Report for Short Term Scientific Mission

CA15224

Applicant: Beryl Eusemann

Home Institution: Friedrich-Loeffler-Institut, Institute of Animal Welfare and Animal Husbandry
Host/Host Institution: Prof. Alejandro Rodríguez Navarro / Universidad de Granada, Departamento Mineralogía y Petrología

Start and End of STSM: 05.02.2018 – 16.02.2018

1. Purpose of the STSM:

The aim of this STSM was to deepen the cooperation which exists between Alejandro Rodríguez Navarro's (University of Granada) and our working groups since the EU COST meeting in Ljubljana in 2017. ARN group had analyzed the composition and bone strength of tibiotarsi of some of the laying hens of our experimental study. Therefore, I was interested in learning the techniques which they used for the analyses: Thermogravimetric analysis, Fourier-transform infrared spectroscopy, X-ray diffraction, three-point bending test and scanning electron microscopy.

Moreover, we aimed to analyze more samples as not all treatment groups had been analyzed so far.

Lastly, we wanted to plan a possible publication about our results and to write an abstract for the 45th European Calcified Tissue Society Congress in Valencia.

2. Description of the work carried out during the STSM:

2.1 First week – learning the methods

During the first week of this STSM Alejandro's working group showed me the methods which they use to assess bone strength and bone composition.

2.1.1 Preparation of the bones for analyses

All bones are stored at -20°C before analyses and sent on dry ice to Alejandro's lab. Usually, tibiotarsi are taken for analyses.

Before beginning with the analyses, all bones are weighed and the length as well as the minor and major diameter of the long bones are measured. After these measurements, the strength of the bone is measured via three-point bending test (see below). Thereafter, a part of the center of the diaphysis of about 1.5 cm is taken out with a Dremel cutting tool. This part is cut longitudinally into four parts. These parts are called slices. For each slice, the bone marrow with the medullary bone, if present, is separated from the cortical bone and both parts are stored in different tubes. Each cortical slice is then weighed and its length, width and thickness are measured. Afterwards, a three-point bending test of the slices is performed (see below). Finally, three of the cortical slices are pulverized for infrared spectroscopy and thermogravimetric analysis while the fourth slice is stored intact for X-ray diffraction. Furthermore, two round slices, about 0.5 cm thick, are taken out of the diaphysis of the whole bone for scanning electron microscopy. After the whole preparation procedure, all parts are stored at -20°C until the analyses are performed.

2.1.2 Three-point bending test

Two different three-point bending tests are performed with the bones: a three-point bending test of the whole bone and a three-point bending test of the bone slices. For the whole bones, a weight with the ability to produce a maximal force of 500 N is installed and the tibiotarsus is laid down on the test apparatus. The weight is approaching the bone with a defined velocity and, once it has reached the bone, applies pressure on the bone until it breaks. The strength which is needed to break the bone is recorded. Moreover, the program calculates the strength per area to account for the fact that thicker bones may be stronger just because of their size and not because of a higher stability of the tissue. For this calculation, it is necessary to provide the program with information about the bone diameter.

For the three-point bending test of the bone slices, the same principle and the same machine are used, but the weight used for the bone slices is only able to produce a maximal force of 50 N. For the calculation of the strength per area, it is necessary to provide the program with information about the mean thickness of the slice.

2.1.3 Fourier-transform infrared spectroscopy (FTIR)

The Fourier-transform infrared spectroscopy is used to qualify and quantify substances which are present in the bone. The principle behind this technique is that different

molecules absorb different frequencies of radiation, such as infrared radiation. This absorption can be measured and thus, the composition of materials can be analyzed. With the Fourier-transform infrared spectrometer, the sample can be analyzed pulverized or intact. For analyses of the tibiotarsus, cortical bone is pulverized. Before the first sample is analyzed, the background has to be measured to account for any substances around the detector which give a signal. This background is subtracted automatically from the spectrum of the following samples. Afterwards, the sample is put against the measuring window. The measurement is started. All bone samples are analyzed with 32 spectra. The generated infrared spectrum, with wavenumbers from 4000 cm^{-1} to 400 cm^{-1} (a unit for the frequency), is processed with a program which has been developed by the working group in order to get the relative amount of the main bone chemical components. In the bone the peaks of the spectrum indicate the presence and amount of the following components: water, lipids, proteins, carbonates and phosphates as well as the mineral crystallinity and collagen cross-linking.

