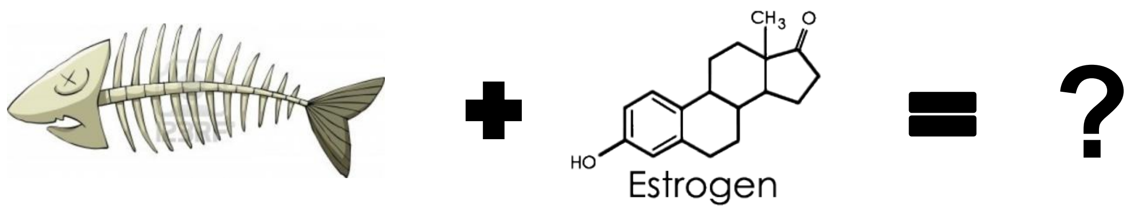
Universidade do Algarve

Faculdade de Ciências e Tecnologia

Estrogen and genistein modulation of bone homeostasis in the teleost, sea bass *Dicentrarchus labrax*: a proteomic approach



Rui Miguel Rodrigues dos Santos

Tese

Mestrado em Biotecnologia

Supervisors: Prof. Deborah M Power

Dr. Antonio Ibarz Valls

2015

Universidade do Algarve

Faculdade de Ciências e Tecnologia

Estrogen and genistein modulation of bone homeostasis in the teleost, sea bass *Dicentrarchus labrax*: a proteomic approach

Rui Miguel Rodrigues dos Santos

Tese

Mestrado em Biotecnologia

Supervisors: Prof. Deborah M Power

Dr. Antonio Ibarz Valls

Verified: Ithenticate plagiarism detector – similarity score with previously published work 12%.

ExcellentPassed: Prof DM Power

Declaração de autoria de trabalho

2015

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Este é um trabalho de tese de mestrado e o conteúdo é da exclusiva responsabilidade do autor:_____________________________________

A Universidade do Algarve não tem o direito, de arquivar e publicitar este trabalho através de exemplares impressos reproduzidos em papel ou de forma digital, ou por qualquer outro meio conhecido ou que venha a ser inventado. Não têm o direito de divulgar através de repositórios científicos e de admitir a sua cópia e distribuição com objetivos educacionais ou de investigação, não comerciais.

Acknowledgements

Esta tese foi realizada no Centro de Ciências do Mar (CCMAR), Universidade do Algarve e no departamento de fisiologia e imunologia, faculdade de Biologia da Universidade de Barcelona.

Agradeço à professora Deborah Power pela oportunidade que me deu em realizar este projecto sob sua orientação e supervisão; por toda a sua disponibilidade e amabilidade em ajudar-me, pela motivação dada em cada reunião de esclarecimento, pela oportunidade de poder realizar parte do projecto na Universidade de Barcelona e especialmente por acreditar em mim e me fazer crescer tanto a nível pessoal como profissional.

Ao professor Toni por aceitar orientar parte do meu projecto tese durante a minha estadia em Barcelona; sempre disposto a ajudar e a ensinar de forma a realizar um excelente trabalho. Um agradecimento especial ao Sérgio e aos seus colegas, António e Ignaci, por me ajudarem sempre que necessário e por tornarem a minha estadia muito mais agradável.

Muito obrigado à Patrícia, Soraia e André por me terem deixado fazer parte da sua equipa de trabalho e pela ajuda e concelhos na realização deste projecto tese. Também um especial agradecimento ao Bruno pela grande ajuda na análise bioinformática dos dados obtidos.

Agradeço a todo o pessoal dos laboratórios 3.33 e 2.28 que de alguma forma fizeram parte deste percurso e me ajudaram a alcançar os meus objectivos.

Obrigado aos meus pais, irmã, família e amigos que adoro.

Obrigado á Sofia que nunca deixou de me apoiar em todo o percurso deste projecto.

Copyright ©

Abstract

The skeleton in vertebrates is in constant turnover and functions as a reservoir of minerals. The regulation of bone turnover in vertebrates is a complex process and diet and hormones have a central role. Estrogen, a steroid, which signals via multiple nuclear and membrane receptors, is important in the turnover of bone in vertebrates. In humans lack of estrogen causes osteoporosis (bone thinning). The way in which estrogen regulates bone turnover is still relatively poorly described. The present thesis reports an experiment performed to assess how estrogen and the phytoestrogen, genistein, affect the bone proteome of a teleost, the sea bass, *Dicentrarchus labrax*. For the experiment an intraperitoneal injection of 17-β-estradiol (E_2) (5mg/Kg body mass), Genistein (5mg/Kg body mass) and coconut oil (the vehicle) was administered to 30 immature sea bass (10 for each treatment) for 5 days and samples of blood and bone were collected. Plasma parameters like calcium, estrogen and vitellogenin were significantly ($p<0.05$) increased by E_2. Genistein only increased vitellogenin. The two treatment did not modify bone metabolism and tartrate-resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP) did not change significantly between treatment groups. Vertebral bone proteome was established and a total of 285 protein spots were detected and used for comparison between experimental groups. Analysis of the gels showed that 8 and 22 protein spots were differentially expressed ($p<0.05$) in vertebra from E_2 and genistein treatment respectively. Of the 8 protein modified by E_2, only 4 were identified. In the genistein treatment, of the 22 proteins differentially expressed only 10 were identified and 2 were the same as found in the E_2 group Tropomyosin alpha-4 chain (TPM4) and Myosing binding protein C cardiac type like (MYPCL3). Identification and biological process were described using Uniprot, NCBI and gene ontology. Proteins differentially expressed in both treatments that were down-regulated were related to calcium ion binding, muscle contraction, cell adhesion, transport, protein targeting and homeostasis. In conclusion E_2 and genistein did not modify indicators of bone turnover but modifications in the bone proteome occurred. A final step still required is the validation of the proteome results by Western blotting of selected proteins.

Keywords: Sea bass, 17-B-estradiol, Genistein, Bone turnover, Bone proteome

Resumo

O esqueleto em vertebrados é um tecido extremamente importante e está envolvido em diversas funções como movimento, suporte e protecção dos órgãos. Este é um tecido hematopoiético que serve de reservatório de minerais uma vez que é constituído essencialmente por cálcio e fósforo. O esqueleto é um tecido extremamente dinâmico que está em constante renovação através de um processo complexo denominado de remodelação óssea, processo este que envolve vários factores como por exemplo a acção de diversas hormonas e também a própria alimentação.

O estrogénio é uma hormona esteróide que faz parte do sistema endócrino e tem como principal função a regulação do sistema reprodutivo de fêmeas em vertebrados e também de machos embora com menor efeito. Esta hormona interactua com as células através da ligação a receptores nucleares e membranares, receptores de estrogénio, que por sua vez promovem a transcrição de vários genes importantes no desenvolvimento, homeostasia e metabolismo do organismo. Além dos estrogénios regularem o sistema reprodutivo de vertebrados, também são extremamente importantes no processo de remodelação óssea em vertebrados. Em humanos a falta de estrogénios devido a mutações ou simplesmente devido à menopausa (decréscimo da produção de estrogénios nas mulheres), provoca o aparecimento da doença osteoporose (desgaste de massa óssea). Embora existam já muitos estudos sobre esta hormona e o seu papel em vários processos biológicos, a forma pela qual os estrogénios regulam a remodelação óssea está ainda muito pouco caracterizada.

Existem vários compostos designados de disruptores endócrinos que têm a capacidade de interferir com o sistema endócrino, como por exemplo a genisteína. A genisteína é um fitoestrogénio com uma estrutura semelhante á do estrogénio e, como tal apresenta também ela efeitos estrogénicos. No entanto, pouco se sabe a cerca da função estrogénica deste composto nomeadamente o mecanismo de acção e a que concentração este composto é capaz de despoletar funções similares ás da hormona estrogénio.

Neste estudo, foi realizado uma experiência em robalo imaturos (*Dicentrarchus labrax*), no qual foram preparados 3 grupos de tratamento com 10 peixes cada: o

primeiro grupo recebeu uma injecção de 17-B-estradiol (E_2) (5mg/kg de massa corporal) com o veículo (óleo de coco), o segundo grupo recebeu uma injecção de genisteína (5mg/kg massa corporal) mais o veículo e um terceiro grupo, designado de grupo controlo que recebeu apenas uma injecção de veículo. Amostras de sangue e osso (vértebra) foram colectadas para análise cinco dias depois do início dos tratamentos. Nas amostras de sangue foram analisados os parâmetros plasmáticos de cálcio, estrogénio e vitelogenina (marcador molecular utilizado para detectar a presença de estrogénio), tendo estes dois últimos, o estrogénio e a vitelogenina, a finalidade de verificar se o implante dos tratamentos E_2 e genisteína foram recebidos com sucesso pelos peixes. Verificou-se nos resultados que o tratamento com E_2 aumentou os níveis de cálcio, estrogénio e vitelogenina no plasma, enquanto o tratamento com genisteína apenas aumento os níveis de vitelogenina ($p<0.05$).

Com o intuito de estudar como estes compostos regulam o metabolismo ósseo, foi medida a actividade das enzimas "tartrate-resistant acid phosphatase" (TRAP) e "alkaline phosphatase" (ALP), marcadores moleculares para as células osteoclásticas (degradação de osso) e osteoblásticas (formação de osso) respectivamente. Os resultados mostraram que ambas as actividades de TRAP e ALP não foram significativamente alteradas pelos tratamentos de E_2 e genisteína.

Em outra vertente deste estudo foram caracterizadas as alterações no proteoma da vértebra provocadas pelos tratamentos utilizando-se a electroforese de 2ª-dimensão (2D). Da análise dos géis de electroforese do proteoma da vértebra resultou a detecção de 285 proteínas por gel. Destas 285 proteínas, 8 foram consideradas como diferencialmente expressas pelo tratamento com E_2 e 22 pelo tratamento com genisteína ($p<0.05$). Das 8 proteínas modificadas pelo tratamento E_2, apenas 4 foram identificadas: Tropomyosin alpha-4 chain; 14-3-3 protein zeta/delta-like; Myosin-binding protein C, cardiac-type-like and Alpha-1-antitrypsin. No tratamento com genisteína, de 22 proteínas modificadas apenas 10 foram identificadas: Tropomyosin alpha-4 chain; Protein disulfide-isomerase; Lipocalin; Uncharacterized protein; Myosin light chain 3; Dermatopontin; Tropomyosin alpha-1 chain; Actin-related protein 3-like isoform 1; Fatty acid-binding protein and Myosin-binding protein C, cardiac-type-like. Estes resultados mostram que 2 das proteínas identificadas e modificadas são comuns nos 2 tratamentos, E_2 e genisteína. A identificação e o processo biológico das proteínas descrito neste estudo, foi determinado através da utilização de bases de dados como o

Uniprot (http://www.uniprot.org), NCBI (http:// http://www.ncbi.nlm.nih.gov/) e Gene ontology (GO) (http://geneontology.org/). As proteínas consideradas diferencialmente modificadas nos tratamentos, apresentam uma menor expressão em relação ao tratamento controlo e estão relacionadas com processos biológicos que envolvem ligação de cálcio, contracção do músculo, adesão celular, transporte, direccionamento de proteínas e homeostasia. De modo a validar os resultados obtidos na análise do proteoma, será necessário realizar um Western Blotting direccionado às proteínas que foram modificadas pelos tratamentos.

Em conclusão ao nível dos parâmetros avaliados no plasma, no tratamento com estrogénio verificou-se um aumento dos níveis plasmáticos de cálcio, estrogénio e vitelogenina, enquanto no tratamento com genisteína apenas houve um aumento dos níveis de vitelogenina. Em relação aos factores analisados na vértebra, ambos os tratamentos mostraram ter efeitos semelhantes uma vez que os dois tratamentos não alteraram o metabolismo do osso (TRAP e ALP), e induziram uma redução do nível de expressão de determinadas proteínas sendo que 2 delas são iguais em ambos os tratamentos.

Table of Contents

CHAPTER 1 1

General Introduction 1

1. Introduction 2

1.1. Global View 2

1.2. The vertebrate skeleton 3

1.3. Cellular organisation of bone 4

1.4. Bone Turnover 5

1.5. Bone structure (Proteome) 6

1.6. Estrogen 7

1.7. Nuclear Hormone Receptors 8

1.7.1. Nuclear Receptor Structure 8

1.7.1.1. Region A / B 9

1.7.1.2 Region DNA Binding Domain (DBD) 9

1.7.1.3. Domain D or Hinge Region 9

1.8. Estrogen receptor 10

1.8.1. Estrogen receptor isoforms 10

1.8.1.1. Isoforms ERα or ERβ 10

1.8.2. Mechanism of action 11

1.9. G protein-coupled estrogen receptor (GPER) 12

1.10. Endocrine Disruptors (EDCs) 13

1.11. Genistein 13

1.12. Bone & Proteome and treatments 14

2. Work Plan 15

CHAPTER 2 17

Estrogen and genistein modulation of bone homeostasis in the teleost, sea bass *Dicentrarchus labrax*: a proteomics approach. 17

1. Introduction 19

2. Material and Methods 20

2.1. Experimental procedure and sampling 20

2.2. Plasma analysis 21

2.2.1. Quantification of calcium plasma levels 21

2.2.2. Radioimmunoassay (RIA) for hormones 21
2.2.3. Plasma vitellogenin relative levels 22
2.3. Tartrate-resistant acid phosphatase (TRAP) and Alkaline phosphatase (ALP) activity 22
2.4. Vertebra proteome analysis 23
2.4.1. Protein Extraction 23
2.4.2. Two-dimensional gel electrophoresis (2-D) 23
2.4.3. Gel image analysis 24
2.4.4. Statistical analysis 25
2.4.5. Database search and Protein identification 25
3. Results 26
3.1. E_2 and Genistein tissue responsiveness 26
3.1.1. Effects of E_2 and Genistein on plasma parameters 26
3.1.2. TRAP and ALP activities in sea bass vertebra 28
3.2. Analysing the vertebra proteome 28
3.2.1. Detection of putative differentially expressed proteins 28
3.2.2. Identification of putative differentially expressed proteins 32
3.2.3. Group of proteins by putative of biological process 34
4. Discussion 36
4.1. E2 and genistein in bone homeostasis 36
4.1.1. Plasma analysis 36
4.1.2. TRAP/ALP 37
4.2. Sea bass proteome 37
5. Conclusion 43
6. References 44

CHAPTER 1

General Introduction

1. Introduction

1.1. Global View

The skeleton in vertebrates is a very important structure that permits movement, provides support and protects soft internal tissue, serves as a reservoir of minerals and is haematopoietic. It is an extremely dynamic structure because it is in constant renewal through several complex processes that are regulated by various hormonal factors such as estrogen, calcitonin and also diet.

