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**Comparison of the transcriptional responses of skeletal muscle and bone to a flooding dose of leucine in the gilthead sea bream (*Sparus aurata*).**

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**Abstract**

Skeletal muscle, cartilage and bone must function in a co-ordinated fashion during locomotion and growth. In the present study on the gilthead sea bream (*Sparus aurata*) we tested the hypothesis that muscle and bone differ in their responsiveness to stimuli eliciting fast growth, providing a potential mechanism for generating the skeletal deformities observed in aquaculture. To investigate transcription regulation in skeletal muscle and bone we stimulated protein synthesis using a flooding dose of the branched chain amino acid leucine and compared the results with saline-injected controls. To increase the amount of available sequence information for gene expression analysis a de novo transcriptome was assembled using publicly available Next Generation Sequencing libraries from embryo, fast skeletal muscle, bone and cartilage. The resulting 5 million reads were assembled into 125,646 isotigs representing around 16,000 unique genes, including most components of the Pi3k/Akt/mTor signalling pathway. Principal Components Analysis was able to distinguish the transcriptional responses between leucine and saline injected controls in skeletal muscle, but not in bone. General Linear Modelling revealed significant temporal changes in gene expression following leucine injection including the tissue-specific markers *sparc*, *bglap* (bone), *mlc2* and *myod2* (muscle) and gene transcripts associated with Pi3k/Akt/mTor signalling, *p70sk6*, *akt2*, *ampka* and *mtor*. Skeletal muscle showed more pronounced and rapid changes in transcript abundance than bone to the same pro-growth signal. The observed differences in transcriptional response are consistent with the idea that fast growth results in a miss-match between muscle and bone development and may contribute to a higher incidence of skeletal deformities.

Keywords: NGS, muscle, bone, expression, Leucine, mTOR, fast growth, aquaculture, skeletal deformities.

**1. Introduction**

Bone, cartilage and skeletal muscle are the three main components of the musculoskeletal system. The bones give "stability" to the fish body, permit movement, provide physical protection to some organs and have a central role in $Ca^{+2}$ storage and ionic homeostasis (Guerreiro et al., 2007). The trunk muscle is segmented and arranged in myomeres (Altringham and Ellerby, 1999) transmitting contractile force via tendons, to the axial skeleton, resulting in the bending of the fish body and propulsion (Altringham and Ellerby, 1999). Cartilage has a more static function, it forms the new framework for endochondrial bone with a high turnover (eg. fin rays), provides elasticity to the fish skeleton and is involved in a variety of functions including locomotion (Symmons, 1979; Benjamin et al., 1992).

During growth increasing muscle mass needs to be coordinated with bone formation and mineralization. A mismatch in growth rates between tissues could theoretically result in a poorly mineralized bone attempting to support the increasing force generated by muscle, leading to the development of bone deformities (Lysfjord et al., 2002). Such deformities directly affect muscle structure, swimming performance and, ultimately, have an impact on fish growth and welfare (Waagbø et al., 2005). For example, mineralization problems in farmed Atlantic salmon (Salmo salar) were observed to increase during smoltification with adverse impacts on fish welfare (Fjelldal et al., 2006).

The gilthead sea bream (Sparus aurata) is a member of the Sparidae family and is intensively farmed in Southern European countries with an annual production of 120,000 metric tonnes (FAO, 2008). Bone deformities have a significant impact on gilthead sea bream production, with nearly 80% of fry and juveniles affected (Andrades et al., 1996; Boglione et al., 2001; Boglione et al., 2013). Various types of skeletal deformities have been described, including an incomplete jaw, open operculum, abnormal caudal fin and deformities in the $2^{nd}$ and $6^{th}$ vertebrae of the backbone (Moretti et al., 1999; Boglione et al., 2001; Boglione et al., 2013).

Branched chain amino acids, particularly leucine, are powerful stimulators of protein synthesis in mammals (Anthony et al., 2001) and fish (Kawango et al., 2015; Ren et al., 2015), and suppress protein breakdown in humans (Louard et al., 1995). Pi3k/Akt/mTor signalling is the main pathway regulating protein synthesis during muscle growth in fish (Seiliez et al., 2008; Johnston et al., 2011; Garcia de la serrana et al., 2012a). Recent studies in mammals have also identified the Pi3k/Akt/mTor pathway as an important promoter of growth and development in bone (Xian et al., 2012) with key roles in

chondrocytes (Phornphutkul et al., 2008) and osteoblasts (Sun et al., 2013) in response to pro-growth signals such as leucine (Kim et al., 2009).