2.1.4 Thermogravimetric analysis (TGA)

The thermogravimetric analysis is used for quantifying the amount of water, organic matter, phosphate and carbonates in the bone. All these substances are lost at different temperatures. The weight loss is used to determine the relative amount of each component. A small amount (about 40 mg) of cortical bone or bone marrow is put in small silver capsules which have been weighed before. The charged capsules are weighed again and the difference between the two weights indicates the weight of the bone material in the capsule. Afterwards, the charged capsules are heated in a muffle furnace at 200°C for 30 minutes and, after having cooled down, weighed again. The weight difference gives the amount of water in the sample because all the water vaporizes at 200°C. The charged capsules are now heated at 600°C for 30 minutes and weighed again. The weight difference gives the amount of organic matter which is combusted at 600°C. Finally, all charged capsules are heated at 900°C and weighed for the last time. The weight difference shows the amount of carbonates which decompose above 600°C. Finally, the residual weight is the phosphate mineral which is stable at 900°C.

2.1.5 X-ray diffraction (XRD)

Alejandro's working group uses two dimensional X-ray diffraction to determine the orientation of the hydroxyapatite crystals making the bone mineral. The principle behind this method is that crystal planes diffract X-rays at specific angles. By measuring the variation of the intensities of diffracted X-rays, detailed information about the orientation of crystals can be obtained. The intact bone slices are used for this analysis. In order to analyze

several bones at once, i.e. without having to start the diffractometer for each bone, Alejandro's working group mounts 30 bone slices on a ruler, with a distance of 1 cm between two slices. This ruler is installed in the diffractometer and, with a special program which has been written for this purpose, moves 1 cm after each measurement, so that all 30 bones are analyzed one after the other. The duration of the analysis of one bone slice is 1 minute. The generated 2D diffraction pattern can be used for the determination of the orientation of the crystals in each bone sample. The degree of orientation of the crystals in the bone mineral indicates the maturity of the bone tissue. A high degree of orientation is characteristic of a mature bone.

2.1.6 Scanning electron microscopy (SEM)

Scanning electron microscopy is used to see the structure of the bone, e.g. whether medullary bone is present and how thick the cortex is. For this purpose, the round bone slices, about 0.5 cm thick, which were taken out from the diaphysis (see 2.1) are embedded in epoxy resin, polished and coated with carbon. Then, the slices are observed on a scanning electron microscope using back-scattering imaging. With this image, the thickness of the cortex and distribution of medullary bone can be measured as well as other characteristics of the bone (porosity, microcracks). In our case, we used SEM to see in which treatment groups medullary bone was present.

2.2 Second week – Analyzing our bone samples

During the second week of this STSM, we started to analyze bone samples of my working group which had been sent to Granada by my colleagues. In our experiment at FLI in Germany, we investigated the effect of egg production and 17ß estradiol on bone health in laying hens. Therefore, we had an experimental setup with two different layer lines: the high performing layer line WLA (320 eggs per year) and the low performing layer line G11 (200 eggs per year). In each layer line there were four different treatment groups:

Group S had been given an implant which prevented egg production

Group E had been given an implant containing 17ß estradiol

Group SE had been given both implants and therefore did not lay eggs but had a similar concentration of 17ß estradiol in plasma compared to control hens

Group C were untreated control hens

To find out how these treatments and how laying performance influenced the composition and strength of the bones, Alejandro's working group had already analyzed tibiotarsi of

control hens of both layer lines as well as group S of the layer line WLA. The samples which we analyzed during this STSM were tibiotarsi of groups E and SE of the layer line WLA in order to get results of all treatment groups of this layer line.