Estrogen is a steroid hormone that regulates the female reproductive system in vertebrates. In the last decade the hormone estrogen has been intensely studied in order to understand its function in other processes than reproduction such as bone homeostasis in mammals. The interaction of estrogen with cells is regulated by nuclear receptors, the estrogen receptors (ER), that promote transcription of genes when they bind to the response elements in target genes that are implicated in development, homeostasis and metabolism. There are two types of ER in tetrapod and at least three have been found in fish (ER α and two ERβ). The mode of action of ERs is complex because these receptors form dimers (homo- dimers or hetero - dimers) that interact with DNA. Despite this ER receptors, estrogens can act also by membrane receptors (GPER) that mediates a non-genomic response (Samartzis et al. 2014).

The importance of estrogen for skeletal homeostasis is evident when the impact on the skeleton of menopause is considered. At menopause when the production of estrogen ceases problems such as osteoporosis (bone thinning) become common. Although the lack of estrogen has been clearly associated with osteoporosis the way estrogen regulates bone remodeling is still poorly characterised.

A number of organic and inorganic substances interfere with the endocrine system. Such substances when present in the environment can interfere with animal physiology and are known as endocrine disruptors. These compounds act in the body mimicking and competing with hormones by binding to their specific receptors. Certain compounds such as genistein have a similar structure to the hormone estrogen and are considered natural endocrine disruptors (EDCs). Genistein is an isoflavone belonging to the phytoestrogen group and can be found in vegetable products such as soy. Increasing interest in phytoestrogens has occurred over the last decade as a potential means to

remediate the decline of estrogens in women as they age, as such they have become a target for Biotechnology. In addition, because endocrine disruptors (EDCs) are present in the diet and other household products their potential impact on public health has become an issue of interest (Pinto, Estêvão, and Power 2014). Another fact that allies Biotechnology to this study is the theme of animal production and more specifically, aquaculture, the production of fish for human consumption. In the development of modern aquaculture the basic compounds of fish feed are fishmeal and fish oil that are now very expensive and of limited availability. The further growth of the aquaculture sector will require development of alternative fish feeds and this is under development and fish meal is being substituted by plant material such as soy oil and soy meal.

Taking into consideration the rich source of phytoestrogens in fish feed there is an urgent need to study and evaluate EDCs in plant material. It is important to understand what dosages and under what conditions EDCs pose a threat to fish production but also to the end consumer, the public.

1.2. The vertebrate skeleton

Vertebrates have a highly organized body plan and possess a vertebral column (Warren, F, and K 1994), a skull to protect the brain, a muscular system that allows complex movement and a central nervous system consisting of the brain and spinal cord. The vertebral column and skull are part of the skeleton that is composed of two types of tissue, bone and cartilage. The vertebrate skeletal system consists of a skull, spine and limbs, although elements may be absent or vestigial in some species (Warren et al. 1994). The skeleton can be divided into two parts: the exoskeleton, fish scales and the endoskeleton that supports, protects and permits movement.

The evolution of the skeleton in vertebrates has been shaped by different physical and chemical properties of the medium they inhabit (land, sea or air). Air is much less dense than water which promotes a lower resistance to movement but water provides buoyancy which facilitates locomotion of aquatic living beings (Warren et al. 1994) and has moulded the external and internal anatomy of vertebrates.

The vertebral column is a characteristic of all vertebrates and has a key role in locomotion and support. In water gravity is lower than on land, so the vertebral column

in fish has a more important function in locomotion (Hall 2005). The vertebral column of fish functions to produce the thrust necessary to overcome the drag of water and also has to be strong enough to withstand compression forces during movement and prevent body "shrinkage" when the muscle fibers contract. The vertebral column in fish is adapted to its role in movement and it allows fish to move with their characteristic ripple movement.

At the cellular level, fish bone appears to resemble terrestrial vertebrate bone, although the disposition of cells in the bone differs. The skeleton is composed of a several bone types eg. fibrous, dermal chondroid and the chondrocytes in cartilage and osteoblasts, osteoclasts and osteocytes in bone are responsible for skeletal turnover and balance breakdown and synthesis so that homeostasis is maintained (Warren et al. 1994).

1.3. Cellular organisation of bone

Osteoblasts are mature cells formed from stem cells called mesenchymal cells (MSC). Osteoblasts are not capable of division but are metabolically active and are responsible for the synthesis of organic bone matrix components such as collagen type I, osteopontin, osteocalcin, among others (Judas et al. 2012). Non-collagenous proteins such as osteocalcin are responsible for mineralization and promote the complex between collagen and hidroxyapatite crystals. Osteoblasts also produce multiple growth factors that play a fundamental role in bone matrix as well as in osteoclast activity (Hall 2005). Osteoblasts regulate both bone formation and remodelling and this can be monitored by measuring alkaline phosphatase. Alkaline phosphate (ALP) has a key role in bone mineralization although its exact function is still not fully understood (Golub and Boesze-battaglia 2007).

Osteoclasts are cells that play a significant role in resorption and remodeling of bony tissue. Mononuclear precursors originate from the bone marrow, join the bone matrix forming multinucleate osteoclasts (Judas et al. 2012). Bone resorption is a complex process coordinated by hormones and cytokines that cause osteoclasts to secrete proteolytic enzymes that are involved in the destruction of bone matrix. Tartrate-

resistant acid phosphatase (TRAP) is highly expressed by osteoclasts and is a marker of osteoclast activity in vertebrates (Guerreiro et al. 2002).

1.4. Bone Turnover

Bone is an active and dynamic tissue that is in constant remodeling throughout life. The main processes occurring during development is primarily the growth of bone, and this occurs until the bone/skeleton achieves its final shape and size. In adults the skeleton is fully formed and the bone turns over and the main metabolic activity is bone remodelling (Amadei et al. 2006).

Remodelling is a complex process involving the removal of old bone (resorption) and its replacement with new bone, the overall action is to degrade bone or replace damaged areas (Figure 1.1). The factors that underlie bone remodelling include mechanical stress, cytokines, hormones, prostaglandins, growth factors that act on or are released by bone specific cells, the osteoblasts and osteoclasts (Hall 2005). Little is known about what triggers remodelling but the first step involves recruitment of osteoclasts to remodel bone via physical and hormonal stimuli. The differentiation of osteoclast progenitor cells occurs only by cell - cell interaction with osteoblasts. Fully differentiated osteoclasts resorb bone by releasing protons to generate an acid environment and released proteolytic enzymes degrade the organic phase of the bone. Another hypothesis of bone remodelling is the the existence of a receptor, osteoprotegerin (OPG) synthesized by osteoblasts that prevents binding of receptor activator of nuclear factor kappa-B ligand (RANKL) to receptor activator of nuclear factor kappa-B (RANK) and suggests osteoblasts control bone remodeling by activating or inactivating osteoclasts (Amadei et al. 2006).

The process of bone remodeling is considered to be a process by which damaged areas of bone can be removed and the bone structure optimized in favor of its "mechanical action" (Judas et al. 2012). The existing model for bone remodelling suggests that bone is first remodelled from a metabolic perspective and mechanical considerations are less important. The structure and functional adaptations of bone are normally in equilibrium with the metabolic function however, if for some reason this balance is not achieved the metabolic function prevails over structural needs.

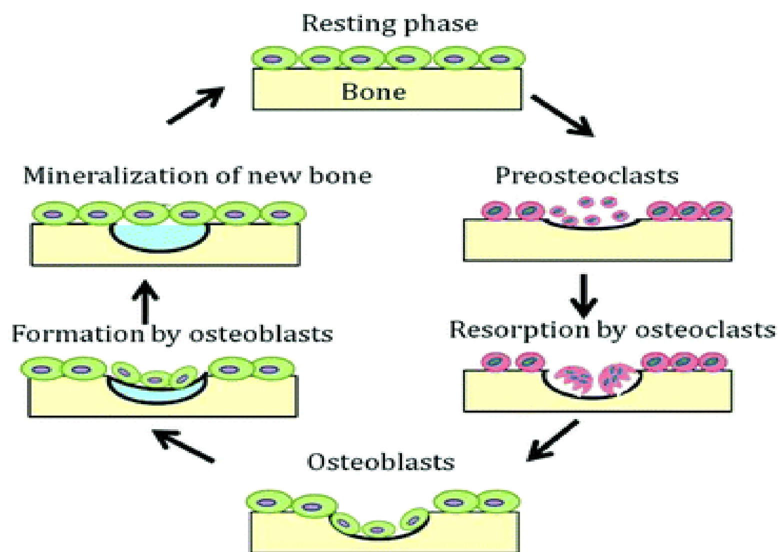


Figure 1.1. Scheme showing a model of bone turnover. The model consist of a phase of resorption by osteoclasts and then a second phase involves the formation of new bone by osteoblast (Mawani et al. 2012).

1.5. Bone structure (Proteome)

Bones are rigid organs that form the skeleton of vertebrates, they are complex structures and consist of multiple components such as matrix protein, collagen fibers, mineral component and bone cells. Other tissue found in bones includes nerves, blood vessels, cartilage and bone marrow. Although some knowledge exists about the formation of bone, bone remodelling and associated metabolism, the bone proteome has been difficult to study because of the complex and rigid structure of bone.

Nonetheless some notions about the bone proteome exist. Collagen is an extremely important component of the extracellular matrix of bone. Collagen is a fibrous protein and there are at least five isotypes of collagen and the main form of collagen in bone is collagen type I. Several other non-collagenous proteins are present in bone and are important for stabilization, calcification, and metabolism. The proteins in bone may originate from plasma and others are synthesized by bone cells. The most abundant proteins are osteocalcin, osteonectin and other less abundant proteins include bone proteoglycans, and constituents of cartilage adhesive proteins such as fibronectin, laminin, various growth factors, among others (Anjos et al. 2013).

The present project will build on results of the host group about calcium homeostasis (Guerreiro et al. 2002), and the role of parathyroid hormone-related protein PTHrP in bone turnover (Anjos et al. 2013). The role of estrogens and phytoestrogens in

the turnover of bone and their impact on the fish vertebrate proteome will be established and the results compared with studies in other vertebrates.

1.6. Estrogen (Biosynthesis)

Estrogens are a group of steroid compounds derived from cholesterol with a polycyclic structure (Figure 1.2) and are the primary female sex hormones in vertebrates. Their biosynthesis involves a complex series of enzymatic reactions mainly characterized by catalyzing oxidase P450 (CYPs), dihydro-steroid dehydrogenase (Payne and Hales 2004).

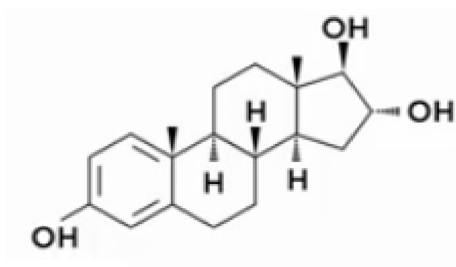


Figure 1.2. Estrogen molecule derived from cholesterol, contains four carbon atoms.

Three main estrogens have been identified; i) estrone (E1), ii) estradiol (E2) and iii) estriol (E3). The estrogens arise via catalysis of the androgens, testosterone and androstenedione, by aromatase (arom P450, Figure 1.3).

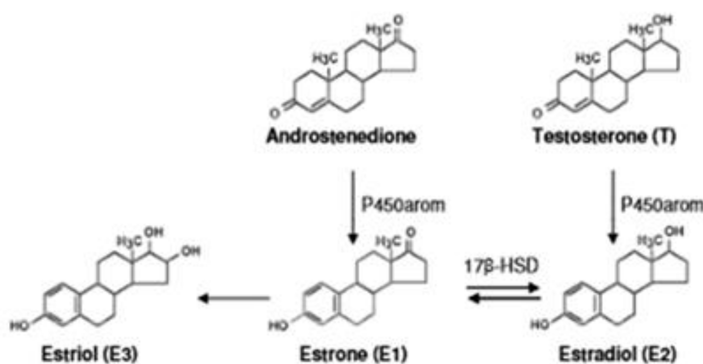


Figure 1.3. Formation of Estriol, Estrone and Estradiol from the androstenedione and testosterone compounds. This reaction is catalysed with P450 aromatase.

1.7. Nuclear Hormone Receptors

Nuclear hormone receptors are a class of proteins that when activated by a ligand, act as a transcription factor and regulates gene expression by interacting with specific DNA sequences, the hormone response element (HRE). The receptor recognizes and binds to HREs in the target gene and then switches on or off gene transcription within the target cell nucleus. This process involves transcription and translation of proteins which is a process of slow response. These genomic responses can control the development and differentiation of the skin and bone and also regulate reproductive tissues among other things.

1.7.1. Nuclear Receptor Structure

A nuclear receptor has a modular structure with distinct regions and autonomous function (Figure 1.4). Nuclear receptors are composed of a variable N-terminal region, a highly conserved sequence for DNA binding, a hormone binding region and well conserved C-terminal domain (Aranda and Pascual 2001).

- Variable amino terminus (NH2, A / B);
- DNA binding domain (DBD) or conserved C region;
- Hinge Region (D);
- A conserved ligand binding domain (LBD) or E region;
- A C-terminal (COOH) region (F).

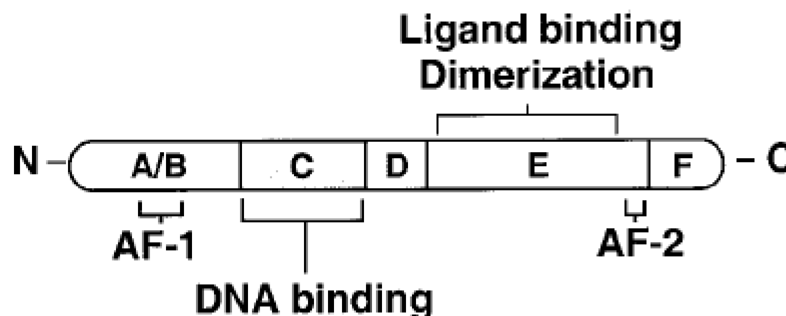


Figure 1.4. Schematic representation of the general structure of a nuclear receptor. This structure is composed of a variable N-terminus (A/B) which can contain a AF-1 domain, a DNA binding domain, DBD (C), a hinge region (D), a ligand binding, LBD (E) and a COOH end region (F) usually containing a AF-2 domain. (Aranda and Pascual 2001)

1.7.1.1. Region A / B

The region A / B of a nuclear receptor is considered the most variable both in size and sequence and usually contains an AF-1 domain. The AF-1 domain weakly activates gene transcription in the absence of a ligand (ligand independent). Several isoforms of steroid receptors frequently exist that result from alternative promoter usage or splicing and generate proteins that differ in the A / B region.

1.7.1.2 Region DNA Binding Domain (DBD)

DBD is the most conserved domain of nuclear receptors and recognizes specific DNA sequences termed hormone response elements (HRE) in the target gene. The domain contain two zinc "fingers" composed of nine cysteines and other well conserved residues that are essential for high affinity binding to DNA. Four cysteines form the first "finger" and generate the P box region that recognizes the DNA. The other zinc "finger" is denominated "D box" and is involved in receptor dimerization (Aranda and Pascual 2001).

1.7.1.3. Domain D or Hinge Region

The D domain has low conservation between the receptor isoforms and is situated between the DBD and LBD domains allowing rotation of the DBD.