The first objective of the present study was to increase the available nucleotide sequence resources for gene expression studies in gilthead sea bream by generating a de novo assembly using publicly available EST and 454-Next Generation Sequencing (NGS) data from embryos, bone, cartilage and fast skeletal muscle (Garcia de la serrana et al., 2012b; Florbela et al., 2013; Calduch-Giner et al., 2013). Phylogenetic analyses enabled paralogues associated with the teleost-specific whole genome duplication to be identified and specific primers developed to study the transcriptional regulation of bone and muscle growth markers and components from the Pi3k/Akt/mTor and associated signalling pathways. In order to further investigate the bone-muscle growth miss-match hypothesis of skeletal deformities gene expression signatures were compared over time in groups of fish injected with either saline or a flooding dose of the branched chain amino acid leucine.

## 2. Material and methods

### *2.1. Gilthead sea bream de novo transcriptome and bioinformatic analyses*

Publicly available 454 Titanium-libraries for skeletal muscle (ERP000874), bone, cartilage (ERP002185), embryo (SRX076735) and all ESTs available for gilthead sea bream (Sparus aurata) were retrieved from NCBI Short Reads Archive (www.ncbi.nlm.nih.gov/sra) (October 2013). Isotigs were constructed using Newbler 2.5 assembler (Roche, 454 Life Sciences) in a Debain Linux system, IBM x3755 8877, with 8 CPU cores (4 x dual-core AMD Opteron), 64-bit, 2.8 GHz processor with 128 Gb of RAM maintained by the University of St Andrews. Isotig identification, gene ontology annotation, pathway mapping by KEGG Automatic Annotation Server (KAAS) (http://www.genome.jp/tools/kaas/), full-length coding sequences (CDS) and paralogue detection were performed as previously described (Garcia de la serrana et al., 2012b). Phylogenetic analysis for paralogue identification and nomenclature were performed as previously described (Garcia de la serrana et al., 2012b).

### *2.2. Digital gene expression analysis*

The 454-libraries from juvenile gilthead sea bream skeletal muscle, bone and cartilage were generated from the same animals enabling a direct comparison of transcript abundance between tissues. Reads used from bone, cartilage and skeletal muscle libraries were separately mapped against the gilthead sea bream de novo transcriptome using GS Reference Mapper (Roche, 454 Life Sciences). Differences in number of reads mapped from each tissue were analysed using R-based script as previously described (Garcia de la serrana et al., 2012b).

*2.3. Leucine and control injections and sampling*

120 gilthead sea bream juveniles of 108.0 ± 0.8g body mass and 17.7 ± 0.04cm fork length (Mean ± SE) were maintained in the CCMAR Ramalhete field station in Faro, Portugal. Animals were distributed between 6 individual 200L fibreglass tanks (30 animals per tank) in an oxygenated, open circuit seawater system and maintained at 24.8 ± 0.2$^{o}$C at constant salinity (36 ± 0.3ppt) in duplicated experiments. Fish were fasted overnight (16h) and anesthetized with tricaine methane sulfonate-MS222 (20mg/l, SIGMA, Dorset, UK) and given a single intraperitoneal injection of 1ml solution containing 150mM (177mg/kg body mass) leucine (leu) (SIGMA) or a saline control, pH 7.4 as previously described in Owen et al., 1999 for salmon. Individuals from both treatments were injected in parallel in groups of 5 at intervals of 20 minutes. Fish were sacrificed before the treatment (0h) and at 1h, 3h, 7h, 12h and 24h post-injection using an overdose of tricaine methane sulfonate-MS222 (50mg/l) (SIGMA) followed by sectioning of the spinal cord. Fast skeletal muscle at 0.5 fork length and a clean section of the backbone were rapidly dissected, flash frozen in liquid nitrogen and stored at -80$^{o}$C until further analysis.

*2.4. Ethical statement*

All animal experiments were carried out in compliance with the Guidelines of the European Union Council (86/609/EU) and Portuguese legislation for the use of laboratory animals. All animal protocols were performed under a Group-1 license from the Direcção-Geral de Veterinaria, Ministerio da Agricultura, do Desenvolvimento Rural e das Pescas.

*2.5. Quantitative real-time PCR*

Total RNA and cDNA synthesis from individual bone and skeletal muscle samples were performed as previously described (Garcia de la serrana et al., 2012a). The qPCR procedures were compliant with the minimal information requirements for publication of quantitative PCR guidelines (Bustin et al. 2009). Primers were designed with Net primer (www.premierbiosoft.com/netprimer/) with melting temperatures of 60$^{o}$C, and where possible crossed an exon-exon junction. Sequences for *ribosomal protein S6 kinase 1* (*p70sk6*), *5'-AMP-activated serine/threonine-protein kinase catalytic subunit alpha* (*ampka*) and *mechanistic target of rapamycin serine/threonine-protein kinase* (*mtor*) were retrieved from the de novo gilthead sea bream transcriptome (sequences retrieved are summarized in Supplementary File 1) while other primers were design based on public sequences (Supplementary File 2). Primers sequences, amplicon size, efficiency and melting temperature for qPCR are listed in Supplementary File 2. All qPCR reactions, primer pair efficiency estimation and controls were performed as previously described (Garcia de la serrana et al., 2012a). The stability of four reference genes *r18*, *β-actin*, *elongation factor 1 alpha* (*ef1α*) and *ribosomal protein L27* (*rpl27*) (Supplementary File 2) was tested using Bestkeeper software (Michael et al. 2004). Analysis demonstrated that *ef1a* was the most stable reference gene across tissues and time points and was selected to calculate the relative expression of each gene using the Pfaffl method (Pfaffl, 2001).

