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Morphological Study and Mineral Analysis of the Lower Mandible of Adult Atlantic Salmon (*Salmo salar*) from Scotland with Mandibular Deformation

Estudio Morfológico y Análisis de Minerales de la Mandíbula Inferior de Salmones del Atlántico (*Salmo salar*) Adultos con Deformación Mandibular, Provenientes de Escocia

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SUMMARY: Mandibular deformity is a condition that affects the jaw bone of adult salmon and has been observed in Norway and Chile, causing weight loss, poor quality of farmed fish and increased mortality. The causes range from high temperatures of the state of eggs, to poor nutrition phosphorus or vitamin C. This work aims to analyze this deformity by histochemical and mineral analysis technique during an episode presented in centers of the Scotia Sea. Jaw and spinal segments of 21 Atlantic salmon in Scotland were used. These samples were classified into three groups: Group 1: Severely deformed. Group 2: Mildly affected. Group 3: Normal controls. Four jaws per group were fixed in 10 % formalin and embedded in Paraplast, sections of 5 microns were performed using a Microm® microtome histochemical technique Von Kossa was used for the detection of calcium deposits, which highlights the calcium osteoid black and red color. For proximate analysis, and in order to obtain and compare levels of calcium, phosphorus, zinc and magnesium in total 9 bone jaws (6 affected with DM and 3 controls) and 9 body sections the Mann-Whitney test was used to compare these values between misshapen salmon and controls. To correlate values, jaw and body segment a Spearman corrrelation was applied. Fish group 1 presented a ventral deviation of the alveolar bone body. In fish group 2 prominence of the visible joint on both sides or unilaterally was observed. Comparing the values of % Ca, % P, % Mg and Zn jaws with DM and healthy ones with Mann Witney method it was found that the values of these minerals vary between salmon and controls affected. There was a significant difference in the percentage of P, which indicates that there is less P in affected fish vertebrae. Spearman correlation noted that the percentages of the minerals studied in dental bone and vertebral segments are uncorrelated. Rather, Von Kossa distribution indicates that Ca/P is not homogeneous in the dental bone, as a result of mineral resorption from the skeleton including the operculum, articular bone and dental towards kype. This paper states that Von Kossa histochemical technique showed significant differences between deformed fish and controls and also showed differences between the various segments of the dental bone. The alveolar bone is a dynamic structure adapted to continuous histological changes may be involved in MD, phosphorus deficient diets, coupled with the initial formation of Kippe.

KEY WORDS: Dentary bone; Salmon skeleton; Mandibular deformation**;** ***Salmo salar*****; Von Kossa.**

INTRODUCTION

The skeletal deformities affecting viscerocranium adult salmon have been reported in countries such as Norway and Chile. The mandible deformity (MD) has been observed in many species of fish for over 100 years, but, in most cases, the cause is unknown. Studies in Norway, in 1990 concluded that the possible cause could be: high temperature for the state of ova (Alekseyev *et al*., 1995) and the use of oxytetracycline (Baeverfjord *et al*., 1998). In the case of Chile, it has been reported that this disease may be due to the quality of imported eggs or to problems in food formulation.

Other factors have been mentioned that could be involved in the MD, for example: water quality because, low oxygen or high CO_2 levels, metabolic acidosis can cause tissue and bone demineralization (Alekseyev *et al*.), parasitism (Baeverfjord *et al*.) and hereditary factors (Bruno & Poppe, 1996; Figueroa Alvarez, 2004). Previously, we

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have suggested evolutionary factors indicating that this abnormality is the result of an evolution heterocrónica salmon, which produces a change in bone structure.

The (MD) is the ventral deviation and/or ventral side of jaw bone of the lower jaw, at a variable angle between 45 ° and 90 ° from its normal position. Associated to this, there occurs a disturbance of bone and joint-angled square, generating a prominence in the area which is visible externally, there is also a cartilage jaw dysplasia. This deformation alters the occlusion of the mouth, thereby preventing the individual eating normally, thus generating a low weight and increased mortality in juvenile and adult fish.