1.7.1.4. Ligand Binding Domain (LBD)

The ligand binding domain has a moderately conserved sequence but its structure is well conserved. It is a multifunctional domain that binds the ligand and also mediates homodimerization and heterodimerization. The two best conserved regions are the motif signature and AF-2 that is responsible for ligand-dependent activation. LBD consists of 12 conserved alpha helixes numbered H1 to H12 and H12 contains AF-2 and plays a crucial role in ligand binding. This domain is also responsible for binding of coactivators and correpressores which are also called co-regulatory proteins. These have

a role in facilitating or inhibiting the transcription of a gene with the connection type of bound ligand/receptor (agonist or antagonist).

1.8. Estrogen receptor

Estrogen receptors are proteins that form part of the group of large nuclear receptors. They are receptors that are activated by the hormone estrogen, specifically estradiol-17B. These receptors when activated by estrogen have the ability to translocate to the nucleus and bind to DNA and activate the transcription of specific genes involved in regulating development, metabolism and homeostasis of the organism. Because of their mode of action estrogen receptors are also called transcription factors.

1.8.1. Estrogen receptor isoforms

The estrogen receptor (ER) can be found in the form of ERα or ERβ and each of these forms is encoded by different genes, ESR1 and ESR2, respectively (Matthews and Gustafsson 2003). ERα and ERβ are proteins with different transcription activities depending on the type of ligand, cell or promoter. Both receptors are expressed in the same cells and thus formation of ERα and ERβ homodimers or heterodimers ERαERβ may occur. Although ERα and ERβ proteins differ they have conserved structure and functional domains (eg. DNA binding domain, ligand binding, dimerization and transcriptional activities) characteristic of the superfamily of nuclear receptors (Nilsson et al. 2001).

1.8.1.1. Isoforms ERα or ERβ

Various forms of the ER have been identified in humans, however their biological functions are still poorly characterised (Figure 1.5). The transcription of mRNA ERα involves a complex process involving at least seven different promoters (Matthews and Gustafsson 2003) . At least two isoforms of ERα, a large isoform, 66 kDa and a small isoform, 46 kDa, occur as a consequence of alternative initiation ATG codons (Matthews and Gustafsson 2003).

In common with ERα the receptor ERβ has a complex regulatory system and the gene contains at least two promoter binding sites. Several isoforms of ERβ formed by alternative splicing have been identified and they may have extensive N-terminal and others have simple insertions and translocations in the C-terminal LBD domain (Enmark et al. 1997). Long 530 aa and short 485 aa (wild-type) ERβ receptor isoforms exit. Several other forms of ERβ were found to have some deficiency in exons in wild-type form and this causes reductions in some areas.

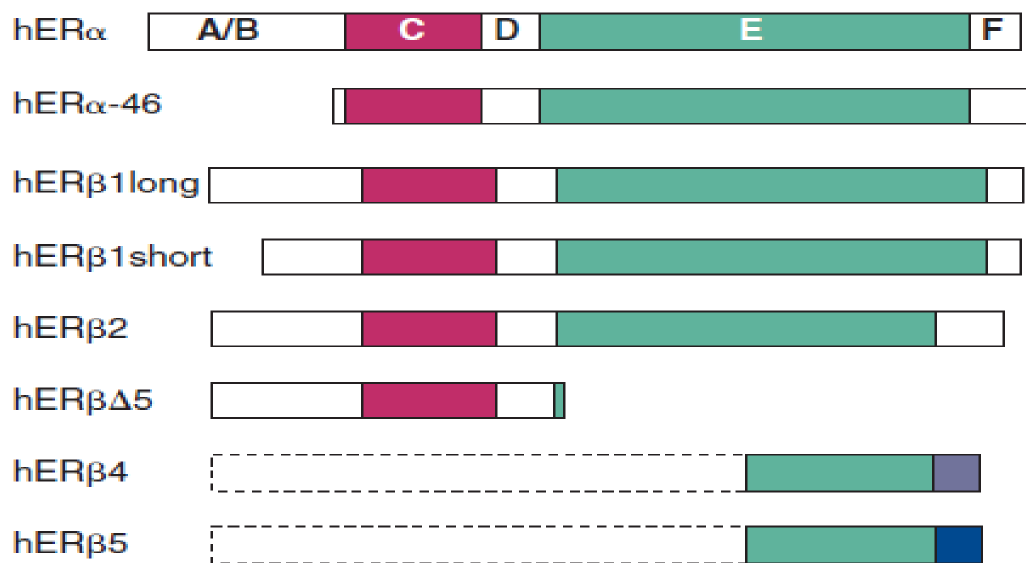


Figure 1.5. Schematic image of the human estrogen receptor α, β and its isoforms. (Matthews and Gustafsson 2003)

1.8.2. Mechanism of action

The estrogen receptors are predominantly localized in the cytosol of the cells. When the ligand estradiol binds to the receptor this promotes their migration to the nucleus where occurs dimerization and subsequent receptor binding to a specific site on DNA (HRE) occurs (Figure 1.6). This binding promotes transactivation of genes and this may be a weak ligand independent N-terminal A / B AF-1 activation or a ligand dependent process via binding to LBD – AF2. The domain LBD in addition to having a cavity for ligand binding also has binding sites for regulatory proteins. These proteins are known as coactivators or correpressores and interact with the receptor in order to facilitate inhibition (antagonist effect) or the transcription of a particular gene (agonist effect) (Aranda et al.2001).

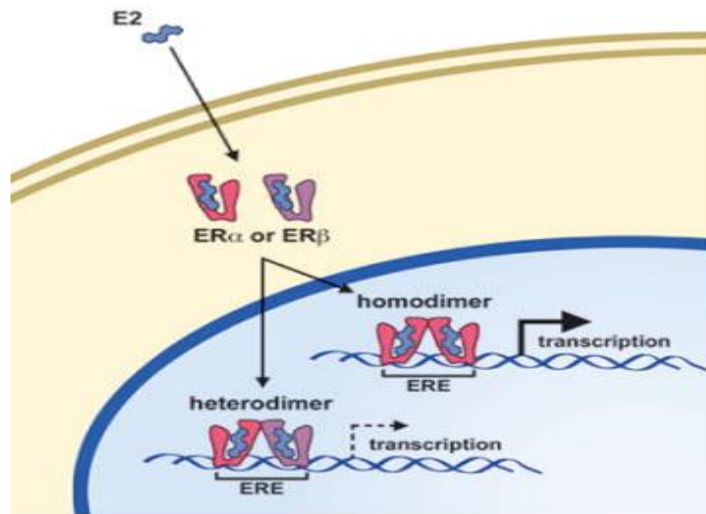


Figure 1.6. Schematic representation of action of the estrogen receptor mechanism in cells (Bean, Ianov, and Foster 2014).

1.9. G protein-coupled estrogen receptor (GPER)

GPER or formerly denominated as G protein-coupled receptor 30 (GPR30) is an integral membrane protein with high affinity for 17-B-estradiol. Estrogen is a hormone involved in several physiological processes trough activation of nuclear receptors ERα and ERβ resulting in a slow genomic response (Majumder et al. 2015). In addition to this classic response it was identified another estrogen receptor that mediates a rapid non-genomic response (GPER) (Samartzis et al. 2014). The genomic actions can take hours or days to do an effect while non-genomic effects occur in seconds or minutes (Pinto et al. 2014). This response is independent of transcriptional activity and is present in a wide variety of cells and tissues. GPER is localized in the endoplasmic reticulum and is signals can promote an intracellular mobilization of calcium, generation of cyclic adenosine monophosphate (cAMP), phosphorylation of many proteins, cell migration and proliferation (Levin 2009). It is know that this protein is widely expressed in mammalian tissues like human and hamster gonads (Majumder et al. 2015). Furthermore this protein was already described to be present in the oocyte of two teleost fishes, atlantic croaker and zebrafish (Pang and Thomas 2009) and also in scales of sea bass (*Dicentrarchus labrax*) (Pinto et al. 2014).

1.10. Endocrine Disruptors (EDCs)

Endocrine disruptors compounds (EDCs) are exogenous substances that act in a similar way to several hormones causing alterations in the physiological role of endogenous hormones. As example are the estrogenic EDCs that can have an estrogenic or anti-estrogenic effect. They can act by mimic or blocking estrogen action and by modifying the ratio concentration of hormone and receptor. Estrogenic EDCs comes from different sources as plants (phytoestrogen), fungi (mycoestrogens) and cyanobacteria, synthetic therapeutic drugs, and also chemical compounds used in industry and agriculture (Pinto et al. 2014). Many of this estrogenic EDCs are considered as persistent pollutants and eventually contaminate the seas and rivers, affecting many organisms as the case of fish. It is know that some endocrine disruptors cause problems in the reproductive system such as reduced fertility, ratio male female, changes in hormone levels, compromise immune response, and many other consequences. Besides fish health another indirect consequence of estrogenic EDCs will be the contamination of environment and the health of other living beings that consumes this contaminated fishes, which is the case of humans (Pinto et al. 2014).

1.11. Genistein

Genistein is an isoflavone that belongs to the category of phytoestrogen as it has a similar structure to estrogen and is a secondary metabolites of plant origin present in legumes and soybeans (Cooke, Selvaraj, and Yellayi 2006) (Liu et al. 2007). Isoflavones are hydrolysed by glycosidases which liberate genistein, glycitein, diadzeín and once hydrolysed these substances may have different effects :

- Estrogenic or anti estrogenic effects;
- Enzyme Inhibitory effects related to the development of cancer;
- Antioxidant.

The estrogen hormone is considered to be one of the key in the balance between bone formation and resorption however there is controversy that is also associated with adverse effects such as increase of breast cancer. Thus several studies have been

undertaken in order to observe whether these substances have positive or negative effects on human health, although doubt still remains if these compounds are beneficial in our diet. Another thing that also needs to be considered is the fact that estrogenic effects of isoflavones are generally much lower than the effect of estrogen.

1.12. Bone & Proteome and treatments

Several studies have demonstrated that estradiol modulates the activity of osteoblasts and osteoclasts by intracellular connections especially estrogen receptor ERα, which in turn trans- activate specific genes (Taranta et al. 2002)(Beck and Hansen 2004). It is therefore considered that estrogen plays an essential role in bone remodelling but however the exact mechanism of how it operates is still elusive (Imai et al. 2009). Osteoporosis is a disease that affects millions of people in the world and females are the one who suffer most from this. With advances in science have also arisen further studies in order to better understand the mechanism of action of osteoporosis and find a path to healing. One technique that has been most used is based on the hormone estrogen replacement since the lack of this hormone is the major cause of the onset of osteoporosis. Although this therapy has shown progress as to the replacement of bone mass is certain that it has also shown some controversy for the use in the treatment of this pathology, in particular by increasing the number of blood clots, endometrial cancer, breast cancer and heart disease (Mayo Clinic 2013). However this hormone replacement therapy is still not explicit due to the elusive mechanism of estrogen action.

This study aims to unravel the mechanism of action of estrogen at the level of the bone proteome. The effect of estradiol treatment was compared to genistein a phytoestrogen and should provide insight into the signalling stimulated by the estradiol and the EDC. Genistein is a compound with estrogenic effects and is present in various food stuffs, its effect on the bone proteome is largely unexplored. The results of the present study may provide insight into the effect of genistein treatment on the bone proteome and if such compounds are beneficial.

2. Work Plan

Task 1. In this project the teleost fish, european sea bass (*D. labrax*) was used as the experimental animal model. Immature sea bass (average wet weight 57 ±4.3 g and average length 17 + 2.2 cm) were kept at 18 ° C in 3 tanks of 200L with 10 fish each and fed twice daily to satiation. Each tank corresponded to one of the three treatments; control, estradiol (E_2) and genistein. One group received an intra-peritonealy injection of E_2 (5mg/kg body mass) in coconut oil, the other received an injection of genistein (5mg/kg body mass) and the control group received only coconut. Fish were exposed to the treatment for five days before collection of samples. Samples of blood and vertebra were collected for analysis (Figure 1.7).

Task 2. Measurement of plasma parameters such as calcium, vitellogenin and estrogen concentrations. Also the activities of tartrate-resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP) was determined to establish how estrogen and genistein affect bone turnover.

Task 3. Optimisation of protein extraction and 2D electrophoresis of vertebral bone protein extracts was carried out. The proteome of vertebral bone was generated by isoelectric focusing (pI 3-10, GE Healthcare) followed by separation on SDS-PAGE polyacrylamide gel electrophoresis (15,5%) (10-200 kDa, Ethan Dalt six vertical system ,GE Healthcare). After staining digital images were captured and the proteomes of the treatment groups compared to the control group. The identity of the modified proteins was determined by gel comparisons of the proteome of the sea bass generated and the sea bream (*Sparus aurata*) previously characterized and published.

Tasks 4. All data collected in the experiment was used for bioinformatics analysis using software like Gene ontology (GO), Uniprot and NCBI to determine the identity of the proteins differentially expressed by the E_2 and genistein treatment.

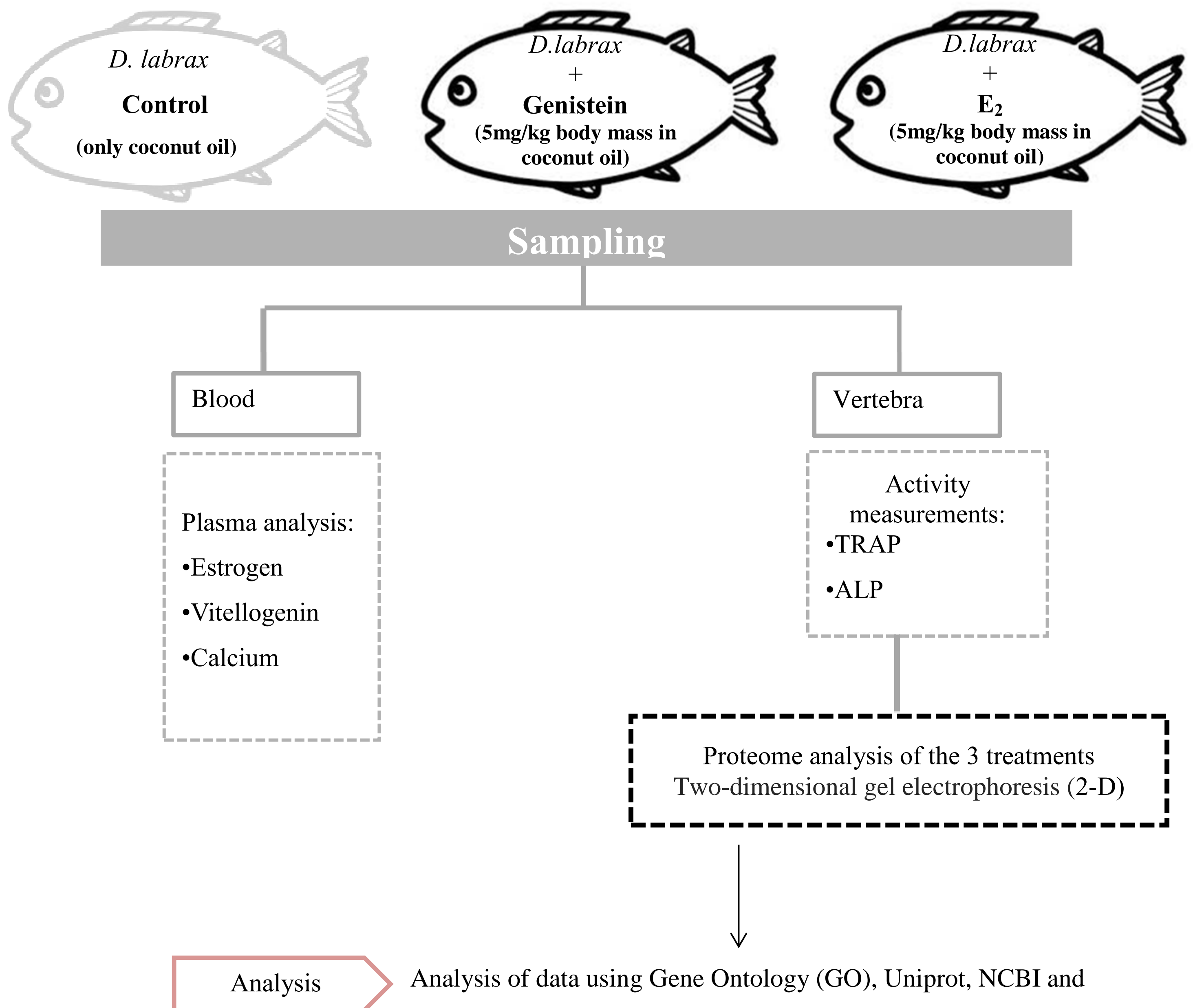


Figure 1.7.Schematic representation of the work plan projected. Three Group of 10 Sea bass (average wet weight 57 +4.3 g and average length 17 + 2.2 cm), received an intra-peritonealy and then killed after 5 days to collect samples of blood and vertebra. Samples of blood used for plasma analysis and vertebra samples to measure TRAP , ALP activity and proteome analysis with 2-DE.