### *2.6. Statistical Analysis*

Statistical analysis of qPCR results was performed using PASW Statistics 21.0 for Mac (IBM). Gene expression was analysed using principal components analysis (PCA) and a general linear model with treatment as the variable, tissue and time as cofactors and fish length as a random factor. The number of reads mapped from each condition was extracted using the R statistical package (www.R-project.org) and Chi-square statistic followed by a Hochberg-Benjamin (false discovery rate, FDR) correction was applied to detect significant differences in the number of reads per isotig as previously described (Garcia de la serrana et al., 2012b). Graphs comparing the contribution of reads from each experimental condition per isotig were constructed using R-script as previously described (Garcia de la serrana et al., 2012b).

## 3. Results

### *3.1. De novo assembly*

Over 5 million reads were assembled into 125,646 isotigs with an average length of 725bp (N50=1073bp). Around 50,000 isotigs were successfully annotated, containing over 16,000 unique genes (Supplementary File 3), equivalent to the 62 to 83% of the protein-coding genes described for other teleost species (www.ensembl.org). Comparison of zebrafish complete proteome against the gilthead sea bream transcriptome allowed us to identify over 4,000 different isotigs containing more than 90% of the coding sequence (Supplementary File 3). A total of 180 paralogue pairs were identified in the gilthead sea bream transcriptome using phylogenetic analyses and named according to the zebrafish nomenclature, significantly increasing the number identified for this species (Garcia de la serrana et al., 2012b; Supplementary File 3).

*3.2. Skeletal muscle, bone and cartilage digital gene expression*

In order to study the major differences in digital gene expression (DGE) between fast skeletal muscle, bone and cartilage reads were mapped against the global transcriptome and normalized by isotig length and library size. Pairwise comparisons of reads mapped were used to infer differential DGE between tissues (Fig. 1).

The top 10 isotigs appearing in the ranked list of the most differently expressed genes between tissues are shown in Table 1 (the complete list can be found in Supplementary File 4). Gene Ontology (GO) analysis of all genes that showed significant differences in expression in fast skeletal muscle (Supplementary File 5) revealed enriched in genes categories related to "muscle contraction" (GO:0006936), "ribosomal subunit" (GO:0044391), "sarcomere" (GO:0030017) and "gluconeogenesis" (GO:0006094). In contrast, categories such as "intermediate filament cytoskeleton organization" (GO:0045104), "neurofilament" (GO:0005883), "structural molecular activity" (GO:0005198) and synapse (GO:0045202) were enriched in bone and "electron transport chain" (GO:0022900), "secretion" (GO:0046903), "respiratory chain" (GO:0070469) and "fibrillar collagen" (GO:0005583) were enriched in cartilage (Supplementary File 5).

*3.3. Effects of a flooding dose of leucine on gene expression*

Gilthead sea bream were injected with a flooding dose of leucine or a saline control to study the response at transcriptional level in bone and muscle for key components or the Pi3k/Akt/mTor and associated signalling pathways including *akt2*, *ampka*, *mtor* and *p70sk6*. Tissue-specific genes for

skeletal muscle (*myod2*, *mlc2*) and bone (*bglap*, *sparc*) plus col1a were also included in the analysis. Principal components analysis (PCA) was used to initially explore the data and establish whether the observed differences in genes expression could be attributed to treatments and/or tissues. Our PCA analysis clearly separated gene expression signatures from the different tissues (Fig. 2A), driven by the influence of the tissue-specific genes (Fig. 2B). PCA analysis also distinguished between leucine injected and control individuals for skeletal muscle, but for bone (Fig. 2A), consistent with a more pronounced impact on transcription in skeletal muscle. General linear modelling demonstrated that leucine injection significantly changed the expression of all Pi3k/Akt/mTor components measured (*mtor*, *akt2* and *p70sk6*) (Fig. 3; Table 2). The expression of *ampka*, a gene with a central role in regulating energy metabolism, showed no clear pattern following leucine injection (Table 2; Supplementary File 6). Skeletal muscle gene markers *myod2* and *mlc2* were expressed at very low levels or were absent in bone tissue (Fig. 4). Myod2 is a member of the myogenic regulatory factor family of transcription factors involved in the activation of myoblasts and initiation of the myogenesis program (Review in Fong and Tapscott, 2014 and Johnston, 2006) while mlc2 is a contractile protein (reviewed in Scruggs and Solaro, 2011). *Mlc2* and *myod2* significantly increased expression in response to leucine injection with a rapid recovery of *mlc2* to pre-injection values, but a sustained elevation of *myod2* (Fig. 4; Table 2). Sparc is secreted by osteoblasts during bone formation, initiates mineralization and form complexes with collagen (Jundt et al., 1987; Yan and Sage, 1999). Bglap is only secreted by osteblasts and plays a crucial role in bone matrix formation representing up to 1-2% of the total bone protein with a strong affinity for calcium (Lee et al., 2007). *Sparc* and *bglap* genes were detected in bone, but not in skeletal muscle, and col1a was present in both tissues (Fig. 5; Table 2; Supplementary File 6). Expression of *sparc* and *bglap* were significantly affected by leucine injection (Table 2; Figure 5), although considerable variability was observed between individuals. The detection of *col1a* in skeletal muscle could be explained by the presence of tendons and the connective sheet surrounding muscle fibres, where *col1a* is an important component (Supplementary File 6). There were significant changes in transcript abundance for Pi3k/Akt/mTor components after only 1h in skeletal muscle but not until 3-7h after injection in bone, consistent with differences in the sensitivities of the tissues to a pro-growth stimulus (Fig. 3).