In Chile, the cranial deformations in Atlantic salmon were observed in two consecutive crops immediately after transfer from freshwater to seawater, shortening cord and vertebrae fracture were also noted. The condition was not repeated after adjusting dietary phosphorus levels and vitamin C. A major risk factor mentioned is the time of transfer, the salmon that entered in fall and winter had a percentage rate of 84 % of affected individuals. Another significant risk factor was the weight of arrival at sea, with the affected individuals with MD who were admitted with lower weights. Disease appears immediately at the time of transfer to sea, reaching a peak incidence admitted nine months following sea introduction.

As is known, the jaw is formed from the first gill arch, during the state of ova. From the first gill arch cartilage forms square (dorsally) and the mandibular cartilage (ventrally), constituting the rudiments of upper and lower jaw. The body does not involute mandibular cartilage as in mammals but persists as such. The mechanism involves the intramembranous ossification of the bone morphogenetic proteins and the activation of a transcription factor called Cbfa1. It is thought that bone morphogenetic proteins from the cephalic instruct epidermis derived mesenchymal cells of the neural crest cells to become bone to cause it directly Cbfa1 expression (Leiva *et al*., 1984; Mardones Loyola, 2004). Cbfa1 seems to activate genes for osteocalcin, osteopontin and other extracellular matrix proteins specific to the bone.

Replacement of fishmeal with plant protein, has resulted in a higher incidence of MD. Animal based feed is rich in protein, a good source of calcium, phosphorus, iron and zinc for salmon, a carnivorous fish. In contrast, plant foods, in which the phosphorus is found as phytate, have disadvantages such as malabsorption. Moreover, the phytate form insoluble complexes with calcium, thereby reducing the bioavailability of calcium necessary for physiological functions. In addition, phytates have a negative effect on the absorption of other minerals such as magnesium, iron and zinc. Phosphorus is an essential component of fish diets. Its deficiency affects not only hard tissue, which is responsible for rickets, leading to malformations, but also influences various aspects of intermediary metabolism, influencing weight gain and feed conversion.

This study analyzes the jaw bone of salmon affected with MD from farms in Scotland during the episode of MD occurred in 2003. Levels were determined Ca, P, Zn and Mg of jaw bone and the start of the flow of spinal segment. Our hypothesis is that the levels of Ca, Zn and Mg in median affected salmon populations will be equal to the control population. But in the case of phosphorus values will be lower in salmon with MD. In the correlation values with alveolar bone backbone these are mutually independent.

MATERIAL AND METHOD

We studied 21 Atlantic salmon, which were in fattening stage in seawater centers in Scotland. Of each salmon using 2 samples, corresponding to the jaw and a body segment (cross section) cut at the first caudal vertebrae, which were frozen at their place of origin for shipment by air to the Embryology Laboratory, Faculty of Medicine. Universidad de Chile, Santiago, Chile.

The 21 heads were classified into three groups according to the following criteria. 1) Severely deformed (Group 1): fish with open mouth, bilateral alteration of square-articular joint and tooth loss. 2) mildly affected (Group 2): abnormal unilateral or bilateral joint square-articular bone and partial alteration of the buccal occlusion 3) normal controls (Group 3): normal-looking jaw, no prominence in the square-articular joint, normal buccal occlusion.

Histological jaws were 12, 3 tissue segments obtained of e ach. The first segment corresponds to the square-articular joint. The second to the middle of committing ramus mandibular jaw bone and cartilage and the third to the mandibular symphysis. These samples were fixed, they were decalcified and embedded in Paraplast, then cut into 5 microns using a microtome Micro®, Von Kossa histochemical technique was used to detect calcium deposits (13) which highlights the calcium color black and red osteoid.

Proximate analysis. In order to obtain and compare levels of calcium, phosphorus, zinc and magnesium in bone a total of 9 jaws were used (6 with MD and 3 controls) and their body sections). Each sample was analyzed individually in the Animal Nutrition Laboratory of the Faculty of Veterinary and Animal Sciences, University of Chile. With the results of the proximate analysis, we compared the mean values of Ca, P, Mg and Zn between jaws affected and controls, and also compared affected fish body sections and controls.

