock of Iceland can affect the levels of fluoride which accumulate in grass and water, as has been evaluated in several locations of the country (Sigvaldason & Óskarsson, 1986). Since fluoride in bedrocks of Iceland is quite even between the regions, it probably only contributes to the age-related accumulation that horses and other herbivores experience throughout their life, having no extra effect where fluoride concentration was high.

Kornelíusdóttir (2010) studied fluoride in waterholes in Iceland. There was no statistical difference between the fluoride found in waterholes between regions and therefore it is highly unlikely that fluoride in water could explain any difference found in bones of the Icelandic horse. However, it was interesting that the one waterhole with values higher than the safe level (1.5 mgF/l) was found in the South region, at Flúðir, with 4.04 mgF/l.

Volcanoes in Iceland have erupted nine times (Veðurstofa Íslands, 2020) since the eldest horse in the study was born (1991), four times in the South region; twice in Hekla and twice in Eyjafjallajökull/Fimmvörðuháls. Five eruptions were located in the central highlands; four in Grímsvötn/Gjálp and one in Bárðarbunga/Holuhraun (Veðurstofa Íslands, 2020). The eruptions in the South did not seem to affect the fluoride accumulation of horses born and living within the region, since fluoride concentrations in all age groups from the South were lowest compared to age groups from the other regions, except for foals from the South, which had the second lowest value in foal age group. Even looking at the age group of 5-12 years in the South, which were at a sensitive developmental stage when eruptions took place in Eyjafjallajökull 2010 and 2011, the average fluoride concentration is lower than in the same age group from the North and East, and significantly lower than in horses of that same age group from the West ($P = 0.0065$). Volcanic ash particles can travel for days suspended in the air, when of a certain diameter (Durant et al., 2010) and could therefore have fallen all over Iceland, (Gislason et al., 2011a). However, Gislason et al., (2011b) reported that a hazardous amount of fluoride fell over the South region, at the beginning of the eruption.

Monitoring of airborne heavy metals from volcanoes and industrial plants in Iceland was conducted on moss from 1990 until 2015 by the Icelandic Institute of Natural History (*Náttúrufræðistofnun Íslands*). Even though fluoride was not monitored, the distribution of sulphur throughout Iceland can be used as a frame of reference on how airborne fluoride could distribute from volcanoes and industrial plants to water and vegetation in the country. It seems that contamination from volcanoes is the most significant factor, and affects the widest range of land, whereas the industrial plants contribute to a continual and localized contamination. Sulphur concentration in moss decreases with increasing distance from the three aluminium smelters in Iceland, indicating that the highest concentrations are around the smelters themselves. The amount of sulphur released in the 2014 eruption in Holuhraun, Bárðarbunga, was however thousandfold greater than the amount released from the three Icelandic aluminium smelters combined (Magnússon, 2018). Nonetheless, it was estimated that constant exposure to airborne heavy metals had greater effects in the long run, than exposure to large amount for shorter time. This could possibly explain why the volcanic eruptions, as of 1991, did not seem to have great impact on fluoride accumulation in the horses of the study. As for the continual exposure from the aluminum smelters, especially for horses in the West region, it seems to have an impact on the horses. Even foals from the West had high fluoride concentration compared to foals from the other regions. However, when the outliers with the highest values measured are removed, the average fluoride concentration for all horses in the West is not significantly higher than that of the other three regions. This indicates that fluoride emissions from the aluminium smelters are of greatest danger when an accident in the production happens during the sensitive growth phase of the horses.

As previously mentioned, an aluminium smelter is also located in the East region, where no samples were unusually high compared to the total average. Samples from this region had their origin at least 30-40 km from the smelter, located in Reyðarfjörður. With further research, it could be hypothesized that if samples from Reyðarfjörður were analyzed for fluoride concentration, the results would be similar to that of the samples collected near the aluminium smelter in Hvalfjörður. Previous results of fluoride monitoring in sheep from Reyðarfjörður favour the hypothesis (Jóhannsdóttir, Flosadóttir & Þórðarson, 2014; Guðmundsdóttir et al., 2019). However, compared to sheep monitoring near the aluminium smelter in Hvalfjörður, we would not expect the values in horses from Reyðarfjörður to be as high as measured in sheep.

The statistical analyses done in this study were bivariate analyses based on age groups, and not on age as a continuous variable. It would be interesting to do a multivariate analysis on fluoride concentration, region and age, as a continuous variable, but it would be preferable to do such analysis on a larger batch of samples. The analysis could possibly inform which variable is a greater influencer in fluoride accumulation of the Icelandic horse, age or origin. A retrospective analysis on sheep monitoring results would be very informative, as no such statistical analysis has been done on the information gathered in the environmental monitoring done around the aluminium smelters.