**4. Discussion**

The gilthead sea bream de novo transcriptome described here significantly improves on previous transcriptomes in terms of the number of isotigs annotated (>50,000), and number of unique genes identified (>16,000) including near full-length transcripts (2,981 with >90% of CDS) (Garcia de la serrana et al., 2012b). The number of paralogues retained from the teleost-specific whole genome duplication is around 20% of gene content (Garcia de la serrana et al., 2014) whereas we could only identify 360 from this 454 assembly equivalent to 24% of the expected number and much less than estimates in some other fish species using the Illumina platform (Mareco et al., 2015).

The DGE analysis was able to capture basic differences between tissues (Fig. 1, Table 1; Files S4 and S5). Skeletal muscle showed a significant enrichment of muscle-specific genes including sarcomere structural components such as *mlc2* or *myosin heavy chain* (*mhc*) (Clark et al., 2002) and myogenic regulators such as *myostatin* (Johnston, 2006) together with an enrichment in genes related to "gluconeogenesis", e.g. *aldolase* and *phosphoglucomutase*, reflecting the predominant glucose-based metabolism in fast skeletal muscle (Izumiya et al. 2008). Bone and cartilage DGE and GO analyses were also consistent with the main physiological roles of these tissues, particularly genes related to secretion, collagen and production of extracellular matrix (Estêvão et al., 2011) (Table1; Supplementary File 4 and 5). However, there was a significant presence of genes related to neurofilaments in the bone libraries and "oxidation-reduction processes" in the cartilage libraries, a strongly anaerobic tissue (Grimshaw and Manson, 2004) suggesting that neural cord and gills were not totally removed during dissection of bone and cartilage samples, respectively.

Previous studies in mammals have already demonstrated the importance of Pi3k/Akt/mTor pathway in bone growth and matrix development (Xian et al., 2012), but this remains unexplored in fish. Our results show that key components of the Pi3k/Akt/mTor pathway are stimulated in bone and skeletal muscle in response to leucine injection, consistent with an increase in protein synthesis. Downstream genes associated with growth in muscle (*myod2* and *mcl2*) and bone (*sparc* and *bglap*) were similarly more highly expressed in leucine-injected than saline-injected controls.

The main finding of the present study was that transcripts showed a faster and more pronounced response to leucine injection in skeletal muscle than bone for the majority of genes analysed (Fig. 2). The reasons behind the distinct time courses of transcriptional responses in bone and muscle are unknown, but could include differences in the dynamics of leucine uptake, amino acid sensing and other regulatory processes. GO terms related to "translational elongation" and "ribosomal subunit"

(Supplementary File 4) were significantly enriched in skeletal muscle compare to bone in our transcriptome, indicating a greater capacity for protein synthesis in the former tissue. Skeletal muscle in fish has a well-known function as a reservoir of amino acids which are seasonally mobilised for migration and to build gonads prior to spawning (Mommsen et al., 1980). This function together with a higher capacity for protein synthesis may also underlie the faster and more pronounced transcriptional response to pro-growth signals in muscle than bone. Bone marrow has a role in regulating bone growth by contributing monocyte-macrophage cells to the osteoclast population, by stimulating osteoclastogenesis and through secretion of IL-11 (Usha and Nandeesh, 2000). Bone marrow is also a primary source of Igf1, one of the key regulators of the Pi3k/Akt/mTor pathway (Xian et al., 2012).

Igf1 receptors in fish muscle stimulate amino acid uptake (Degger et al., 2000), leading to myoblast proliferation and DNA synthesis (Pozios et al., 2005; Duan et al., 2010). Igf1 provokes a rapid proliferative response in sea bream myoblasts in culture within 24h – 36h of treatment (Castillo et al., 2002). It has been suggested that in bone where growth is regulated through the growth hormone receptor in salmon vertebrae whereas the Igf1 receptor is more involved with mineralisation (Wargelius et al., 2005). Furthermore, in vitro experiments with sea bream osteocytes found that proliferative responses to Gh and Igf1 treatment were relatively slow and only observed after 5 d (Capilla et al., 2003).