To compare values of Ca, P, Zn and Mg between deformed salmon and controls we used the Mann-Whitney test, which is based on hierarchies of observations (Rojas *et al*., 2004). Hypothesis: Ho: Mx = My, Ha: ≠ My My. Where: Mx is the median of the salmon population affected with MD. My is the median of the control population. Be an a = 0.05. To correlate the jaw values (X) and body segment (Y) was correlated by Spearman hierarchies where: Ho: X and Y are mutually independent. Ha: X and Y are not mutually independent.

RESULTS

In the samples of Group 1 was noticed a permanently open mouth, with a deviation in some cases ventral and ventrolateral of the lower jaw, describing an angle ranging between 60 ° and 90 ° (Figs. 1 and 2). Asymmetries were evidenced between the cartilages, these differences being related mainly to its diameter, length and dislocations with sudden deviations in trajectory apparent angles with impaired alveolar bones and joints. The mandibular cartilage showed a loss of brightness and translucency. The loss of teeth both above and below becomes apparent.

All fish in group 2 had a slight alteration of the lower jaw. This was evident externally as a protrusion on the square-articular region coinciding with the different degrees of rotation in this joint (Figs. 3 and 4), which in the case of 3 samples was seen only on the left side of the skull, and was observed in 3 samples on both joints. No cases of asymmetry between rami, mandibular cartilage alterations nor ventral deviation of the lower jaw were reported. The samples showed various degrees of alteration in the mouth closure.

The fish in Group 3 had mouths shut (Figs. 5 and 6) the caps covering in full to the gills. Cartilage both right and left jaw was located along the inner face of jaw bone presenting a shiny, semi-translucent and symmetrical dimension between each.

Normal alveolar bone was observed as cellular compact bone tissue with collagen fibers parallel and trabecular tissue osteons typical with numerous mature marrow spaces of variable size, containing within adipose tissue. The bone tissue in some cases showed immature bone sectors, which by characteristic arrangement of the collagen fibers is also known as woven bone. More osteocytes were observed by area, with a random distribution and a more intense stainingof bone matrix. In the centers of ossification

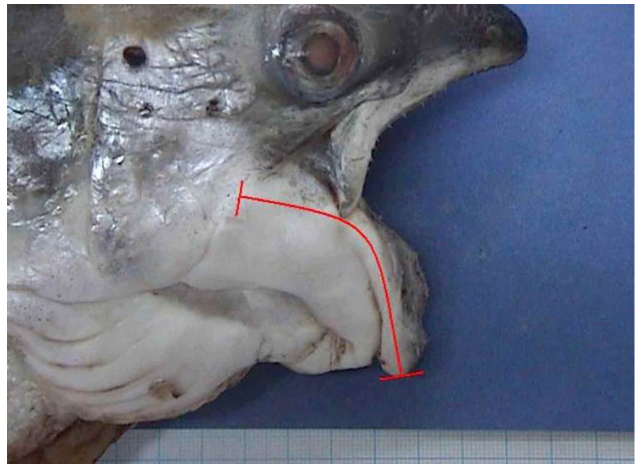

Fig. 1. Group 1, ventral deviation at 90 ° angle. The red line describes the path of the lower jaw from square-articular joint, to the mandibular symphysis.

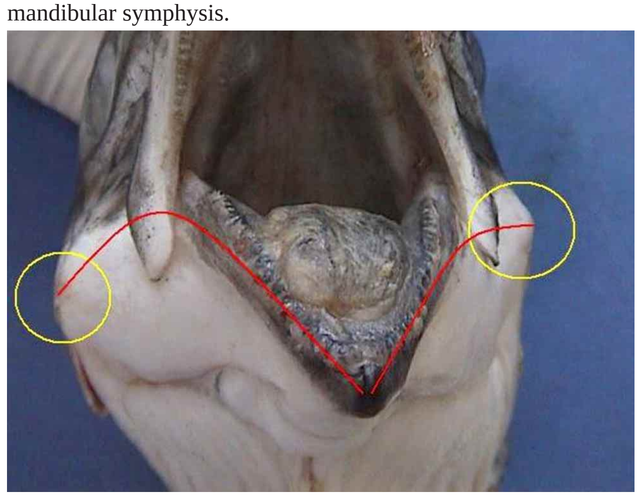

Fig. 2. Group 1, shows a partially open mouth and bilateral protrusion (yellow circle).