CHAPTER 2

Estrogen and genistein modulation of bone homeostasis in the teleost, sea bass *Dicentrarchus labrax*: a proteomics approach.

Estrogen and genistein modulation of bone homeostasis in the teleost, sea bass *Dicentrarchus labrax*: a proteomic approach.

Centro de Ciências do Mar (CCMAR), Universidade do Algarve, Campus de Gambelas, Faro, 8005-139, Portugal

Departament of Physiology and Immunology Facultat of Biology, University of Barcelona, Barcelona, Spain

Keywords: Sea bass, 17-B-estradiol, Genistein, Bone turnover, Bone proteome

1. Introduction

The skeleton in vertebrates is a metabolically active tissue that is in constant renewal (Seibel 2005) through several complex processes that are regulated by hormones such as estrogen, proteins, cells like osteoblast, osteoclast and also diet. The fish skeletal consist in an endoskeleton like mammalians and also an exoskeleton, the fish scales (Pinto et al. 2014). Bone is a tissue that goes through cycles of bone reabsorption in way to maintain its mechanical and metabolic capacities in which the most important is to secure the metabolic process (Judas et al. 2012). This process is called of bone turnover and consists of reabsorption of old bone by osteoclasts and formation of new bone by osteoblasts (Seibel 2005). Bone is rich in minerals and the mineral homeostasis between mammals and fish is different. The terrestrial vertebrates only have one source of minerals, calcium and phosphorous that comes from diet. In fish beyond diet they also can uptake calcium from the environment. Marine water is a great source of calcium and fish only need to uptake phosphorous from diet. For fish that habit in freshwater, the calcium content is very low and they have to mobilize calcium from the mineralized tissues (Pinto et al. 2014).

Estrogen is a steroid hormone that is essential for skeletal growth, development and maintains healthy bones (Syed and Khosla 2005). Estrogen deficiency or decrease after menopause was been associated with an increase in bone resorption (Riggs 2000) and when the homeostasis of bone breaks down, several problems can occur like the disease osteoporosis (Eastell 2005). Although estrogen is considered to be the most important sex hormone in females, estrogen also contributes to the maintenance of bone in males. Biosynthesis of estradiol occurs in fish and it plays a similar role to that observed in all vertebrates (Lange, Hartel, and Meyer 2002). In some species of fish, especially in the reproductive cycle of females, estrogen have the capacity to increase calcium in plasma and decrease it from scales due the demand of vitellogenin, a protein precursor of egg yolk (Pinto et al. 2014).

It is known that estrogen has several functions and deficiency can decrease bone mass but this can be combated by estrogen replacement therapy that inhibits bone turnover and bone resorption in mammals (Beck and Hansen 2004). Estrogen interacts with cells via cytoplasmic or nuclear way, by estrogen receptors (GPER) or (ER). This receptors promote the transcription of target genes directly or indirectly, that regulates

the metabolism and homeostasis of the skeleton. Although all of these functions of estrogen hormone, is mechanism of action in reducing the bone resorption is still poorly characterized (Spelsberg et al. 1999).

A chemical of interest in the context of bone turnover is genistein. Genistein is a phytoestrogen that have similar function with estrogen and competes for the binding of ER (Wang, Sathyamoorthy, and Phang 1996). It is of interest study this phytoestrogen compounds once they are present in the agriculture industry and can contaminate the waters where fish habits. Another reason is that fish in aquaculture are feed with soy oil and so, there is an urgent need to understand if this compounds are prejudicial to fish health. In a way to understand the mechanism of action of genistein, the present study establishes the effect of genistein on bone metabolism and vertebral proteome to then compare it to the control treatment.

Bone response to estrogen and genistein was assessed by measuring markers of osteoclasts and osteoblasts, TRAP and ALP respectively. To access the bone proteome 2D electrophoresis was performed to establish how treatment with estrogen and genistein affects the bone proteome in sea bass, *D.labrax.*

2. Material and Methods

2.1. Experimental procedure and sampling

Manipulation of animals was performed in agreement with international (Guidelines of the European Union Council, 86/609/EU) and national ethical guidelines for animal care and experimentation. The work was carried out under a “Group-I” license from the Portuguese Government Central Veterinary service (Direcção-Geral de Veterinária-DGV, Ministério da Agricultura, do Desenvolvimento Rural e das Pescas) to the Centre of Marine Sciences, CCMAR-CIMAR (permit ref. Ofício Circular no. 99/0420/000/000 of 09/11/2009 from DGV) and conducted by a DGV certified investigator (DMP). The study was approved by the Animal Care and Use Committee of the Centre of Marine Sciences. Immature sea bass *D.labrax* were obtained from local fish farms and maintained at the University of Algarve Ramalhete Marine Station (Faro,

Portugal) in 500 L through-flow seawater tanks at natural temperature and photoperiod for winter and fed with commercial dry pellets at 1% body weight/day.

For 17-B-estradiol and genistein treatments, sea bass were randomly assigned to 3 tanks (n=10/tank) and maintained for one week prior to the start of the experiment under the same conditions as those indicated above, except that the water were maintained at 18ºC. Fish in one tank received an intra-peritoneal (ip) injection of coconut oil vehicle containing 5mg/kg body weight of 17-B-estradiol (E_2, Sigma-Aldrich) hormone. In another tank fish received an ip injection of 5m/kg body weight of genistein in the coconut oil vehicle (Abcam). In the third tank the fishes received a ip injection of the coconut oil vehicle only (control treatment).

Fish were kept for 5 days post injection and then fish anaesthetised with 2-phenoxyethanol in seawater (1:10000 v/v; Sigma-Aldrich, USA), weighed, measured and blood sample collected from the caudal vein using a heparinised syringe. Plasma was obtained by centrifugation of blood. After anaesthesia fish were killed by decapitation. A number of 5 or 4 vertebras were dissected out of fish, immersed in ice cold 1x PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM Kh_2PO_4) containing a protease inhibitor cocktail (1ml/20g tissue; Sigma-Aldrich, US) and dissected free of soft tissue. Cleaned vertebras were snap-frozen in liquid nitrogen and stored at -80ºC until analysis.

2.2. Plasma analysis

2.2.1. Quantification of calcium plasma levels

Total plasma calcium (protein bound plus free) was measured in duplicate in 2.5µl plasma samples from individual fish using colorimetric assays (Spinreact 1001060 and 1001150, Barcelona, Spain) (Guerreiro et al. 2002).

2.2.2. Radioimmunoassay (RIA) for hormones

Sex steroid levels were measured in individual plasma samples by RIA using specific antiserum against E_2 (Guerreiro et al. 2002). All samples from the same

experiment were quantified in duplicate in a single assay after heat denaturing samples to remove binding proteins.

2.2.3. Plasma vitellogenin relative levels

The total plasma protein concentration (mg/ml plasma) was measured in diluted samples (1/75) using the Bradford method with minor modifications for a micro-plate reader (Bio-Rad Protein Assay Kit, Bio Rad, Portugal). Bovine serum albumin (Sigma-Aldrich) was used as the protein standard.

Plasma vitellogenin (Vtg) was detected by SDS-PAGE as previously described (Guerreiro et al. 2002). Briefly, diluted plasma (1/10 in Tris Buffer pH 7.8) was mixed with an equal volume of sample buffer [6 % (w/v) SDS, 6 % (v/v) 2-mercaptoethanol, 40 % (w/v) sucrose, 0.02 % bromophenol blue in 0.125 M Tris–HCl, pH 6.8) boiled for 5 min, centrifuged (60 s, 5000 rpm) and fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 8 % polyacrylamide). Molecular weight marker (BioRad) were run on all the gels. Proteins were detected using Coomassie blue staining, scanned and digital images captured in an Alphalmager Imaging System (Alpha InnotechAlphalmager). The band corresponding to Vtg, at 180K Da (Ibarz, Pinto, and Power 2013), was quantified using ImageJ v.1.48 program after manual correction of artifacts. Vtg quantification is shown as relative pixels (intensity) per square millimeter. Plasma values are given as mean±standard deviation (SD) and compared using a one way ANOVA (SPSS 12.3 statistical software).

2.3. Tartrate-resistant acid phosphatase (TRAP) and Alkaline phosphatase (ALP) activity

To determine if E_2 and genistein modified *D.labrax* vertebral bone metabolism, TRAP and ALP activities were measured. Briefly, one vertebra was removed from each fish and cut into 4 parts and each was placed in a well of a multi-well plate. For the TRAP assay 200ul of 20 mM tartrate in NaAc buffer (0.1 M, pH 5.3) and 100 mM para-nitrophenyl-phosphate (pNPP; Sigma–Aldrich, USA) was added to the tissue and incubated at 22 ° C for 30 minutes. The reaction was stopped by adding 100 ul of 2M

NaOH and the absorbance read at 405 nm. The measurement of ALP enzymatic activity was carried out in a similar way with the exception that the reaction buffer was ALP buffer (100 mM Tris-HCl pH 9.5, 0.1 mm MgCl2, 100mM pNPP and 0.1 mM $MZnCl_2$). Assays to determine TRAP and ALP activity in vertebra were carried out in duplicated.

2.4. Vertebra proteome analysis

2.4.1. Protein Extraction

The protein extraction protocol was performed on 29 samples each composed of 4 to 5 vertebrae from an individual fish. The vertebrae were pulverized individually using liquid nitrogen and a mortar and pestle. The pulverized tissue was transferred to a 1.5 ml eppendorf to which was added 1 ml of lysis buffer (7M urea, 2M Thioreia, 50mM Tris pH 7.9, 4% CHAPS and Protease inhibitor mix (1 ml/20g)) and then tissue homogenated using a manual piston. The homogenates were sonicated twice for 10 s, incubated at 4°C for 2 h and then centrifuged for 1h at 16.500 g. Supernatant was collected and immediately frozen at -20 ºC. The amount of total protein in extracts was quantified using the Bradford method (BioRad, USA).

2.4.2. Two-dimensional gel electrophoresis (2-D)

For the first dimension electrophoresis, 600 ug of protein was diluted in 450 uL of rehydration buffer (7M Urea; Thioreia, 2% CHAPS, DTT, 2% v/v IPG buffer pH 3-10 NL; Amersham Biosciences) containing a trace of bromophenol blue. The solution was then loaded in to IPG strips 24 cm pH 3-10 (GE Healthcare Biosciense AB). Isoelectric focusing (IEF) was carried out using an IPGphor unit (Amersham Pharmacia Biotech, UK) according to the manufacturer's instructions. IPG strips 24 cm pH 3-10 were rehydrated at 50V for 12 h at room temperature and followed by : 500V 1.30ha , 1.30ha 1000V , 2000V 1.30ha , 1.30ha 4000V ; 1:30 to 8000V ; 6h at 48kV and 20h to 50V. IPG strips were equilibrated and reduced in buffer (6M urea, 30 % glycerol, CHAPS, 2% SDS, DTT 100mM) for 15 min and then were alkylated in the same buffer containing 135mM of iodocetamida for further 15 min. Equilibrated IPG strips were then transferred to the SDS-PAGE (12.5% polyacrylamide, 24 x 18 cm) and the proteins

separated using the following conditions: 15 mA/gel for 30 min followed by 30 mA/gel for a further 6h at 20 Cº .The 2-D electrophoresis was performed using a Ethan Dalt six vertical system (GE Healthcare, Barcelona, Spain). Once electrophoresis was finished proteins were fixed for 1 h in 40% v/v methanol containing 10% v/v acetic acid and stained overnight using Comassie blue G (20% v/v methanol; 1.5% v/v phosphoric acid; 12.5% w/v ammonium sulfate; 0.1% w/v Coomassie brilliant blue G-250, Bio-Rad, U.S).

2.4.3. Gel image analysis

Coomassie blue stained gels were digitalized using a scanner GS-800 calibrated densitometer (BIO-RAD, Barcelona, Spain) and digital images (at 300 dpi resolution) captured using Labscan software (BIO-RAD, Barcelona, Spain). The gel images were saved as uncompressed TIFF files. Gel image analysis was carried out using an ImageMaster^{TM}2D Platinum software, version 6.0 (GE, Healthcare, Barcelona, Spain). Five gels each containing a pool of vertebral extracts from 2 fish, were analysed per group (control, 17-B-estradiol and genistein). A consensus proteome was obtained by matching proteins on the 5 gels generated per experimental group using the automatic setting of the software followed by manual matching and editing to remove artifacts. The protein spots were quantified using the percentage volume criterion (%vol), which is automatically calculated by the ImageMaster software. Normalized protein spot values were used to generate a Master gel of each treatment (the average value of a spot present in 5 gels) and then Master gels were compared against control treatment to identify proteins modified by the 17-B-estradiol or genistein treatment. Proteins with a spot volume > 2 (double) (using the Master gels) were considered to be up-regulated and < 2 considered to be down regulated. To enhance the results, spot-by-spot supervision was carried out to correct over-or under-detected spots, and validate candidate protein spots.