Differential responses to powerful pro-growth signals at the transcriptional, protein signalling and cellular levels provide a potential mechanism for the hypothesised mismatch between the growth of muscle and bone under conditions of fast growth in all stages of the life cycle (Boglione et al., 2013; Lysfjord et al., 2002; Lewis et al., 2004; Wargelius et al., 2009) and likely contribute to the high incidence of skeletal deformities observed in aquaculture for this species.

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## 6. CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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**Table 1.**

Top 10 differentially expressed genes in number of mapped reads between bone, cartilage and fast skeletal muscle.

| Tissue | Gene ID | Length (bp) | Muscle (reads) | Bone (reads) | Cartilage (reads) | FDR | Orthologue ID |
|---|---|---|---|---|---|---|---|
| *Muscle* | *Glycogen synthase* | 589 | 6372 | 64 | 220 | 0.0054 | *gys1* |
| | *Myosin Light Chain 2* | 598 | 5896 | 43 | 34 | 0.0064 | *mlc2* |
| | *Myosin heavy chain* | 866 | 5561 | 52 | 18 | 0.0109 | * |
| | *Fructose-bisphosphate aldolase a* | 599 | 5477 | 56 | 23 | 0.0237 | *aldoa* |
| | *Phosphoglucomutase 1* | 301 | 3803 | 12 | 53 | 0.0057 | *pgm1* |
| | *Lysophosphatidylcholine acyltransferase 1* | 183 | 3376 | 0 | 0 | 0.000 | *lpcat1* |
| | *Sarcoplasmic endoplasmic reticulum calcium atpase 1* | 352 | 2991 | 21 | 37 | 0.032 | *atp2a1* |
| | *solute carrier family 25 member a5* | 317 | 2496 | 21 | 14 | 0.00041 | *slc25a5* |
| | *Glycerol-3-phosphate dehydrogenase* | 221 | 2028 | 6 | 2 | 0.0012 | *gpd1* |
| | *Hemoglobin subunit alpha-2* | 269 | 1936 | 22 | 64 | 0.010 | *hba2* |
| *Bone* | *Vimentin* | 2188 | 0 | 509 | 0 | 0.000 | *vim* |
| | *Rab gdp dissociation inhibitor alpha* | 3292 | 0 | 291 | 8 | 0.000 | *gdi1* |
| | *Neurofilament-medium polypeptide* | 1214 | 0 | 226 | 0 | 0.000 | *nefm* |
| | *Asporin* | 2523 | 0 | 222 | 10 | 0.0108 | *aspn* |
| | *Stathmin* | 1771 | 0 | 216 | 2 | 0 | *stmn1* |
| | *Ubiquitin carboxyl-terminal hydrolase isozyme l1* | 1389 | 0 | 194 | 2 | 0 | *uchl1* |
| | *Complexin 1* | 2039 | 0 | 190 | 0 | 0 | *cplx1* |
| | *Probable g-protein coupled receptor 132* | 700 | 0 | 177 | 4 | 0 | *gpr132* |
| | *Synaptosomal-associated protein 25* | 2127 | 0 | 175 | 0 | 0 | *snap25* |
| | *Peripherin* | 1117 | 0 | 175 | 0 | 0 | *prdh* |
| *Cartilage* | *nadh dehydrogenase subunit 1* | 284 | 212 | 429 | 3396 | 0.0002 | *mt-nd1* |
| | *nadh dehydrogenase* | 516 | 108 | 129 | 3218 | 0.007 | *mt-nd5* |

| | | | | | | |
|---|---|---|---|---|---|---|
| *subunit 5* | | | | | | |
| *cytochrome c oxidase subunit iii* | 177 | 34 | 103 | 2481 | 0.000 | *mt-co3* |
| *nadh dehydrogenase subunit 4* | 266 | 87 | 23 | 2327 | 0.0004 | *mt-nd4* |
| *nadh dehydrogenase subunit 6* | 252 | 61 | 89 | 1461 | 0.011 | *mt-nd6* |
| *Tenascin isoform 1* | 3381 | 0 | 2 | 671 | 0.000 | *tnxb* |
| *Coagulation factor xiii* | 4670 | 0 | 2 | 573 | 0.000 | *f13a1* |
| *Calcium-binding tyrosine* | 605 | 0 | 17 | 440 | 0.000 | *cabyr* |
| *Cartilage matrix protein* | 2497 | 0 | 42 | 385 | 0.000 | *crtap* |
| *Calcium-binding phosphoprotein 5 precursor* | 925 | 0 | 8 | 346 | 0.000 | *notch1* |

Gene ID and orthologue name were obtained by blastx against the NCBI nr database. FDR: False Discovery Rate.