To recapitulate, the results show that the average fluoride concentration in the Icelandic horse is low compared to other species, even though relatively high values were observed in few samples. It would therefore seem that horses are not an appropriate indicator species for environmental fluoride burden. As said before, it is believed that deer are at risk of developing dental lesions when they accumulate between 1000 and 2000 ppm F during the early periods of their lives (Vikøren & Stuve, 1996). Based on the comparison of fluoride accumulation between horses and sheep in Iceland, Icelandic horses do not seem to be in danger of developing symptoms of chronic fluorosis, on average, in their daily exposure to environmental fluoride. Even though the highest fluoride concentrations from the West were connected to emissions from the aluminium smelter in Hvalfjörður, the values were still relatively low compared to the highest values found in sheep from the same area (Yngvadóttir et al., 2017; Yngvadóttir et al., 2018). Even though the rate of fluoride accumulations varies between species and even breeds of the same species (Vikøren & Stuve, 1996; Death et al., 2015), it is highly plausible that the Norwegian deer baseline could be indicative of the levels where pathological changes might be seen in teeth or bones of sheep or horses, depending on the age of the animal under environmental fluoride pressure.

All things considered, the results from this study show a significant difference in equine mandibular fluoride concentration between regions and age. However, it is necessary to both improve the distribution throughout the country and increase the number of samples in some age groups. Increasing sample numbers in foals and juveniles (1-4 y.o.) is particularly important since great exposure to fluoride in the early years of the horse seems to have considerable impact on fluoride accumulation in bones. Furthermore, examining dental lesions in connection with fluoride concentrations in horses aged five to twelve years would be ideal to estimate the fluoride accumulation in the early years of the horse, since exposure to fluoride when animals are teething is more probable to cause dental fluorosis. However, examining skeletal lesions in horses in connection to fluoride concentrations would be optimal to make reliable baseline for fluoride accumulation. Even though skeletal lesions would be more prominent in animals which are exposed to fluoride from a young age, skeletal fluorosis can develop in animals in later stages of their life when exposed to great levels of fluoride, even if they did not reside in a highly fluoride dense area when they were young, unlike dental fluorosis. The difference in fluoride concentrations between regions in the present study makes it arguable that horses born in the West would be the most likely to develop signs of either dental or skeletal fluorosis, since the highest values for all age groups are found in that region, indicating great levels of fluoride exposure in the environment from a young age. That said, continuing to increase the knowledge of fluoride concentrations in horses all over Iceland is important to better understand which factors affect the accumulation the most, along with either dental or skeletal condition check of the horses, to estimate which levels of fluoride concentration could be harmful for Icelandic horses, and comparing this to the Norwegian baseline. Even though they are based on a specific sample set, these results do represent fluoride accumulation in horses from four Icelandic regions and five age groups. They can definitely serve as a guideline to some extent, since they are the only present available data of mandibular fluoride concentrations in Icelandic horses, which places emphasis on the whole country.

4.1 Conclusion

- Icelandic horses accumulate less fluoride with age than sheep, making them less appropriate as an indicator species for environmental fluoride contamination.
- Fluoride concentration in Icelandic horses is, on average, below the proposed levels of increased risk for fluorosis for other species.
- Foals in the West region have higher bone fluoride concentration than foals in other regions, indicating environmental contamination in the area.
- Fluoride concentration in horses from the West region is higher in horses residing close to the aluminium smelter in Hvalfjörður.
- The difference in fluoride concentration gets less pronounced with increasing age, possibly representing cessation of skeletal growth.
- Although the results indicate that horses accumulate fluoride at a lower rate than sheep, it can be expected that pathological changes in the horse would be associated with similar levels of fluoride concentration as have been proposed for other species.