2.4.4. Statistical analysis

Plasma parameters Ca, E_2 and TRAP/ALP activity was analysed by one-way analysis of variance (ANOVA) (SigmaStat, v.3.50, Systat Software, Inc, San Jose, CA, USA) after accessing normality and homogeneity of variance. Statistical significance was considered at $p<0.05$ and data is presented as mean ± standard error of the mean (SEM). For proteomic analysis the mean, mean square deviation (MSD) were determinate for all selected normalized protein spots. Comparison between protein spot volume of control, 17-B-estradiol and genistein treated samples was carried out using a Student's t-test and protein spots intensity considered to be significantly different when a difference of 5% occurred. The statistical test fused to identify differentially expressed proteins between experimental groups was performed using the ImageMater™ 2D Platinum software.

2.4.5. Database search and Protein identification

Through the analysis of the proteome with the ImageMaster software some candidate proteins were chosen because they had a significantly ($p< 0.05$) higher or lower level of expression between the different treatments. After a rigorous analysis to identify the pI of proteins using the scale of the isoelectric point (pI) for Immobiline Dry strips (pH 3-10 NL, 24 cm) and molecular weight (Mw) markers for SDS-PAGE it was possible to determine the pI and Mw of each candidate protein.

To proceed with identification of proteins differentially expressed by E_2 and genistein treatments, it was used a method which is based on an approximation, instead submit the proteins spot to MALDI-TOF mass spectrometry analysis. Identification was made by trying to approach the modified proteins to a data of already identified proteins in a similar specie, sea bream *Sparus aurata* (Anjos et al. 2013).This method is based on comparing the Mw/pI of the proteins spot with Mw/pI of the proteins identified in *Sparus aurata* and then gets an amino acid sequence by similarity. A basic local alignment search tool (Blast) that compares proteins of sea bream against a database of mRNA of sea bass was carried out (http://ccmarlnx.ualg.pt:4567/). The blast resulted in the identification of several sequences of mRNA in *D.labrax* and were translated into protein using the ExPASy translate tool (http://www.expasy.org) and ORF finder

program of the National Center for Biotechnology Information (NCBI) (http: //www.ncbi.nlm.nih gov). These translated proteins were analysed and their pI/Mw predicted. Finally with this partial proteome of D.labrax it was possible to access in silíco the sequence of proteins correspond to the spot candidates that were up- or down-regulated in the *D. labrax* proteome. Further characterization of the putative proteins modified by treatment with genistein or 17-B-estradiol was classified by biological process, localization and molecular functions using Uniprot (http://www.uniprot.org), NCBI (http://www.ncbi.nlm.nih.gov/) and Quick GO (http://www.ebi.ac.uk/QuickGO/).

3. Results

3.1. E_2 and Genistein tissue responsiveness

3.1.1. Effects of E_2 and Genistein on plasma parameters

Circulating levels of calcium (Ca), E_2 and vitelogennin abundance in plasma were measured 5 days after the treatment with E_2 and genistein (Table 2.1). Total plasma Ca was significantly ($p<0.05$) increased in E_2 treated fish compared to control fish (Table 2.1). Similarly, plasma E_2 was significantly ($p<0.05$) modified comparing to control fish (Table 2.1). Analysis of plasma protein by SDS-PAGE confirmed that the E_2 and genistein treatment induced a significant ($p<0.001$) increase in circulating levels of Vtg (Figure 2.1 and 2.2).

Table 2.1. Calcium, estradiol levels in plasma from sea bass with or without E2 and Genistein treatment after 5 days.

	Control	**Treated E2**	***p* value**	**Treated Gen**	***p* value**
Calcium(mM)	2,66 ± 0,46	4,00 ± 0,61	*	2,62 ± 0,3	n.s
Estradiol(ng/ml)	1,7±2,27	8,81±6,61	*	0,33±0,26	n.s

Values are given as mean±SE of 9-10 fish per treatment and control and E2/Gen-treatment fish are compared using a one way ANOVA test (SPSS v12.3). ns no significance: *p<0.05.

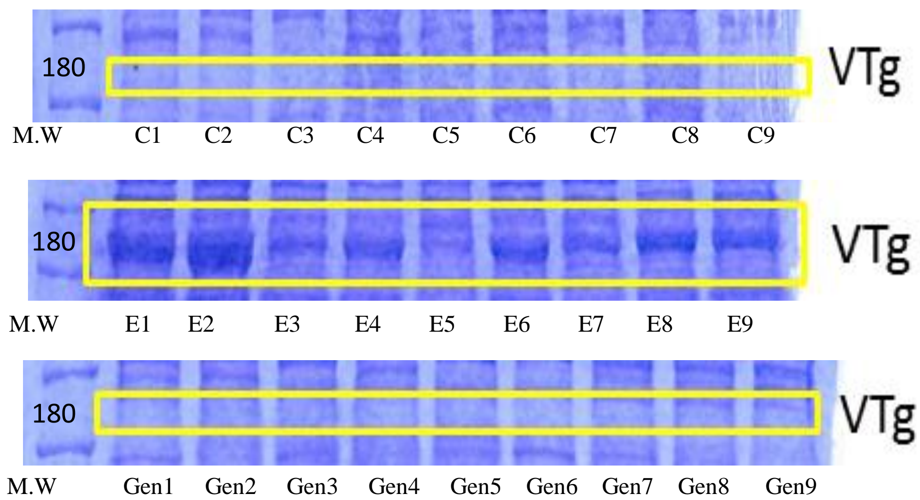


Figure 2.1. Identification of vitellogenin in plasma by SDS-PAGE. Only the region of the gel containing Vtg (approximate molecular weight 180 kDa) is shown in the yellow box. Individual plasma samples from all fish (Ctr, E_2, Gen, n=9) are shown. The molecular weight marker (MW) is present in the first lane of each gel (kDa).

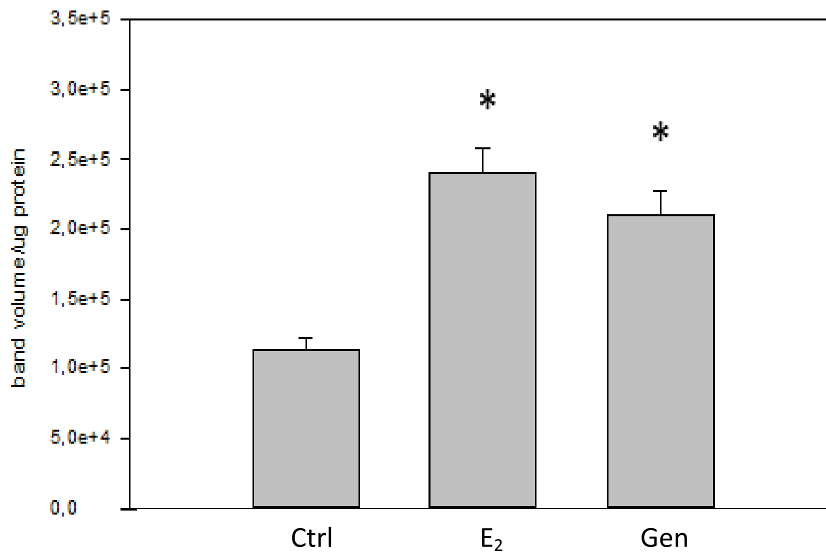


Figure 2.2. Quantification of putative vitellogenin calculated as the band volume outline (area in mm^2) using ImageJ. Vitellogenin was significantly expressed ($p<0.001$) in the E_2 and genistein treatment comparatively to the control group. * Indicates that a statistically significant difference exists ($p<0.001$).

3.1.2. TRAP and ALP activities in sea bass vertebra

TRAP activity in vertebra was not significantly different between any of the treatment groups. ALP activity in vertebra was also not significantly different (Figure 2.3).

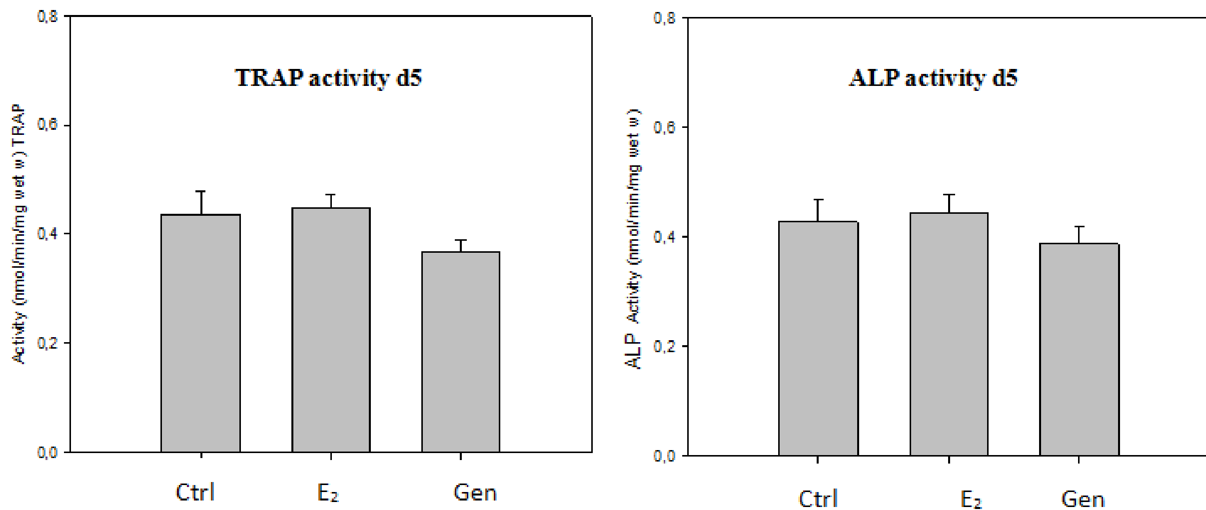


Figure 2.3. TRAP and ALP activity in vertebra of immature sea bass after 5 days of the treatment with E_2, genistein and control. Data presented as mean±-SEM; one-way ANOVA, $p<0.05$; n=8–10. Results are expressed as nmol pNP produced min/mg of wet weight.

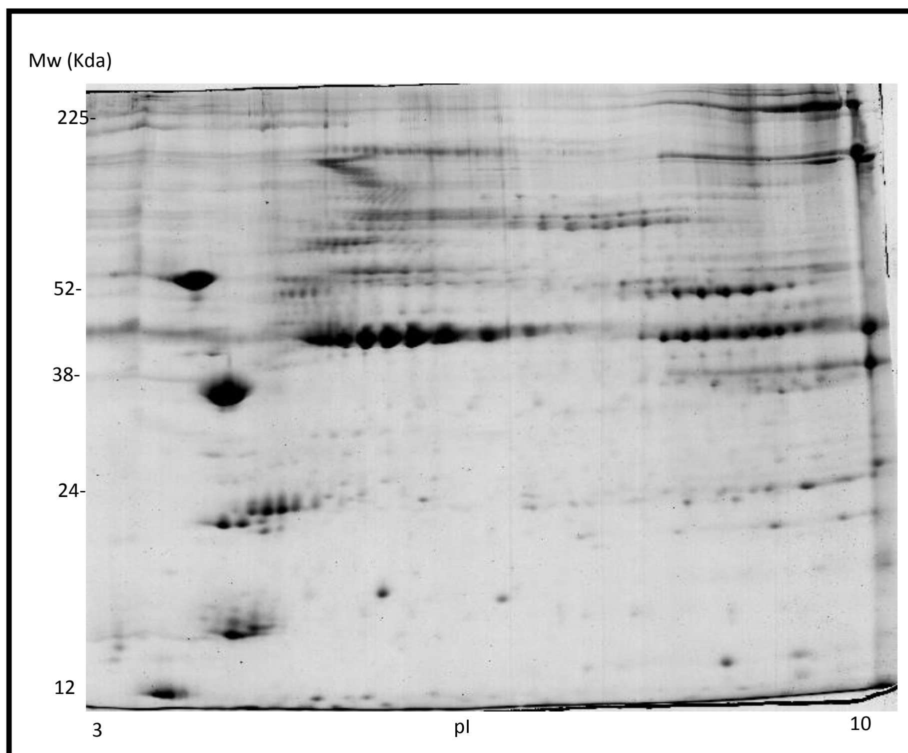
3.2. Analysing the vertebra proteome

3.2.1. Detection of putative differentially expressed proteins

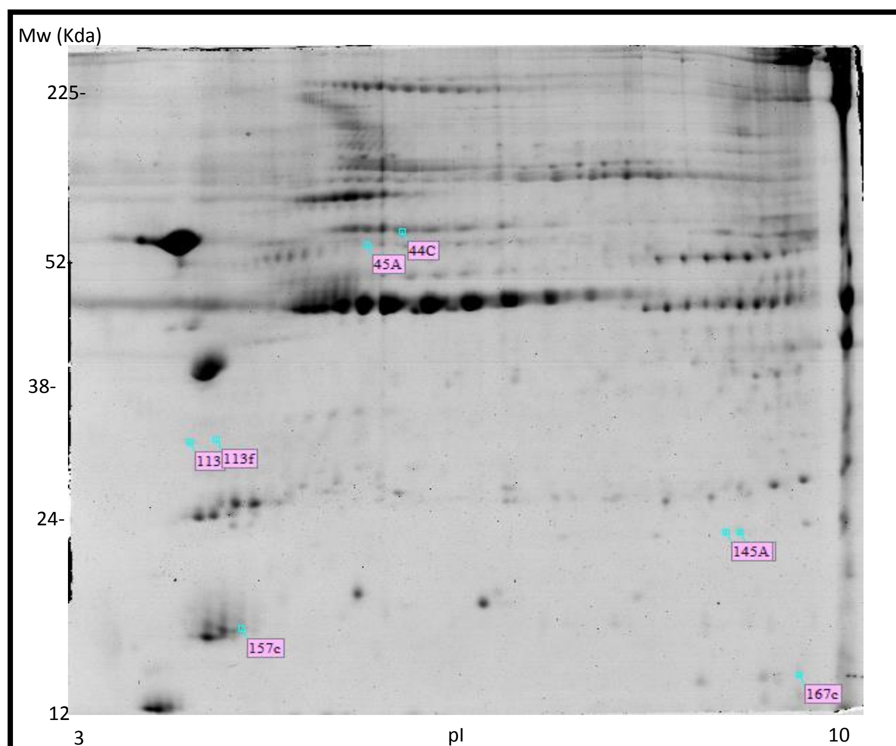
The vertebra proteome profile after 2D electrophoresis using a pI range 3-10 and SDS-PAGE polyacrylaminde concentration of 12.5% is shown in the representative gel images of Control, E_2 and genistein treated fish (Figure 2.4). The number of protein spots detected on each one of the five gels was 285 (5 gels Control, 5 gels E_2 and 5 gels of genistein treatment). Protein spots that were significantly different between treatment groups are labelled on Figure 2.4. There was 8 protein spots that were significantly ($p<0.05$) modified in E_2 versus control gels and 22 protein spots significantly modified ($p<0.05$) in genestein treatment versus control. All of the candidate protein spots were significantly down-regulated in response to E_2 and genistein treatment. Proteins

significantly ($p<0.05$) modified in the E_2 and genistein treatment were shown (Figure 2.5 and 2.6).