* The specific isoform of the myosin heavy chain was not identified with a high enough degree of confidence.

**Table 2.**

Statistical analysis of gene expression in response to leucine or saline injection. P-values for time, tissue, treatment factors and tissue*treatment interaction are shown.

| | Gene ID | Time | Tissue | Treatment | Tissue *Treatment |
|---|---|---|---|---|---|
| *Pi3k/Akt/mTor* | *akt2* | 0.45 | 0.00 | 0.00 | 0.01 |
| | *mtor* | 0.12 | 0.00 | 0.00 | 0.01 |
| | *p70sk6* | 0.02 | 0.67 | 0.00 | 0.01 |
| | *ampka* | 0.05 | 0.63 | 0.01 | 0.01 |
| *Muscle markers* | *myod2* | 0.35 | 0.00 | 0.00 | 0.27 |
| | *mlc2* | 0.31 | 0.00 | 0.04 | 0.02 |
| *Bone markers* | *col1a* | 0.74 | 0.04 | 0.18 | 0.20 |
| | *bglap* | 0.21 | 0.00 | 0.00 | 0.21 |
| | *sparc* | 0.25 | 0.00 | 0.00 | 0.25 |

Ribosomal protein S6 kinase 1 (p70sk6), RAC-beta serine/threonine-protein kinase (akt2), osteonectin (sparc), osteocalcin (bglap), myosin light chain 2 (mlc2), myoblast determination protein 2 (myod2), 5'-AMP-activated serine/threonine-protein kinase catalytic subunit alpha (ampka), mechanistic target of rapamycin serine/threonine-protein kinase (mtor) and Collagen alpha-1(I) chain (col1a).

**Figures legends**

**Fig. 1.** Dot plot pairwise comparison of reads mapped from each tissue to the annotated isotigs. Each dot represents an isotig with reads from one or both tissues. X and Y graph illustrates the relative abundance of reads from each tissue mapped per isotig.

**Fig. 2.** (A) Principal component analysis using the expression of all genes analysed in bone (empty circles for controls and empty diamonds for leucine) and skeletal muscle (full circles for controls and full diamonds for leucine). Dashed ellipse include >80% of the skeletal muscle control samples; continuous ellipse include >80% of the leucine injected skeletal muscle samples (B) Relative weight contribution of each of the genes analysed in the generation of the two PCA components during the principal components analysis and the percentage of variability explained by each of the components. Genes part of the Pi3k/Akt/mTor pathway are indicated in dark grey background, skeletal muscle gene markers are indicated in pale grey background and bone gene markers are indicated in white background. Ribosomal protein S6 kinase 1 (p70sk6), RAC-beta serine/threonine-protein kinase (akt2), osteonectin (sparc), osteocalcin (bglap), myosin light chain 2 (mlc2), myoblast determination protein 2 (myod2), 5'-AMP-activated serine/threonine-protein kinase catalytic subunit alpha (ampka), mechanistic target of rapamycin serine/threonine-protein kinase (mtor) and collagen alpha-1(I) chain (col1a)

**Fig. 3.** Gene expression of Pi3k/Akt/mTor components in response to intra-peritoneal injection of leucine in bone and skeletal muscle. Gene expression values are expressed as arbitrary units (a.u) for RAC-beta serine/threonine-protein kinase (akt2) (A), mechanistic target of rapamycin serine/threonine-protein kinase (mtor) (B) and ribosomal protein S6 kinase 1 (p70sk6) (C) in response to leucine (filled symbols) or saline (open symbols) for bone (circles) and skeletal muscle (squares). Values represent mean ± SE (N=10 fish per time point for each treatment).

**Fig. 4.** Gene expressions of skeletal muscle gene markers in response to intra-peritoneal injection of leucine in bone and skeletal muscle. Gene expression values are expressed as arbitrary units (a.u) for myoblast determination protein 2 (myod2) (A) and myosin light chain 2 (mlc2) (B) in response to

leucine (filled symbols) or saline (open symbols) for bone (circles) and skeletal muscle (squares). Values represent mean ± SE (N=10 fish per time point for each treatment).

**Fig. 5**. Gene expressions of skeletal muscle gene markers in response to intra-peritoneal injection of leucine in bone and skeletal muscle. Gene expression values are expressed as arbitrary units (a.u) for osteonectin (sparc) (A) and osteocalcin (bglap) (B) in response to leucine (filled symbols) or saline (open symbols) for bone (circles) and skeletal muscle (squares). Values represent mean ± SE (N=10 fish per time point for each treatment).

**Supplementary Files**

**Supplementary File 1.** Mechanistic target of rapamycin serine/threonine-protein kinase (mtor), ribosomal protein S6 kinase 1 (p70sk6) and 5'-AMP-activated serine/threonine-protein kinase catalytic subunit alpha (ampka) extracted from the de novo gilthead sea bream transcriptome used for primer design.