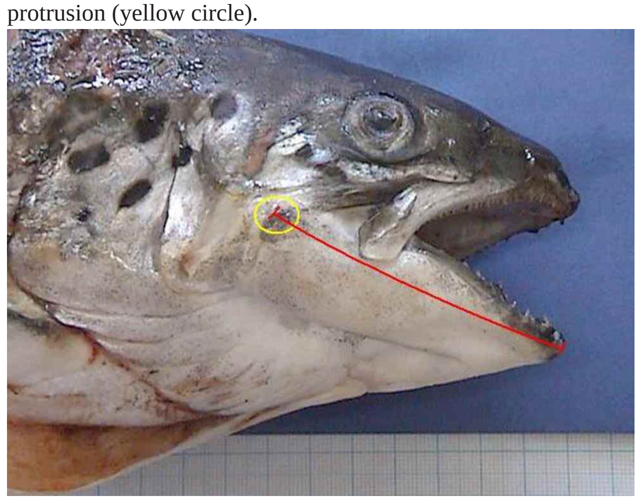

Fig. 3. Group 2. Bilateral protrusion square-articular joint (yellow circle), dental bone symmetry.

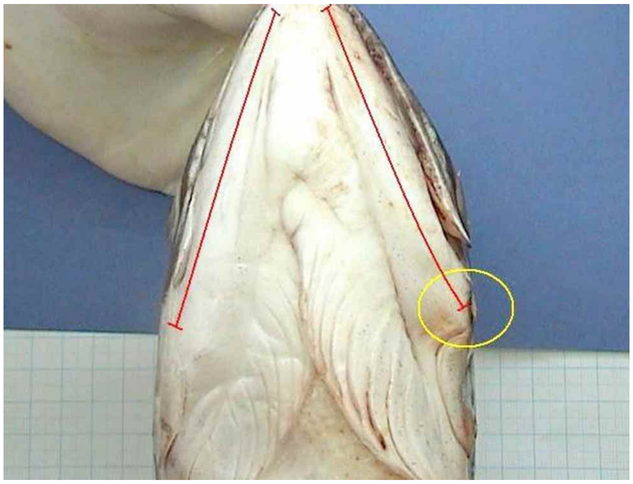

Fig. 4. Group 2, Symmetry is evident in dental bones with a slight unilateral alteration of the left ramus.

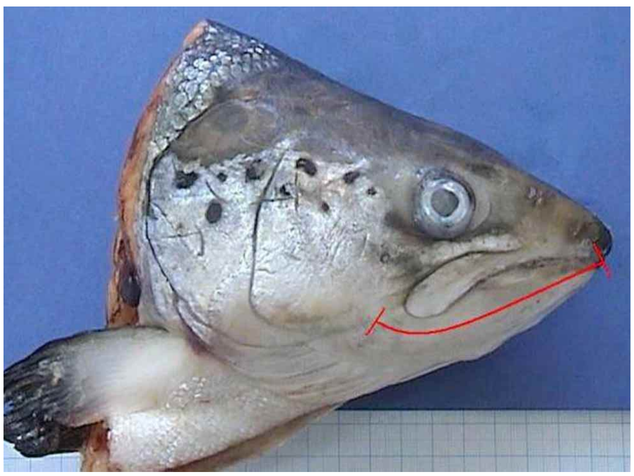

Fig. 5. Group 3, Side view of a normal fish, straight ramus.