A



B



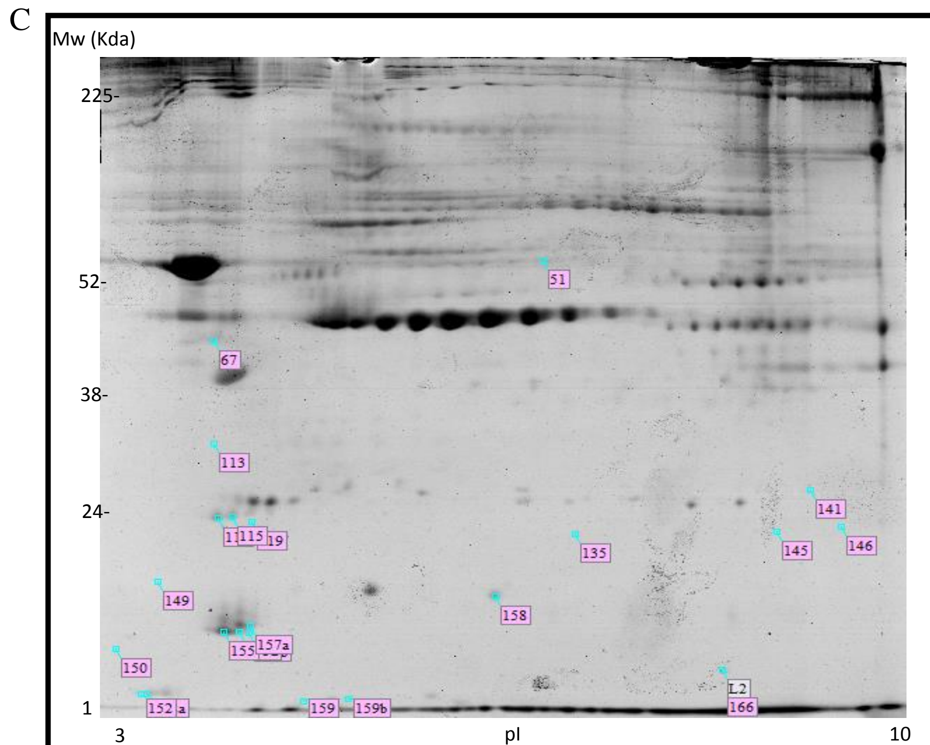


Figure 2.4. 2-DE gel images showing the profile of sea bass *D.labrax* vertebra proteome for control (A), E_2 (B) and genistein (C). (A) Represents the master gel control that was used to match against the E_2 (B) and genistein (C) master gel. Proteins extracted from the vertebral bone of two individual were pooled and 600 ug loaded onto non-linear IPG strips pH 3-10 and following IEF were separated by 12.5% SDS-PAGE. Gels were stained with Coomassie blue as described in the methods. Annotations correspond to proteins that were down-regulated in the different treatments are shown (B and C).

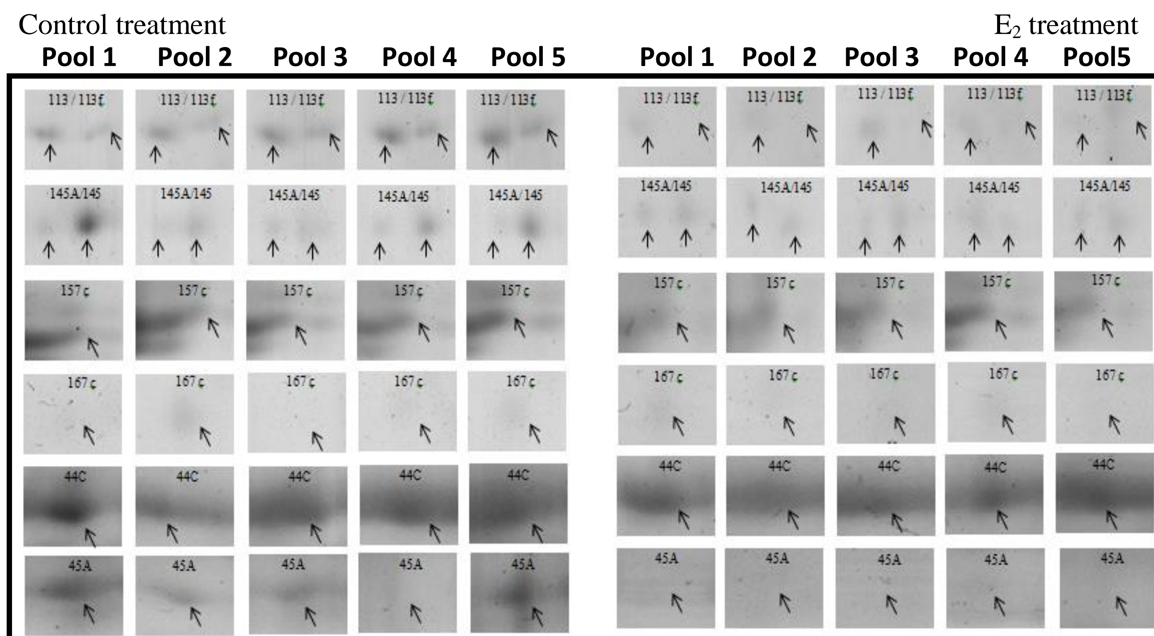


Figure 2.5. Enlarged view of candidate protein spots boxed in Figure 2.5 that are down-regulated by sea bass E2 treatment. The five gels analysed per experimental group are presented (Pool 1 –Pool 5). Black arrows indicate altered protein spots in vertebral proteome of E2 and control treatment; the numbers correspond to the designation given to each of the protein spots. All the candidate protein spots (8) were down-regulated by the E2 treatment.

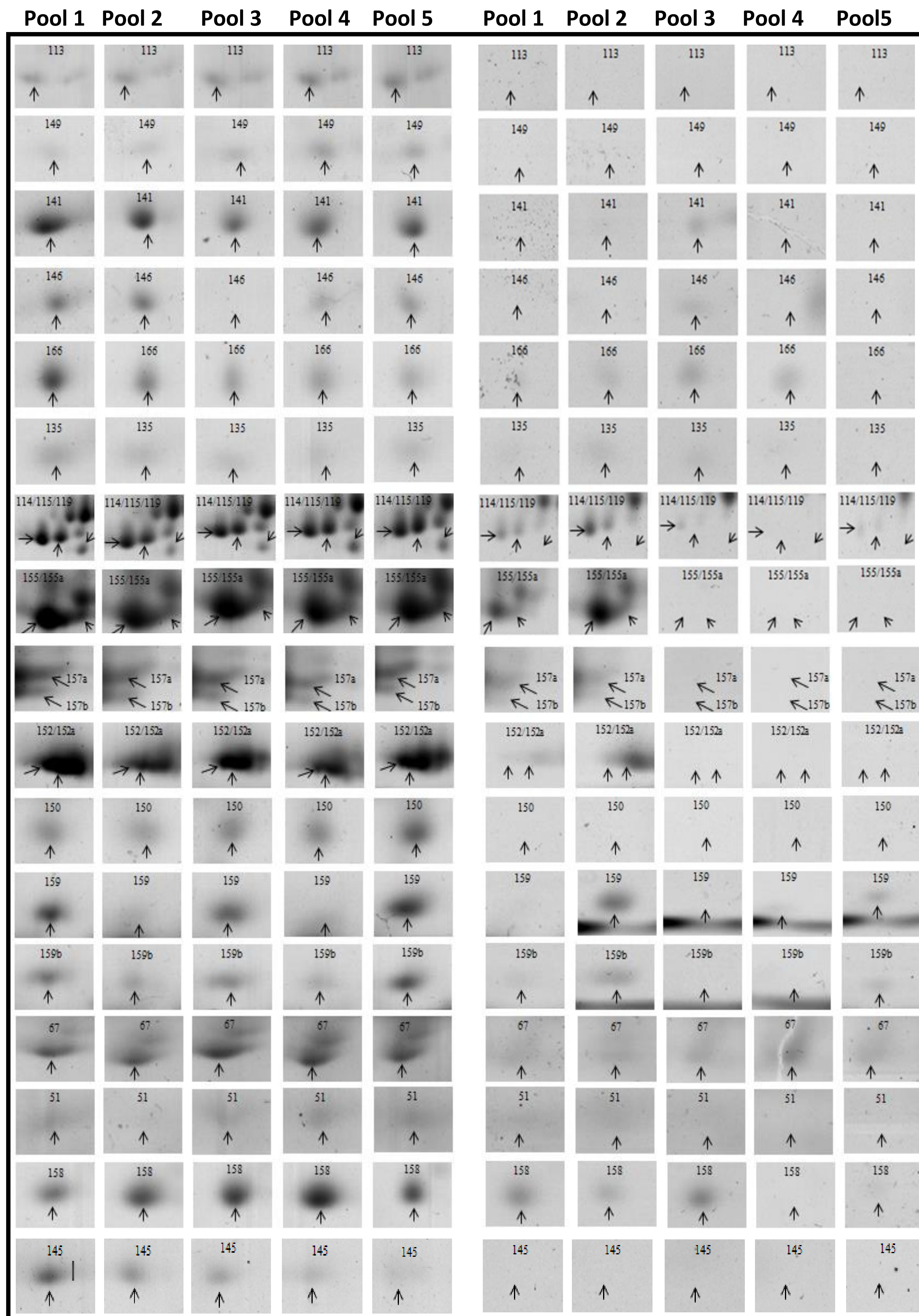


Figure 2.6. Enlarged view of candidate protein spots boxed in Figure 2.6 that are down-regulated by sea bass genistein treatment. The five gels analysed per experimental group are presented (Pool 1 – Pool 5). Black arrows indicate altered protein spots in vertebral proteome of genistein and control treatment; the numbers correspond to the designation given to each of the protein spots. All the candidate protein spots (22) here presented are down-regulated by the genistein treatment.

3.2.2. Identification of putative differentially expressed proteins

The protein candidate spots (8 for E_2 treatment and 22 for genistein treatment) were analysed as described above. Of the 30 differentially expressed proteins in E_2 and genistein relative to the control, only 12 proteins could be completely characterised by comparing them with the proteins of the partial proteome of sea bream (*Sparus aurata*). The others 18 candidate proteins spots were only assigned a pI/Mw based on analysis of the gels but were not identified (Table 2.2 and 2.3). There were 2 common proteins in E_2 and genistein treatment that were both down-regulated relative to control vertebra (figure 2.7).

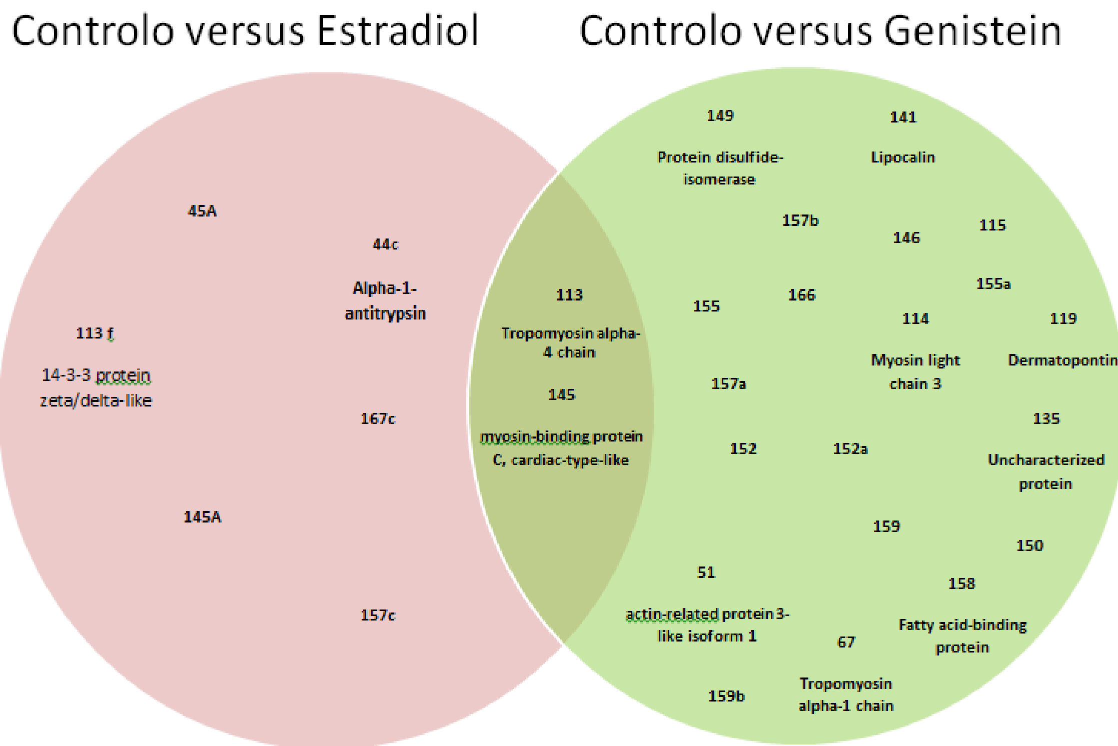


Figure 2.7. Venn diagram representing the candidate protein spots that were differentially expressed between the experimental groups. In total there are 30 candidate spots of which 8 were differentially expressed by E2 treatment and 22 candidate protein spots were differentially expressed by genistein treatment. Two of the differentially expressed proteins; Tropomyosin alpha-4 chain and myosin binding protein C, were common to both the E2 and genistein treatment.

Table 2.2. List of identified proteins that are down-regulated by genistein treatment.

Spot	Protein identity	Mw/pI determined	Mw/pI theoretical (D.Labrax)	Biological process*	GO identifier	Acession no (Uniprot*). /Species
113	Tropomyosin alpha-4 chain	26.5/4.6	28.1/4.61	musclecontraction /osteoblastdifferentiation	GO:0001649/GO:0006936	P67936/*Homo sapiens*
149	Proteindisulfide -isomerase	17.5/4.2	17.8/4.10	metabolic process/cell redox homeostasis	GO:0008152/GO:0045454	U3LS30/*Dicentrarchusl abrax*
141	Lipocalin	21.5/9.3	21.1/9.63	lipidmetabolicprocess/transport	GO:0006629/GO:0006810	C3UZX1/*Perca flavescens*
146	-	19.1/10.2	-	-	-	-
166	-	13.1/8	-	-	-	-
135	Uncharacterize dprotein	19.2/6.5	18.9/6.33	actinfilamentdepolymerization	GO:0030042	G3PY12/*Gasterosteusac uleatus*
114	Myosin light chain 3	21/4.65	16.6/4.40	calciumionbinding	GO:0005509	Q9IB32/*Pennahiaargent ata*
115	-	21/4.75	-	-	-	-
119	Dermatopontin	20.1/5.09	22.3/4.89	negative regulation of cell proliferation/cell adhesion	GO:0008285/GO:0007155	Q9QZZ6/*Mus musculus*
155	-	14.8/4.6	-	-	-	-
155a	-	14.8/4.8	-	-	-	-
157a	-	15/4.9	-	-	-	-
157b	-	14.8/4.85	-	-	-	-
152	-	12/4.2	-	-	-	-
152a	-	12/4.25	-	-	-	-
150	-	14.2/3.6	-	-	-	-
159	-	11.9/5.4	-	-	-	-
159b	-	11.9/5.4	-	-	-	-
67	Tropomyosin alpha-1 chain	39/4.65	18.5/4.68	actinbinding	GO:0003779	P84335/*Liza aurata*
51	actin-related protein 3-like isoform 1	54/5.9	45.2/5.63	actinbinding/ATP binding	GO:0003779/GO:0005524	O73723/*Takifugurubripes*
158	Fatty acid-bindingprotein	16/5.9	14.8/5.46	transport	GO:0006810	S4S3R9/*Sparusaurata*
145	myosin-binding protein C, cardiac-type-like	19/8.6	17.8/8.72	cardiac muscle contraction/ myosin filament assembly	GO:0060048/GO:0031034	Q14896/*Homo sapiens*

Spot identification numbers refers to protein listed in Figure 2.4; 2.5 and 2.6.