**Supplementary File 2.** Primers sequences used for quantitative PCR amplification. E=efficiency, Tm=melting temperature. Ribosomal protein S6 kinase 1 (p70sk6), RAC-beta serine/threonine-protein kinase (akt2), osteonectin (sparc), osteocalcin (bglap), myosin light chain 2 (mlc2), myoblast determination protein 2 (myod2), 5'-AMP-activated serine/threonine-protein kinase catalytic subunit alpha (ampka), mechanistic target of rapamycin serine/threonine-protein kinase (mtor), collagen alpha-1(I) chain (col1a), s18, beta actin (β-actin), elongation factor 1 alpha (ef1α) and ribosomal protein L27 (rpl27).

**Supplementary File 3.** Compressed file containing transcriptome BLAST results, list of unique genes found and phylogenetic trees of paralogues identified in the de novo transcriptome.

Supplementary File 4. Complete list of genes differentially expressed in muscle, bone and cartilage.

**Supplementary File 5.** Gene Ontology analysis of differently expressed genes in muscle, bone and cartilage.

**Supplementary File 6.** Gene expressions of skeletal muscle gene markers in response to intra-peritoneal injection of leucine in bone and skeletal muscle. Gene expression values are expressed as arbitrary units (a.u) for 5'-AMP-activated serine/threonine-protein kinase catalytic subunit alpha (ampka) (A) and collagen alpha-1(I) chain (col1a) (B) in response to leucine (filled symbols) or saline (open symbols) for bone (circles) and skeletal muscle (squares). Values represent mean ± SE (N=10 fish per time point for each treatment).



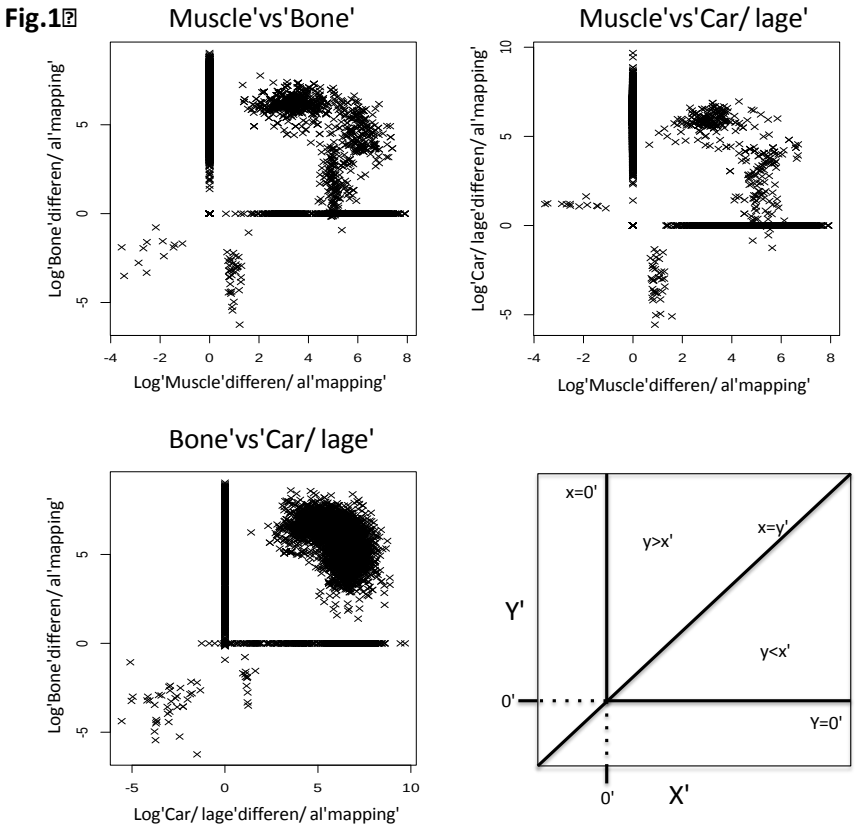
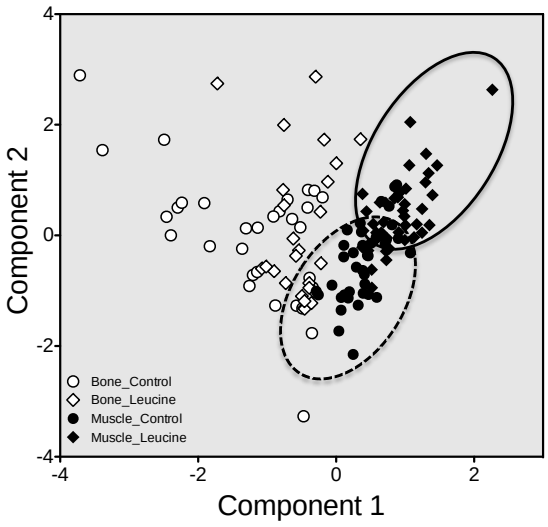