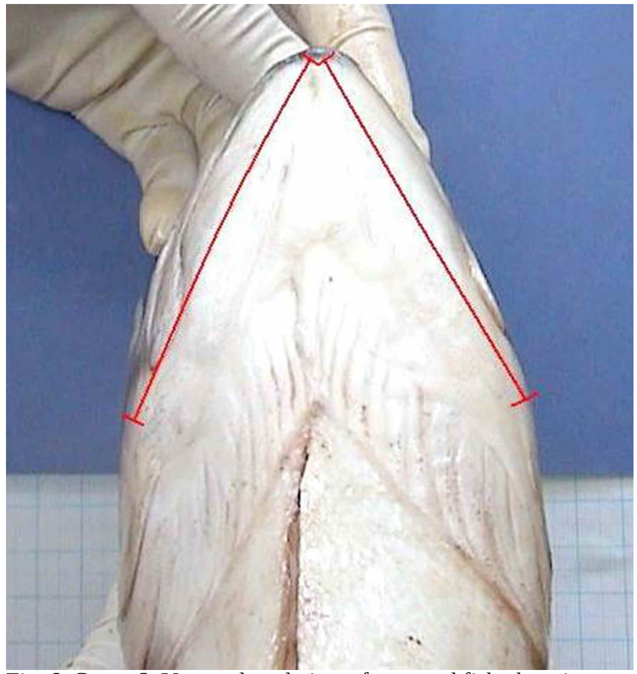

Fig. 6. Group 3, Ventro-dorsal view of a normal fish, there is complete symmetry of mandibular rami and no protrusion.

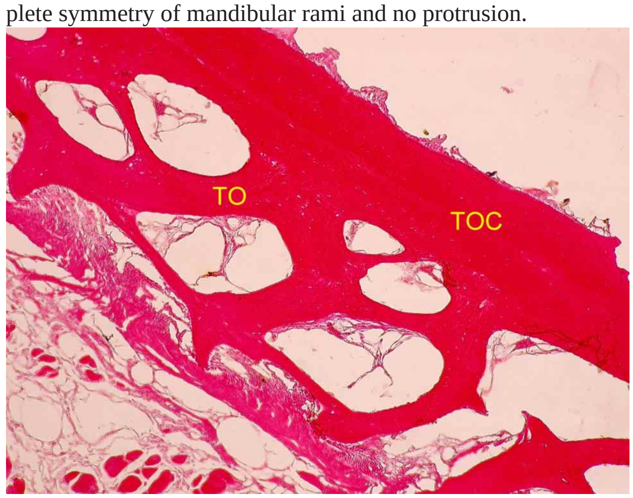

Fig. 7. Group 1. Square-articular joint. Observed uncalcified bone (OCD). Von Kossa technique 200X.

osteoclastic activity and cartilage were observed. Between bone tissuefibrosis is evident in the marrow spaces of the bones, which indicates a reactive process by osteogenic cells.

In the alveolar bone with evidence 1 The osteoid calcification and absence (Fig. 7). In Group 2, the region of the mandibular symphysis was well calcified, however, the region near square-articular joint presented a poor calcification of jaw bone with osteoid (uncalcified) (Fig 8). In the region of endosteal actively functional osteoblasts we observed related to the formation of collagen fibers in the area. In Group 3, the dental bone appeared well calcified (Fig 9).

When comparing the values of % Ca, % P, % Mg and Zn of the jaws with MD and healthy with the Mann Whitney method. shows that there is variation among salmon with MD and controls.

In the statistical analysis of spinal segments compared deformed salmon and controls, and the values of % Ca, % P, Mg and Zn %, this indicates no significant difference between the medians of Ca, Mg and Zn, but if there is difference in the case of phosphorus, it is accepted that that there would be differences between medians of % P in body segments of affected and healthy fish. In this case higher % P existing in the body of salmon Group 3 (control).

Correlated with coefficient of Spearman's correlation hierarchies, values of bone mineral deformed tooth mineral values and their respective spine. For all mineral (% Ca, % P, % Mg and Zn) proved two mutually independent variables, namely, mineral values affected jaws are uncorrelated with mineral values of their respective body segments with a a = 0.05 %.

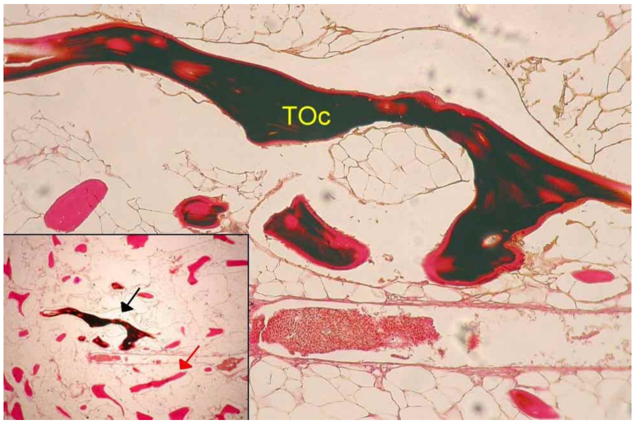

Fig. 8. Group 2. Alveolar bone formed by compact bone tissue (TOC) and trabecular bone (TO) with and without calcification. Von Kossa technique 200X (Box 50X).