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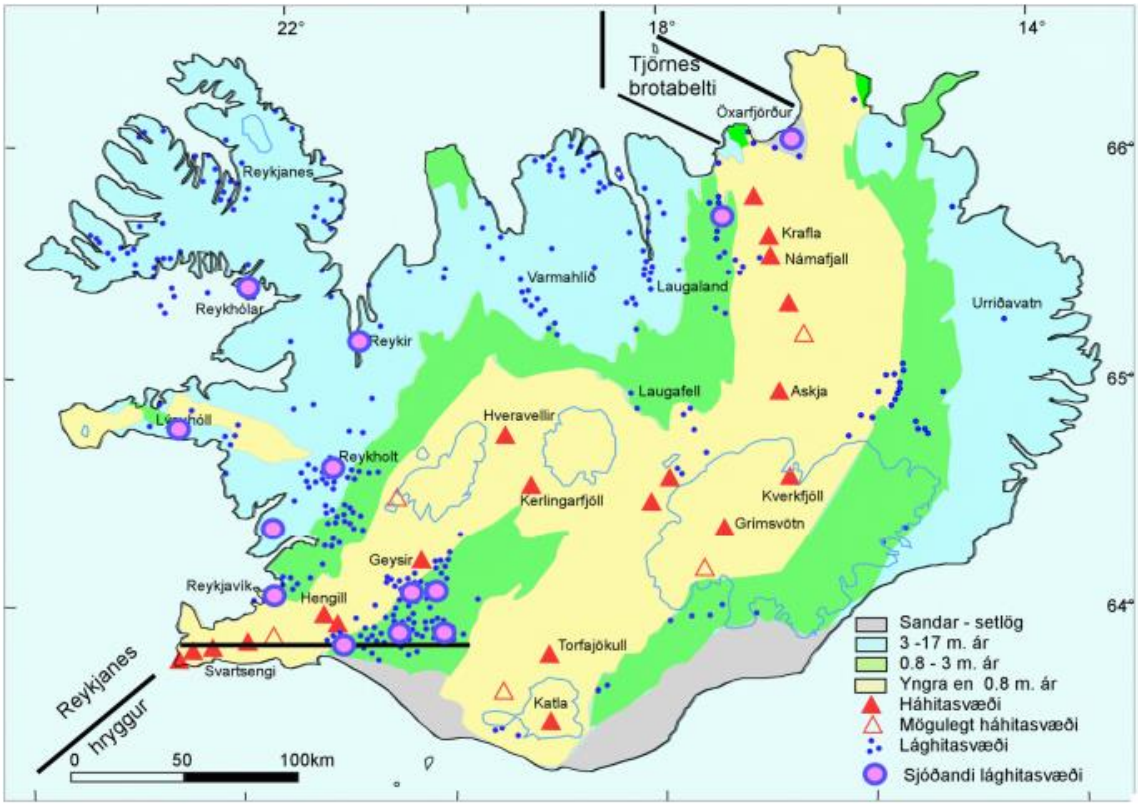
Appendix I



Icelandic map of boiling low-temperature areas (pink and blue circles), high-temperature areas (red triangles), possible high-temperature areas (empty triangles) and low-temperature areas (blue dots). Map adapted from Kristmannsdóttir, H. (2008). *Jarðhitaauðlindir*. Ferðamálasetur Íslands.

Appendix II

Results of fluoride concentrations in 223 Icelandic horses

*Horses divided to four regions; South (1), West (2), North (3) and East (4)

** Horses divided to five age groups; Foals (1), 1-4 y.o. (2), 5-12 y.o. (3), 13-20 y.o. (4) and 21+ y.o. (5).

***Gender of the horses; females (F) and males (M). Gender is not included for foals

Region*	Age group**	Age	Fluoride concentration (ppm)	Gender***
1	1	0	51	
1	1	0	54	
1	1	0	48	
1	1	0	43	
1	1	0	61	
1	1	0	48	
1	1	0	37	
1	1	0	48	
1	1	0	48	
1	1	0	40	
1	2	2	112	M
1	5	21	288	F
1	4	15	245	F
1	4	13	280	M
1	5	23	352	F
1	3	9	254	F
1	3	9	209	M
1	4	19	318	F
1	3	10	254	F
1	2	4	218	M
1	4	15	257	M
1	3	8	153	M
1	4	18	224	F
1	5	26	284	F
1	5	21	335	F
1	5	21	346	F
1	5	26	203	F
1	4	20	246	M
1	2	3	108	M
1	2	3	115	F

Region*	Age group**	Age	Fluoride concentration (ppm)	Gender***
1	3	10	238	M
1	2	4	230	M
1	5	21	377	M
1	3	6	164	M
1	3	6	172	M
1	5	25	176	M
1	3	7	192	F
1	3	7	128	F
1	3	5	173	M
1	2	4	126	F
1	3	5	181	F
1	3	5	177	M
1	3	12	208	M
1	3	10	166	M
1	3	7	131	M
1	3	6	174	M
1	3	5	148	M
1	3	5	152	F
1	2	4	160	M
1	3	5	223	M
1	5	23	308	M
1	3	6	206	M
2	4	15	588	M
2	4	18	271	F
2	3	8	279	M
2	5	20	296	M
2	1	0	69	
2	4	15	426	M
2	3	5	205	F
2	3	8	357	M
2	3	8	333	F
2	3	6	268	M
2	3	9	298	M
2	5	26	319	F
2	2	2	71	F
2	1	0	45	
2	4	18	453	F
2	4	15	453	F
2	3	6	395	M
2	3	12	343	F