*The accession number and biological process were accessed using Uniprot (http://www.uniprot.org) and are from a similar sequence of the protein identified.

Spot identification underlined correspond to the proteins that couldn't be characterised, once it was not possible to find homologue between the generated partial proteome of D.Labrax in silíco and S.bream.

Table 2.3. List of identified proteins that are down-regulated in E_2 treatment.

Spot	Protein identity	Mw/pI determined	Mw/pI theoretical (D. Labrax)	Biological process*	GO identifier	Acession no (Uniprot*). /Species
113	Tropomyosin alpha-4 chain	26.5/4.6	28.1/4.61	muscle contraction/osteoblast differentiation	GO:0006936/GO:0001649	P67936/*Homo sapiens*
113f	14-3-3 protein zeta/delta-like	27/4.4	26.8/4.73	protein targeting/regulation of cell death	GO:0006605/GO:0010941	*P63104/ Homo sapiens*
145	myosin-binding protein C, cardiac-type-like	19/8.6	17.8/8.72	cardiac muscle contraction/ myosin filament assembly	GO:0060048/GO:0031034	Q14896/*Homo sapiens*
145A	-	19/8.5	-	-	-	-
157c	-	15/5.05	-	-	-	-
167c	-	12.9/9.6				
44C	Alpha-1-antitrypsin	59/5.6	30.2/6.12	homeostasis/ regulation of proteolysis	GO:0007599/GO:0030162	P01009/*Homo sapiens*
45A	-	54/5.55				

Spot identification numbers refers to protein listed in figure 2.4; 2.5 and 2.6.

*The accession number and biological process were accessed using Uniprot (http://www.uniprot.org) and are from a similar sequence of the protein identified.

Spot identification underlined correspond to the proteins that couldn't be characterised, once it was not possible to find homologue between the generated partial proteome of D.Labrax in silíco and S.bream .

.

3.2.3. Group of proteins by putative biological process

Classification of biological process of proteins identified as differential expression in treatments is presented in (Table 2.2 and Table 2.3). In the E_2 treatment 8 proteins were down-regulated but only 4 of them were identified. It was only possible to determine Mw/pI of the other 4 protein spots in gel. Two protein spots identified in E_2 were also the same in genistein treatment and are related with muscle contraction; tropomyosin alpha-4 chain (TPM4; spot 113) and myosin binding protein C, cardiac type (MYBPC3; spot 145). The other two proteins identified in the E_2 treatment that were down-regulated were 14-3-3 protein zeta delta/like and alpha-1-antitrypsin (A1AT) that are related to protein targeting and homeostasis, respectively. The other proteins that were down-regulated by E_2 treatment were not identified and MALDI-TOF

and spectra analysis are required to obtain a putative protein identity so that the biological process can be determined. In genistein treatment there was 22 proteins down-regulated relative to the control and only 10 were putatively identified. Two of the identified proteins were also down-regulated by E_2 treatment. Another two proteins that were identified, protein dissulfate isomerase (PDI; spot 149) and lipocalin (spot 141), are involved in metabolic processes. Three proteins, tropomyosin alpha 1-chain (TPM1; spot 67), actin related protein 3-like isoform 1 (ARPCS; spot 51) and an uncharacterized protein (spot 135) are related with actin filaments and actin binding. There was one down-regulated protein that is related to calcium binding (myosin light chain 3; MYC3;spot 114) and two proteins, dermatopontin (DPT; spot 119) and fatty acid binding protein (FABPs; spot 158), that are related with cell adhesion and transport, respectively. The others 12 protein spots differentially expressed could not be identified.

4. Discussion

The bone is a structure extremely important in the life of vertebrate because it serves as support, protection, helps in movement among other functions. Exploration of the proteome of bone is extremely difficult due to its structure, however it is extremely important to obtain a proteome to understand the various mechanisms that regulate this structure and how the process of bone remodelling and bone homeostasis is controlled. In this context estrogen was administered, a hormone that plays an important role in bone homeostasis (Jeong and Choi 2011) and also genistein, a phytoestrogen, which is considered an endocrine disruptor that interferes with the endocrine system of organisms. This phytoestrogens compounds such as genistein have been relatively poorly studied (Urushitani et al. 2002).

4.1. E2 and genistein in bone homeostasis

4.1.1. Plasma analysis

The fishes used in this study were immature sea bass *D. labrax* as they have undifferentiated gonads and the levels of sex steroids in plasma are low. Estrogen and vitellogenin (Vtg) were measured to verify the efficacy of the E_2 injection in the sea bass. Vtg is an indicator of the biological response to E_2 treatments. It is also important see if estrogen mobilized Ca from calcified tissue as have already been reported. Levels of both Ca and Vtg was significantly increased by E_2 treatments as has previously been described (Guerreiro et al. 2002), (Al-Jandal et al. 2011); (Ibarz et al. 2013). Estrogen levels are also increased by E_2 treatment and not by genistein but maybe this increase was caused by the injection of the implant. The only plasma parameter increased by genistein was Vtg and this response has already been described in studies carried out with other teleost fishes like rainbow trout *Onchorynkus mykiss*, and sea bream *Sparus aurata* (Bennetau-Pelissero et al. 2001) (Guzmán et al. 2004).

4.1.2. TRAP/ALP

TRAP and ALP activity are enzymatic markers used to detect osteoclast and osteoblast cell activity, respectively. With the values of TRAP and ALP it can be predicted if E_2 and genistein treatment promotes bone formation or bone resorption. The results show that none of the treatments significantly ($p < 0.05$) modified TRAP or ALP activity. In other studies teleost fish treated with an estrogen receptor modulator (SERM) also did not exhibit modified TRAP and ALP activity (Vieira et al. 2012). Other results from fish treated with E_2 also show no difference in the activity of TRAP and ALP (Guerreiro et al. 2002) even when the tissue was scale, which is part of the integumentary skeleton.

4.2. Sea bass proteome

A vertebra proteome of sea bass was generated to study the molecular effects of E_2 and genistein treatments on bone. The objective was to observe the differential expression of proteins between the different treatments. The differential expression was assessed using the classical method of 2-DE and then the gels were analysed with ImageMaster that gives the volume (%) of the protein spots. Bone is a structure that is difficult to extract and an additional difficulty was that when the vertebra was dissected out it is hard to remove all the adhering muscle and nervous tissues. In a way to reduce this problem and make the samples of protein extraction more homogeneous pools of protein from 2 independent fish (eg. fish 1 and fish 2) vertebrae were used to run in 2-DE. From all the 285 spot proteins a total of 8 proteins and 22 were differential expressed by E_2 and genistein treatment respectively. The spots were selected had a threshold considered to be differential expressed either 2 fold higher expression or 2 fold lower expression. The identification of the proteins that had a modified expression with E2 treatment or Genistein treatment was based on a proteome previously established for the sea bream (Anjos et al. 2013). The consequence of this strategy was that not all the sea bass proteins identified were represented on the proteome previously published (Anjos et al. 2013). In the present study immobiline drystrips had a pI range of 3-10 and the strips in the previously study was 4-7. The candidate spot proteins

identified in the sea bass bone proteome with a determined pI smaller or bigger than that obtained in the sea bream could not be identified.

Genistein is a phytoestrogen compound and is expected to had similar functions with estrogen. In this study this seems to happen once two of the proteins that were dow-regulated are the same in both treatments (TPM4 and MYBPC3). Moreover there are proteins identified in E_2 treatment with similar functions to proteins identified in genistein treatment which is the case of TPM1, TMP4 and ARPCS. Most of the proteins found in sea bass were related to bone structure, bone metabolism and have calcium ion binding properties.

Tropomyosin alpha 1/alpha 4 and Actin related protein like isoform 1

TPM1, 4 and ARPCS are both proteins belong to a large family of integral components of actin filaments. This proteins are involved in several biological process like structural function and have already been described in mammalian bone and fish bone (Pastorelli et al. 2005); (Silva et al. 2011); (Hurst et al. 2004). In bone, tropomyosin 1 and 4 have the main function to stabilize actin microfilaments and regulating is interactions with others proteins such as actin complexes (Silva et al. 2011). Although is not clear, it has been described that tropomyosin 1 and 4 are present in osteoblasts where TMP1 is up-regulated in pre-osteoblasts and down-regulated in mature osteoblasts while TPM4 is dow-regulated in pre-osteoblasts (Silva et al. 2011). The presence of these proteins in osteoclasts has been more studied, and it is known that TPM1, TPM4 and ACRP1 are both expressed in podosomes (structures present in osteoclasts that are responsible for the adhesion and resorption of bone) (Hu et al. 2011); (Hurst et al. 2004). These proteins stabilize the rings of actin for the reabsorption of bone. In a study was found that increase of tropomyosin by estrogen deficiency led to increased bone resorption in mice (Pastorelli et al. 2005). Up-regulation of TPM1 and TPM4 also seems to be related to the skeleton deformation in seabream (*Diplodus sargus*) (Silva et al. 2011). Another study described that administration of E_2 caused a reduction in TPM1 protein in the proteome of bone mice (Pastorelli et al. 2005). This data seems to be in agreement with the results of the present study since TPM4 was reduced by E_2 and genistein and TPM1 and ARPC1 dow-regulated by genistein. Once these proteins appear to be important in bone resorption, this may suggest that these two

compounds known to reduce bone resorption are actually dow-regulating these proteins to counter is effects.

14-3-Protein zeta/delta-like

This protein belongs to the family of 14-3-3 protein that are involved in protein targeting, regulation of cell death and apoptotic effects (Ponnikorn et al. 2011). This protein has already been described in mesenchymal stem cells of mice (Dong et al. 2014) and in bone of *sparus aurata* (Anjos et al. 2013).

Alpha-1-antitrypsin

Alpha-1-antitrypsin is a multifunctional protein with anti-inflammatory and inhibitory functions. Alpha-1-antitrypsin was found to be down regulated by E_2 treatment. This protein was already described to have the capacity to reduce osteoclast activity and bone resorption (Cao et al. 2011). Reabsorption of bone is accomplished by cytokines and inflammatory response (Cao et al. 2011). The anti-flamatory power of alpha-antitrypsin 1 seems to reduce the response inflammatory and reduce bone loss in mice (Cao et al. 2011).

Cardiac myosin binding protein C type like

Protein involved in muscle contraction and has a structural role. This protein is expressed in the heart but is also expressed in some stages of neonatal skeletal muscles and also appears to be required for the formation of skeletal sarcomeres during myofibrillogenesis (Lin et al. 2013). There is little information about how E_2 or genistein acts on the protein since the presence of this protein in bone is not clear.

Protein disulfide isomerase (PDI)

Protein present in the endoplasmic reticulum of eukaryotic cells and whose main function is folding of many proteins through disulfide bonds (Fu, Wang, and Zhu 2011). PDI is also responsible for the intracellular binding of certain molecules such as steroid hormones (estrogen) and endocrine disruptors (Fu et al. 2011). This protein as already been found in osteoclastic and osteoblastic cells and have been suggested to have a role of apoptosis in the osteoclastic cells (Zhu et al. 2010), and to be the initiator of 1,25 (OH) 2D3 signals that lead to effects on maturation of osteoblasts (Chen et al. 2010).

Moreover studies showed that E_2 can bind to PDI (Fu et al. 2011). PDI shares homology with the binding domain of ERα and forms complexes. This protein can enhance the interaction of ERα to specific sequences of DNA but the same don't happen with ERβ (Xiong et al. 2012); (Chen et al. 2010). There are studies in which the knock-out of PDI had led to an increase in ERα (Chen et al. 2010), however how E_2 regulates whether or not the expression of PDI protein is unknown.

Lipocalin

Belongs to the lipocalin family of proteins which transport small hydrophobic molecules such as steroids, lipids and is expressed in many tissues with various functions such as apoptosis, inflammation and iron binding (Costa et al. 2013); (Fried and Greenberg 2012). This protein has been found in bone tissue, liver and immune cells of rat (Zhang et al. 2014). A study in rats showed that lipocalin 2 is expressed in osteoblasts and rats over expressing lipocalin 2 had low levels of deposition of bone matrix and also reduction in the level of differentiation in osteoblasts. Moreover, over-expressing of lipocalin causes an increase in osteoclasts and TRAP activity (Costa et al. 2013).

In this study lipocalin protein was reduced by genistein compound. This may suggest that phytoestrogen genistein is dow-regulating lipocalin to increase bone formation.

Myosin light chain 3

Protein that belongs to the family of myosins which are responsible for mobility of actin. Little is known about this protein in bone although it has been described for vertebral bone of fish (Anjos et al. 2013). In a study of *sparus aurata* this protein was reduced after administration of PTHrP (Anjos et al. 2013). The function of this protein is unknown but is related with calcium ion binding. Homologues of this protein have also been described for vertebral bone of fish (Silva et al. 2011).

Dermatopontin

Derpatopontin is a protein present in the extracellular matrix and can be found in bone or cartilage. There are studies that show that this protein is induced by the stimulation of nuclear steroid receptors and is possibly involved in the differentiation of multipotential stroma cells into osteoblasts (Takeuchi 2010); (Pochampally et al. 2007). In a study it was reported that dermatopontin was able to induce stem cells into osteoblasts during skeletal would healing (Coan, Lively, and Van Dyke 2014) however the effects of estradiol and genistein in the regulation of this protein is still unknown.

Fatty acid binding protein

FABP is responsible for the transport of fatty acids and other lipid substances protein. It is a protein involved in metabolic processes and there are several studies reporting particularly this protein in mammals although it has also been found in the vertebral bone of fish (Silva et al. 2011). It was previously reported that osteoblastic cells and adipocytes comes from the same progenitor, mesenchymal stem cells (Diascro et al. 1998). A study in bone marrow cells of mice showed that E_2 can increase osteoblast proliferation and reduce the expression of adipocytes (Dang et al. 2002). In the present study, administration of genistein has decreased the expression of fatty acid binding protein. This expression regulation by the genistein had already been reported in a study done in adipocytes in which there was also a reduction of ERα and ERβ (Park et al. 2009). All these information described above may suggest that genistein inhibits

fatty acid binding protein to reduce the number of mesenchymal stem cells that differentiate into adipocytes and then increase osteoblast proliferation.