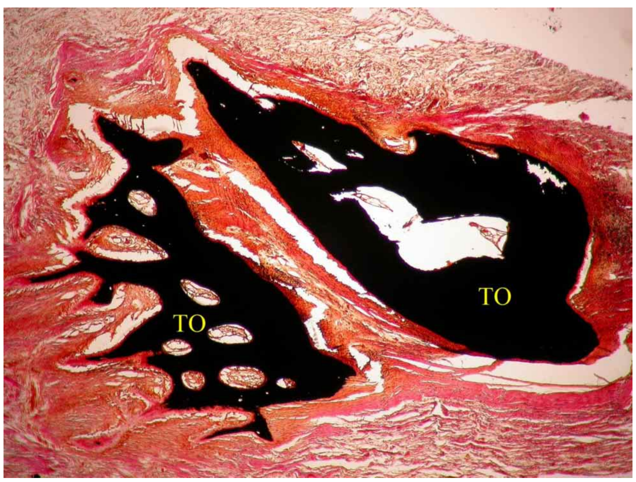

Fig. 9. Group 3. Square-articular joint. Bone tissue is observed (TO) black color, indicating its good calcification. Von Kossa technique. 200x.

Table I. Percentage on dry matter basis of calcium (Ca), phosphorus (P), magnesium (Mg) and zinc (Zn) of Scotland salmon lower jaw.

	%Ca	%P	%Mg	Zn (mg/kg)
01CA	4.8	2.4	0.09	185.2
12CA	5.0	2.4	0.10	116.0
15CA	5.2	2.3	0.10	207.5
14MA	4.0	2.5	0.08	105.1
17MA	5.1	3.0	0.09	151.2
19MA	6.0	3.7	0.10	110.8
03U	4.4	2.7	0.08	121.8
11U	7.5	2.4	0.10	112.0
16U	6.0	3.4	0.10	140.3

Furthermore, the same coefficient values correlated Total minerals (deformed tooth bone and controls) and their respective segments of the spine also concluded mutually independent variables, using a = 0.05 %.

Table II. Percentage on dry matter basis of calcium (Ca), phosphorus (P), magnesium (Mg) and zinc (Zn) of Scotland salmon body segments.

	%Ca	%P	%Mg	Zn (mg/kg)
01CA	1.90	1.7	0.09	63.8
12CA	1.55	1.3	0.08	42.4
15CA	1.67	1.4	0.10	49.8
14MA	1.58	1.3	0.08	29.8
17MA	2.10	1.6	0.08	47.0
19MA	2.20	1.5	0.10	89.8
03U	1.70	2.0	0.08	46.8
11U	1.56	1.9	0.07	60.0
16U	2.20	1.9	0.10	55.8

Table III. Jaws affected v / s controls. T calculated for each mineral and critical values of the rejection regions for Ho, with a = 0.05.

Mineral	*T* calculated	$\omega\alpha/2$	$\omega 1\text{-}\alpha/2$
Ca	5.5	<2	>16
P	8.0	<2	>16
Mg	8.5	<2	>16
Zn	10.0	<2	>16

Table IV. Jaw segments affected v / s controls. T calculated for each mineral and critical values of the rejection regions for Ho, with a = 0.05

Mineral	*T* calculated	$\omega\alpha/2$	$\omega 1\text{-}\alpha/2$
Ca	8,5	<2	>16
P	0	<2	>16
Mg	11,5	<2	>16
Zn	8,0	<2	>16

DISCUSSION

The fish in Group 1, expressed a serious alteration of jaw bone of both mandibular branches, creating a ventral and ventrolateral deviation of jaw bone body with mandibular asymmetry between the branches, thus compromising the skeletal system as cartilage (mandibular cartilage), which agrees with the descriptions in Atlantic salmon post-smolts (Bruno & Poppe; Ducy *et al*., 1997; Figueroa Alvarez). Fish in Group 2 shows the prominence of the joint visible on both sides, and sometimes unilateral, which agrees with Bruno & Poppe, who point out that 10 % of fish with MD had unilateral malformation .