Region*	Age group**	Age	Fluoride concentration (ppm)	Gender***
2	3	7	162	M
2	5	25	1583	F
2	4	20	1192	M
2	4	14	1082	F
2	3	6	474	F
2	5	26	321	M
2	4	19	312	F
2	1	0	224	M
2	5	25	220	F
2	4	18	264	F
2	4	15	644	M
2	4	14	378	M
2	3	12	812	M
2	5	23	269	F
2	1	0	137	
2	1	0	120	
2	1	0	94	
2	1	0	103	
2	1	0	99	
2	1	0	86	
2	1	0	87	
2	1	0	81	
2	2	1	64	M
2	2	4	167	M
2	3	7	176	M
2	3	6	117	M
2	2	1	259	M
2	2	1	304	M
2	4	18	259	M
2	4	18	336	F
2	5	25	272	M
2	2	4	145	M
2	1	0	25	
2	5	21	245	M
2	2	1	74	M
2	3	6	250	F
2	4	13	208	F
2	4	17	216	M
2	4	17	198	M
2	5	24	237	M

Region*	Age group**	Age	Fluoride concentration (ppm)	Gender***
2	4	17	343	M
2	3	11	291	M
2	4	15	284	M
2	2	4	176	M
2	4	16	241	M
2	3	11	260	M
2	4	13	167	M
2	5	22	348	F
2	3	11	216	F
2	4	13	214	F
2	5	24	255	M
2	4	18	239	F
2	3	12	195	F
3	1	0	41	
3	1	0	48	
3	1	0	84	
3	1	0	42	
3	1	0	48	
3	5	24	307	F
3	3	7	261	F
3	3	6	244	F
3	4	14	332	F
3	3	7	227	F
3	4	13	483	F
3	1	0	48	
3	1	0	33	
3	1	0	26	
3	1	0	29	
3	1	0	37	
3	1	0	34	
3	1	0	44	
3	1	0	49	
3	1	0	42	
3	1	0	38	
3	3	10	196	M
3	4	19	246	F
3	4	17	214	F
3	4	19	70	M
3	4	17	197	M
3	4	16	251	F

Region*	Age group**	Age	Fluoride concentration (ppm)	Gender***
3	4	20	298	M
3	4	14	563	F
3	4	13	261	F
3	3	7	314	F
3	4	16	433	F
3	4	20	217	F
3	3	6	253	F
3	3	8	224	F
3	5	21	241	F
3	4	18	284	F
3	5	22	318	F
3	3	12	223	F
3	4	13	245	F
3	3	6	256	F
3	4	19	317	F
3	2	3	219	F
3	2	3	191	F
3	4	16	297	F
3	5	24	544	M
3	5	28	302	F
3	5	27	322	M
3	4	15	463	F
3	5	24	327	F
3	5	22	180	M
3	5	22	258	M
3	5	26	299	F
3	3	10	29	M
3	5	29	302	F
3	4	16	292	F
3	2	3	218	M
3	3	8	441	M
3	3	6	444	F
3	4	20	326	F
3	4	18	441	F
3	4	18	391	F
3	5	28	369	M
4	4	19	464	F
4	4	19	327	F
4	3	10	402	F
4	1	0	47	

Region*	Age group**	Age	Fluoride concentration (ppm)	Gender***
4	1	0	50	
4	1	0	51	
4	1	0	56	
4	1	0	43	
4	3	6	183	M
4	3	9	230	M
4	4	19	239	M
4	3	11	263	M
4	3	8	215	M
4	4	17	202	M
4	4	19	263	F
4	4	14	254	F
4	5	23	245	F
4	5	21	221	F
4	4	15	287	M
4	5	26	274	F
4	5	21	374	F
4	4	20	385	F
4	3	7	288	M
4	5	22	328	M
4	5	23	288	F
4	3	7	334	M
4	5	25	242	F
4	5	20	270	M
4	4	14	337	M
4	4	15	250	F
4	3	10	188	M
4	4	15	264	M
4	4	17	245	M
4	5	22	308	F
4	4	18	228	M
4	5	21	326	M
4	3	12	222	M