5. Conclusion

E_2 and genistein treatment have the capacity to regulate the homeostasis and modify the vertebrae proteome of sea bass *D. labrax*. E_2 also appears to be more effective in the homeostasis of bone once the levels of estrogen and Ca were increased, although the increase of E_2 may be derived from the injection of the implant. All of the candidate proteins spots identified were down-regulated, indicating that both E2 and genistein probably act in a similar way. This idea is given further support by the identification of the same proteins in both treatment group that are differentially expressed. The reason why all the proteins were down-regulated is unclear because it was expected at least some of proteins would be detected that were up-regulated. The effect of genistein on the bone proteome was notable and despite its absence of effect on biochemical parameters 22 proteins were differentially expressed by this treatment.

To validate all of these results more studies are required including MALDI-TOF analysis to permit identification of the proteins modified by E2 and genistein. Further studies will be crucial to unmask the function of E2 and genistein in bone turnover.

6. References

Al-Jandal, N.J., Whittamore, J.M., Santos, E.M., and Wilson, R.W., 2011. "The influence of 17β-estradiol on intestinal calcium carbonate precipitation and osmoregulation in seawater-acclimated rainbow trout (Oncorhynchus mykiss)." The Journal of Experimental Biology 214(16):2791–98.

Amadei, S.U., Silveira, Vanessa Á.S., Pereira, A.C., Carvalho, Y.R., and Rocha, R.F., 2006. "A influência da deficiência estrogênica no processo de remodelação e reparação óssea." Jornal Brasileiro de Patologia e Medicina Laboratorial 42(1):p. 5–12.

Anjos, L., Gomes, A.S., Redruello, B., Reinhart, R., and Canario, A.V.M., 2013. "PTHrP-induced modifications of the sea bream (sparus auratus) vertebral bone proteome." General and Comparative Endocrinology 191(June):102–12.

Aranda, A., and Pascual, A., 2001. "Nuclear hormone receptors and gene expression." Physiological Reviwes 81(3):1269–1304.

Bean, L.A., Ianov, L., and Foster, T.C., 2014. "Estrogen receptors, the hippocampus, and memory." Neuroscientist 20(5):534–45.

Beck, M.M., and Hansen, K.K., 2004. "Role of estrogen in avian osteoporosis." Poultry Science 83:200–206.

Bennetau-Pelissero, C., Breton, B., Bennetau, B., Corraze, G., Le Menn, F., Davali-Cuisset, B., et al., 2001. "Effect of genistein-enriched diets on the endocrine process of gametogenesis and on reproduction efficiency of the rainbow trout Oncorhynchus mykiss." General and Comparative Endocrinology 121(2):173–87.

Cao, Jay J., Gregoire, Brian R., Sun, L., and Song, S., 2011. "Alpha-1 antitrypsin reduces ovariectomy-induced bone loss in mice." Annals of The New York Academy of Sciences 1240:E31–5.

Chen, J., Olivares-Navarrete, R., Wang, Y., Herman, T.R., Boyan, B.D., and Schwartz, Z., 2010. "Protein-disulfide isomerase-associated 3 (Pdia3) mediates the membrane

response to 1,25-dihydroxyvitamin D3 in osteoblasts.” The Journal of Biological Chemistry 285(47):37041–50.

Coan, H.B., Lively, M.O., and Van Dyke, M.E., 2014. “Dermatopontin in the extracellular matrix enhances osteogenic differentiation of adipose-derived mesenchymal stem cells.” Herbert Open Access Journais 1(1):2.

Cooke, P.S., Selvaraj, V., and Yellayi, S., 2006. “Genistein, estrogen receptors, and the acquired immune response.” The Journal of Nutrition 704–8.

Costa, D., Lazzarini, E., Canciani, B., Giuliani, A., Spanò, R., Marozzi, K., et al., 2013. “Altered bone development and turnover in transgenic mice over-expressing lipocalin-2 in bone.” Journal of Cellular Physiology 228(11):2210–21.

Dang, Z.C., Bezooijen, V., Karperien, M., Papapoulos, S.E., and Lowik, C.W.G.M., 2002. “Exposure of KS483 cells to estrogen enhances osteogenesis and inhibits adipogenesis.” Journal of Bone and Mineral Research 17:394–405.

Diascro, D.D., J.R., Vogel, R.L., Johnson, T.E., Witherup, K.M., Pitzenberger, S.M., et al., 1998. “High fatty acid content in rabbit serum is responsible for the differentiation of osteoblasts into adipocyte-like cells.” Journal of Bone and Mineral Research 13:96–106.

Dong, X., Zhu, F., Liu, Q., Zhang, Y., Wu, J., Jiang, W., et al., 2014. “Transplanted bone marrow mesenchymal stem cells protects myocardium by regulating 14-3-3 protein in a rat model of diabetic cardiomyopathy.” International Journal of Clinical and Experimental Pathology 7(7):3714–23.

Eastell, R., 2005. “Role of oestrogen in the regulation of bone turnover at the menarche.” Journal of Endocrinology 185(2):223–34.

Enmark, E., Pelto-Huikko, M., Grandien, K., Lagercrantz, S., Lagercrantz, J., Fried, G., et al., 1997. “Human estrogen receptor b-gene structure, chromosomal localization, and expression pattern.” Journal of Clinical Endocrinology and Metabolism 82(12l):4258–65.

Fried, S.K., and Greenberg, A.S., 2012. "Lipocalin 2: a 'sexy' adipokine that regulates 17β-estradiol and obesity." Endocrinology 153(4):1582–84.

Fu, Xin-M., Wang, P., and Zhu, B.T., 2011. "Characterization of the estradiol-binding site structure of human protein disulfide isomerase (PDI)." Plos One 6(11):e27185.

Golub, E.E., and Boesze-Battaglia, K., 2007. "The role of alkaline phosphatase in mineralization." Orthopaedics 18:444–48.

Guerreiro, P. M., Fuentes, J., Canario, A.V.M and Power, D.M., 2002. "Calcium balance in sea bream (Sparus aurata): the effect of oestradiol-17beta." The Journal of Endocrinology 173(2):377–85.

Guzmán, J.M., Sangiao-Alvarellos, S., Laiz-Carrión, R., Míguez, J.M., Del Rio, M.P.M., Soengas, J.L., et al., 2004. "Osmoregulatory action of 17beta-estradiol in the gilthead sea bream sparus auratus." Journal of Experimental Zoology. Part A, Comparative Experimental Biology 301(10):828–36.

Hall, B.K., 2005. Bones and cartilage: developmental and evolutionary skeletal biology. London: Elsevier Academic Press.

Hu, S., Planus, E., Georgess, D., Place, C., Wang, X., Albiges-Rizo, C., et al., 2011. "Podosome rings generate forces that drive saltatory osteoclast migration." Molecular Biology of the Cell 22(17):3120–26.

Hurst, I.R., Zuo, J.,Jiang, J., and Holliday, L.S., 2004. "Actin-related protein 2/3 complex is required for actin ring formation." Journal of Bone and Mineral Research 19(3):499–506.

Ibarz, A., Pinto, P.I.S., and Power, D.M., 2013. "Proteomic approach to skin regeneration in a marine teleost: modulation by oestradiol-17β." Marine Biotechnology (New York, N.Y.) 15(6):629–46.

Imai, Y., Youn, M.Y., Kondoh, S., Nakamura, T., Kouzmenko, A., Matsumoto, T., et al., 2009. "Estrogens maintain bone mass by regulating expression of genes controlling function and life span in mature osteoclasts." Annals of the New York Academy of Sciences 1173:E31–9.

Jeong, Jae-H., and Choi, Je-Y., 2011. “Interrelationship of runx2 and estrogen pathway in skeletal tissues.” BMB Reports 44(10):613–18.

Judas, F.,Palma, P., Falacho, R.I., and Figueiredo, h., 2012. "Estrutura e dinâmica do tecido ósseo." Clínica Universitária de Ortopedia dos HUC-CHUC and Faculdade de Medicina da Universidade de Coimbra.

Lange, I.G., Hartel, A., and Meyer, H.H.D., 2002. “Evolution of oestrogen functions in vertebrates.” The Journal of Steroid Biochemistry and Molecular Biology 83(1-5):219–26.

Levin, E.R., 2009. “Plasma membrane estrogen receptors.” Trends in Endocrinology and Metabolism 20(10):477–82.

Lin, B., Govindan, S., Lee, K., Zhao, P., Han, R., Runte, K.E., et al., 2013. “Cardiac myosin binding protein-C plays no regulatory role in skeletal muscle structure and function.” Plos one 8(7):e69671.

Liu, R., Hu, Y.,, Li, J., and Lin, Z., 2007. “Production of soybean isoflavone genistein in non-legume plants via genetically modified secondary metabolism pathway.” Metabolic engineering 9(1):1–7.

Majumder, S., Das, S., Moulik, S.R., Mallick, B., Pal, P., and Mukherjee, D., 2015. “G-protein coupled estrogen receptor (GPER) inhibits final oocyte maturation in common carp, Cyprinus carpio.” General and comparative endocrinology 211(2015):28–38.

Matthews, J., and Gustafsson, Jan-A., 2003. “A Subtle balance between ERα and ERβ.” Molecular Interventions 3(5):281–92.

Mawani, Y., Cawthray, J.F., Chang, S., Sachs-Barrable, K., Weekes, D.M., and Wasan, K.M., 2012. “In vitro studies of lanthanide complexes for the treatment of osteoporosis.” The International Journal for Inorganic, Organometallic and Bioinorganic Chemistry.

Mayo Clinic. 2013. "Treatments and drugs." Retrieved (http://www.mayoclinic.org/diseases-conditions/osteoporosis/basics/treatment/con-20019924).

Nilsson, S., Makela, S., Treuter, E., Tujague, M., Thomsen, J., Andersson, G., et al., 2001. "Mechanisms of Estrogen Action." Physiol Rev 81(4):1535–65.

Pang, Y., and Thomas, P., 2009. "Involvement of estradiol-17β and its membrane receptor, G protein coupled receptor 30 (GPR30) in regulation of oocyte maturation in zebrafish, Danio rerio." Gen Comp Endocrinol 161(1):58–61.

Park, H.J., Della-Fera, M.A., Hausman, D.B., Rayalam, S., Ambati, S., and Baile, C.A., 2009. "Genistein inhibits differentiation of primary human adipocytes." The Journal of Nutritional Biochemistry 20(2):140–48.

Pastorelli, R., Carpi, D., Airoldi, L., Chiabrando, C., Bagnati, R., Fanelli, R., et al., 2005. "Proteome analysis for the identification of in vivo estrogen-regulated proteins in bone." Proteomics Journal 5(18):4936–45.

Payne, A.H., and Hales, D.B., 2004. "Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones." Endocrine Reviews 25(6):947–70.

Pinto, P.I.S., Estêvão, M.D., and Power, D.M., 2014. "Effects of estrogens and estrogenic disrupting compounds on fish mineralized tissues." Marine Drugs 12(8):4474–94.

Pochampally, R.R., Ylostalo, J., Penfornis, P., Matz, R.R., Smith, J.R., and Prockop, D.J., 2007. "Histamine receptor H1 and dermatopontin new downstream targets of the vitamin D receptor." Journal of Bone and Mineral Research 22:1338–49.

Ponnikorn, S., Panichakul, T., Sresanga, K., Wongborisuth, C., Roytrakul, S., Hongeng, S., et al., 2011. "Phosphoproteomic analysis of apoptotic hematopoietic stem cells from hemoglobin E/β-thalassemia." Journal of Translational Medicine 9(1):96.

Riggs, B. L., 2000. "The mechanisms of estrogen regulation of bone resorption." The Journal of Clinical Investigation 106(10):1203–4.

Samartzis, E.P., Noske, A., Meisel, A., Varga, Z., Fink, D., and Imesch, P., 2014. “The G protein-coupled estrogen receptor (GPER) is expressed in two different subcellular localizations reflecting distinct tumor properties in breast cancer.” Plos One 9(1):e83296.

Seibel, M.J0,. 2005. “Biochemical markers of bone turnover: part I: biochemistry and variability.” Clin Bbochem Rev 26(4):97–122.

Silva, T.S., Cordeiro, O., Richard, N., Conceição, L.E.C., and Rodrigues, P.M., 2011. “Changes in the soluble bone proteome of reared white seabream (Diplodus sargus) with skeletal deformities.” Comparative Biochemistry and Physiology. Part D, Genomics & Proteomics 6(1):82–91.

Spelsberg, T. C., Subramaniam, M., Riggs, B.L., and Khosla, S., 1999. “The actions and interactions of sex steroids and growth factors/cytokines on the skeleton.” Molecular Endocrinology (Baltimore, Md.) 13(6):819–28.

Syed, F., and Khosla, S., 2005. “Mechanisms of sex steroid effects on bone.” Elsevier, 688–96.

Takeuchi, T., 2010. “Structual comparison of dermatopontin amino acid sequences.” Springer 65(5):874–79.

Taranta, A., Brama, M., Teti, A., Luca, V., Scandurra, R., Spera, G., et al., 2002. “The selective estrogen receptor modulator raloxifene regulates osteoclast and osteoblast activity in vitro.” Bone 30(2):368–76.

Urushitani, H., Shimizu, A., Katsu, Y., and Iguchi, T., 2002. “Early estrogen exposure induces abnormal development of fundulus heteroclitus.” The Journal of Experimental Zoology 293(7):693–702.

Vieira, F.A., Pinto, P.I., Guerreiro, P.M., and Power, D.M., 2012. “Divergent responsiveness of the dentary and vertebral bone to a selective estrogen-receptor modulator (SERM) in the teleost sparus auratus.” General and Comparative Endocrinology 179(3):421–27.

Wang, T.T.Y., Sathyamoorthy, N., and Phang, J.M., 1996. "Molecular effects of genistein on estrogen receptor mediated pathways." Carcinogenesis 17(2):271–75.

Warren, F.W., Lien, F., and K., 1994. Functional Anatomy of the vertebrates. 2 nd ed. Saunders College Publishining.

Xiong, Y., Manevich, Y., Tew, K.D., and Townsend, D.M., 2012. "S-glutathionylation of protein disulfide isomerase regulates estrogen receptor α stability and function." International Journal of Cell Biology 2012:273549.

Zhang, Y., Foncesa, R., Deis, J.A., Guo, H., Bernlohr, D.A., and., Chen, X., 2014. "Lipocalin 2 expression and secretion is highly regulated by metabolic stress, cytokines, and nutrients in adipocytes." Plos One 9(5):e96997.

Zhu, Z., Xue, Li-M., Han, T., Jiao, L., Qin, Lu-P., Li, Yu-S., et al., 2010. "Antiosteoporotic effects and proteomic characterization of the target and mechanism of an er-Xian decoction on osteoblastic UMR-106 and osteoclasts induced from RAW264.7." Molecules (Basel, Switzerland) 15(7):4695–4710.