With regard to histochemical analysis, fish with MD showed demineralization in bone joint and in the distal alveolar bone, which does not coincide with the description by Witten & Hall (2002), that indicates good mineralization of jaw bone in normal adult fish, describing the fish bone as a compact tissue, laminar, with typical osteons.

In the area near the mandibular symphysis of these fish, areas of calcification could be seen with intense cell proliferation and new bone formation coinciding with a state kype training, which is an early sign of secondary sexual character. The comments can relate to Tchernavin who notes that during the migration of fish upstream, Atlantic salmon undergo a series of changes in the morphology of the proximal splanchnocranium, described especially in males (Sugiura *et al*., 2004), observed in both sexes of Atlantic salmon, a significant demineralization in the vertebrae. This decrease of calcium would result from a demineralization, i.e. the removal of minerals without the degradation of the organic matrix of bone. Other authors note that all adult male salmon (salmon, spend a winter in the ocean and then return to breed in spring), show good kype development at the distal end of the lower jaw, as an extension of jaw bone, extending and curving dorsal.

The animals studied showed no visible growth kype, therefore males showed no external secondary sexual characteristics. However certain individuals showed histology, thin bone extensions anastomosed. For this reason, it is possible that they correspond to males experiencing early histological changes of mandibular transformation, before external anatomical sexual changes.

In normal jaw, bone is formed by two teeth (right and left). Its development is related to the embryonic mandibular cartilage formed by the first gill arch. This cartilage does not involute and as the fish grows, is included in the medial or lingual dental bone. It is also noted that the cartilage acts as a model to form dental bone, which at the stage of ova (350 UTA) bars cartilaginous gill arches (mandibular included) are differentiated bone ossification and dental tissue begins adjacent mesenchymal. This is intramembranous ossification and cartilage surrounding the mandibular progressively to eventually ossify its posterior segment, forming the joint bone (Toften & Jobling, 1996). Thus, the bone is then articulated to the square. If the temperature increases and accelerates chondrogenesis deformations appear likely (Tchernavin, 1944). Factors such as high temperature in the egg stage related to abnormal bone formation could also be related to early dental deformation in fish in the present study.

Food products of animal origin are rich in protein and phosphorus, ideal for salmon. The power plant has phosphorus in the form of phytate, which does not have good absorption properties. Furthermore, phytate form insoluble complexes with calcium, which reduces its bioavailability. Phytates also inhibit the absorption of other minerals such as Mg, Fe and Zn. It could be concluded that MD can be caused flour based food plant.

The proximate analysis was performed in order to obtain values of bone mineral in this species, showed lower percentages than those in work are for other species. Shearer & Hardy (1987) reported vertebral levels of P (with fat) in rainbow trout average of 8.8 % and 6.2 % deficiency. Ogino & Takeda (1978) reported that rainbow trout vertebra normally containing 6.5 % P and deficient vertebrae contains 3.2 % P (not fat-free). In salmon were reported to contain 7.8 % of P in the vertebra normal (lean) and 3.3 % of P in fish vertebrae P deficient (Watanabe *et al*., 1980). Roberts *et al*. (2001) mentioned in their studies values of 12.6% P (fat free, based on dry matter). Values found in this study showed much lower percentages than those published.

By comparing the values of % Ca, % P, % Mg and Zn of the jaws with MD and healthy with Mann Whitney accepted hypothesis mentioned that these mineral values do not vary between affected salmon and controls. In terms of percentage of Ca in the vertebrae there was no difference, but there was a significant difference in the percentage of P, indicating that there is less P in affected fish vertebrae. This could indicate the presence of a disease that affects the fish altogether, such as for example, a nutritional deficiency or demineralization as indicated by Kacem & Meunier (2003), more so than a specific localized alteration or injuries to the area.

The Spearman correlation indicated that the percentages of the minerals studied in dental bones and spinal segments are uncorrelated, which shows that the values of the minerals found in the different sectors of the fish can not be compared because they are related. The mineral analysis values of the spine were lower compared with the dental bone, probably due to the fact that in the chemical analysis of the backbones intervertebral discs were included, ligaments, cartilage, notochord, muscle and fatty tissue that could be completely removed by dissection.