**Fig.2**

**A**




**B**

| Gene name | Component 1 | Component 2 |
|---|---|---|
| *akt2* | 0.9 | 0.0 |
| *p70sk6* | 0.4 | 0.6 |
| *mtor* | 0.7 | 0.5 |
| *ampka* | 0.2 | 0.7 |
| *mlc2* | 0.8 | -0.1 |
| *myod2* | 0.7 | 0.0 |
| *bglap* | -0.7 | 0.6 |
| *col1a* | 0.1 | 0.4 |
| *sparc* | -0.7 | 0.6 |
| % Variability explained (accumulated) | 36.5 | 57.6 |

**Fig.3"**

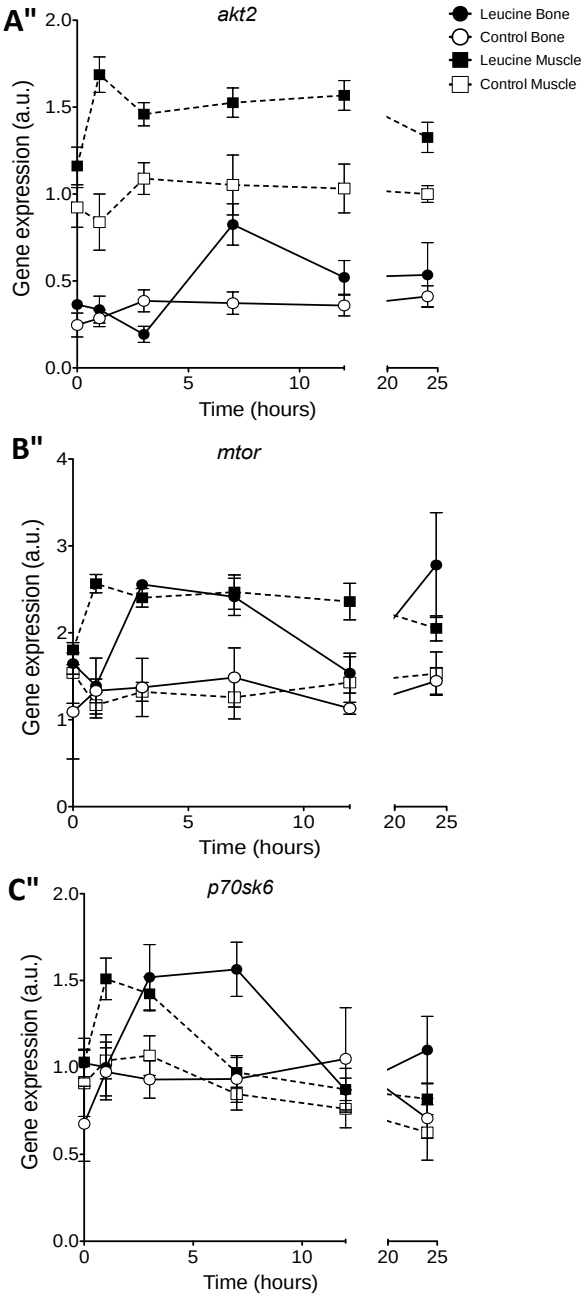


**Fig.4"**

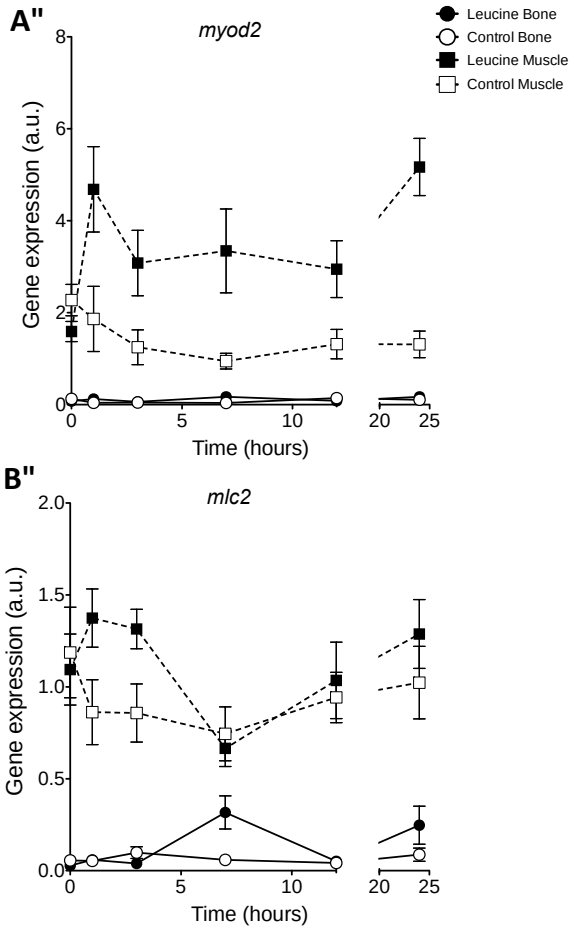


**Fig.5"**