We determined breaking strength of the whole bone and of the bone slices with the three-point bending test and prepared the tibiotarsi for all other analyses. As there was no time left for the remaining analyses of tibiotarsi, these were performed without my help after this STSM. In addition to the tibiotarsus, we analyzed the trabecula intermedia of the sternum in order to get values of a bone which is very close to the keel bone. The trabecula intermedia was analyzed with TGA and FTIR during this STSM.

Beside the work in the laboratory we had a look at and performed statistical analyses of all the results which we had so far. These were the results from groups S and C of the layer line WLA as well as of group C of the layer line G11.

3. Description of the main results obtained:

Performing the statistical analysis of the samples which had already been analyzed before this STSM, we got the following results:

3.1 Comparison of bone strength and composition between control hens of both layer lines

There were significant differences between control hens (group C) of the high performing layer line WLA and the low performing layer line G11. Tibiotarsi of WLA were significantly longer ($p<0.001$), thicker in diameter ($p<0.01$) and heavier ($p<0.05$) compared to tibiotarsi of G11. The bone strength of the whole bone, when corrected for area, was higher in G11 than in WLA ($p<0.05$). In contrast, the strength of the bone slices was higher in WLA, both as breaking strength ($p<0.05$) as well as when corrected for area ($p<0.05$). Cortical bone of G11 showed a higher degree of mineralization compared to cortical bone of WLA ($p<0.05$) which showed a higher degree of organic matter ($p<0.001$). Also medullary bone of G11 showed a higher degree of mineralization ($p<0.001$) and a lower degree of organic matter ($p<0.001$) compared to medullary bone of WLA. G11 cortical bone mineral had a greater crystallinity ($p<0.05$) and higher degree of carbonate substitution ($p<0.01$) which are all indicative of increased bone mineral maturity. This could indicate that bone metabolism (turnover rate) was greater in high performing hens due to the higher calcium demand for eggshell formation.

3.2 Comparison of bone strength and composition between group S and group C of the layer line WLA

We found significant differences between bone breaking strength and bone composition between the hens which had been prevented from egg laying (group S) and control hens (group C) of the layer line WLA. Bone diameter of tibiotarsi was greater in group S compared to group C ($p<0.05$). Bone breaking strength of the whole bone as well as of the bone slices was higher in group S compared to group C ($p<0.001$ for both). The degree of mineralization of cortical bone was significantly lower in group S hens compared to group C hens ($p<0.05$). Moreover, the hydroxyapatite crystals in group C showed a higher degree of orientation ($p<0.05$) which is indicative of increased bone mineral maturity. Medullary bone was present in tibiotarsi of group C but not of group S hens.

4. Future collaboration with the host institution (if applicable):

This STSM has deepened the collaboration between Alejandro's and our working group and we are definitely going to collaborate in the future. We have planned that we are going to send them the last bone samples which still have to be analyzed, i.e. the bones of the treatment groups S, E and SE of the layer line G11.

5. Foreseen publications/articles resulting from the STSM (if applicable):

During this STSM we submitted an abstract to the 45th European Calcified Tissue Society Congress which takes place in Valencia from 26th to 29th May 2018. The topic of the abstract are the differences in bone strength and composition between the control hens of both layer lines (WLA and G11), i.e. the comparison of a high performing layer line with a low performing layer line. We are still waiting for an answer concerning the acceptance of this abstract.

Moreover, we submitted an abstract to the 15th European Poultry Conference which takes place in Dubrovnik from 17th to 21st September 2018. In this abstract we present the results of the radiography which we have performed at FLI in Celle together with the results of the analyses which were performed in Granada of group S and group C of the layer line WLA.

Furthermore, we are planning to write a paper about our results. Before starting to write this manuscript, we first want to get all the results, also of the samples which have not been analyzed so far, in order to be able to include all treatment groups of both layer lines in the publication.