A comparison of dental deformed bones and controls by Mann Whitney test indicated that there is no difference between calcium, phosphorus, zinc and magnesium, which may conclude that there is a lower percentage of minerals in the MD. This could be explained by the fact that the percentage of mineral fully considered both dental bones while the histology is made on an affected area, which covers an area of low amount of calcium deposits. Besides the distribution of Ca and P is not homogeneous in dental bone at the start of sexual differentiation, product mineral resorption from the skeleton including the operculum, articular bone and dental toward kype (Shearer & Hardy; Sugiura *et al*.).

This paper establishes and confirms that deformation affecting mandibular alveolar bone, joint, and hyomandibular square adult salmon. The Von Kossa histochemical technique showed significant differences between controls and deformed fish, and also showed differences between the segments of jaw bone. No differences were found between bone mineral and deformed dental checks, but less phosphorus was found in salmon affected vertebrae.

ROJAS, R. M.; RAMÍREZ, M. E & DEL SOL, M. Estudio morfológico y análisis de minerales de la mandíbula inferior de salmones del atlántico (*Salmo salar*) adultos con deformación mandibular, provenientes de Escocia. *Int. J. Morhphol., 34(2)*:1097-1104, 2016

RESUMEN: La deformación mandibular es una patología que afecta al hueso dentario de salmones adultos, se observó en Noruega y en Chile, ocasionando disminución de peso, baja calidad de peces cultivados y aumento de la mortalidad. Las causas varían desde temperaturas elevadas al estado de ovas, hasta alimentación deficitaria en fósforo o vitamina C. Este trabajo tiene como propósito analizar esta deformación mediante una técnica histoquímica y de análisis de minerales durante un episodio presentado en centros de mar de Escocia. Se utilizó la mandíbula y segmento vértebral de 21 salmones del Atlántico de Escocia. Estas muestras se clasificaron en tres grupos Grupo 1: Severamente deformes. Grupo 2: Levemente afectados. Grupo 3: Controles normales. Cuatro mandíbulas por grupo fueron fijadas en formalina al 10 % y se incluyeron en paraplast, se realizaron cortes de 5 µm utilizando un micrótomo Microm®. Se utilizó la técnica histoquímica de Von Kossa para la detección de depósitos de calcio la cual destaca al calcio de color negro y el osteoide de color rojo. Para el análisis químico proximal, y con el propósito de obtener y comparar niveles de calcio, fósforo, zinc y magnesio en los huesos se utilizó un total de 9 mandíbulas (6 afectadas con DM y 3 controles) y sus 9 secciones corporales. Para comparar estos valores entre salmones deformes y controles se utilizó la prueba de Mann-Whitney. Para correlacionar los valores de mandíbula y segmento corporal se hizo una correlación por jerarquías de Spearman. Los peces del grupo 1, presentaron una desviación ventral del cuerpo del hueso dentario. En los peces del grupo 2 se observó la prominencia de la articulación visible en ambos lados o unilateralmente. Al comparar los valores de % Ca, % P, % Mg y Zn de las mandíbulas con DM y sanas con el método de Mann Witney se encontró que los valores de estos minerales no varían entre salmones afectados y controles. Hubo una diferencia significativa en el porcentaje de P, lo cual indica que existe menos P en vértebras de peces afectados. La correlación de Spearman señaló que los porcentajes de los minerales estudiados en huesos dentarios y segmentos vertebrales no están correlacionados. Por el contrario, la técnica Von Kossa mostró que la distribución de Ca/ P no es homogénea en el hueso dental producto de la reabsorción mineral desde el esqueleto incluyendo el opérculo, hueso articular y dental hacia la kype. El presente trabajo establece que la técnica histoquímica de Von Kossa fue la que permitió observar diferencias importantes entre peces deformados y controles, además mostró diferencias entre los distintos segmentos del hueso dentario. El hueso dentario es una estructura dinámica adaptada a continuos cambios histológicos pudiendo estar involucrados en la DM, dietas deficientes de fósforo, sumado a la formación inicial del Kippe.

PALABRAS CLAVE: Hueso dentario; Esqueleto de salmón; Deformación mandibular; *Salmo salar*; Von Kossa.

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