SORAIA ISABEL GONÇALVES DOS SANTOS

Comparative Ontogeny of European Sea bass (Dicentrarchus labrax) and Gilthead Sea bream (Sparus aurata) using Potential Biomarkers of Larval Quality

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Mestrado em Biotecnologia

Trabalho elaborado sob orientação de:

Professora Doutora Deborah Mary Power

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Abstract

Gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) are important aquaculture species in the Mediterranean. The hatchery production of these species is of crucial importance as it supplies the juveniles and determines the productivity and quality of the commercialized fish. The aim of the present thesis is to provide an understanding of gilthead sea bream and European sea bass larval ontogeny and the challenges faced during hatchery production. The overall goal of the project is to contribute to improve key performance indicators (KPIs) by developing molecular indicators linked to the quality of this critical production stage. To achieve the objectives, European sea bass and gilthead sea bream larvae from several production batches with four early development larval stages before the juvenile phase, were received from different hatcheries. Each batch was classified as good or bad based on skeletal malformations then larvae were prepared for molecular analysis (quantitative PCR, qPCR) to evaluate genes related to the skeleton, endocrine system, immune system, and metabolic genes. The genes used for qPCR analysis were derived from a preliminary screen of genes that clustered with "good" or "bad" batches of larva and included 9 potential candidate genes for European sea bass and 12 for Sea bream. In general, divergent expression of genes related to good and bad quality were detected at later developmental stages in the gilthead sea bream. In contrast, divergent expression of genes related to good and bad quality were detected very clearly in early to late developmental stages of European sea bass. The qPCR analysis identified 5 core quality biomarker genes strongly linked to skeletal ontogeny in Sea bream (*gk, ostc, op, on* and *m-cad*) and 2 different genes in European sea bass (*try* and *apoa1*). Further analysis on a greater number of larval production batches is required to confirm the robustness and applicability of the biomarker genes to the hatchery stage of production in industry.

Keywords: Aquaculture; commercial hatcheries; larval development; quality biomarkers; qPCR.

Resumo

A dourada (*Sparus aurata*) e o robalo (*Dicentrarchus labrax*) são as duas espécies mais importantes na aquacultura do Mediterrâneo. A produção destas espécies em cativeiro é de extrema importância uma vez que fornecem os juvenis às empresas de aquacultura, sendo durante a sua produção que se determina a produtividade e a qualidade do peixe que irá ser comercializado. A ontogenia das larvas e a qualidade dos juvenis são afetadas por uma série de fatores durante a fase de produção em cativeiro, além disso, os eventos que ocorrem no início do desenvolvimento têm o potencial de impactar na trajetória de desenvolvimento e o desempenho dos estados de desenvolvimento posteriores essencialmente modulando a performance dos juvenis O desenvolvimento de métodos rápidos, simples e de baixo custo para prever a qualidade das larvas e, idealmente, ligá-la ao desempenho das fases posteriores é uma prioridade para as empresas de produção de larvas no Mediterrâneo. Quanto mais cedo durante o ciclo de produção os indicadores de qualidade das larvas puderem ser definidos, maiores serão as poupanças financeiras e de tempo investido, uma vez que os lotes com fraco desempenho podem ser eliminados com antecedência. Por exemplo, algumas anomalias morfológicas externas podem ser detetadas precocemente no desenvolvimento e esses peixes podem ser removidos das culturas ou, se uma elevada proporção de um lote estiver malformada, ou mesmo a cultura pode ser interrompida. No entanto, se as malformações só se tornarem evidentes nos juvenis, isso levará a uma redução significativa no seu valor de venda, apesar de uma despesa semelhante na sua produção em comparação com os juvenis de alta qualidade. Potenciais indicadores de qualidade já são aplicados a larvas de peixes e relacionados com as características morfológicas, metabolismo ósseo, condição nutricional, potencial de crescimento e estado imunológico, mas combinando características morfológicas com a expressão de genes selecionados, pode ser uma nova abordagem poderosa para a classificação precoce da qualidade larval, mesmo antes que os sinais morfológicos se tornem óbvios. Compreender a ontogenia dos peixes relacionada ao potencial de crescimento, desenvolvimento ósseo, estado imunológico e nutricional pode ajudar a identificar os indicadores de qualidade dos peixes.

O objetivo desta tese é fornecer uma resposta com aplicação na indústria de criação de larvas em viveiros, que possa contribuir para a melhoria dos principais indicadores de desempenho (KPIs) por meio de desenvolvimento de indicadores moleculares vinculados á qualidade dessa etapa crítica e sensível da produção. Assim,

larvas de robalo e dourada provenientes de vários lotes de produção de diferentes companhias, com quatro estados larvares de desenvolvimento foram usados para este trabalho. Primeiramente foi criado um manual com os procedimentos de amostragem para ajudar as empresas incubadoras destas espécies que colaboraram connosco a fim de obter uma análise mais homogénea ´minimizando as possíveis diferenças inerentes á origem de cada amostra/larva. Após a receção de cada amostra foi passada por um processo de controlo qualidade, onde foi classificado a viabilidade da amostra antes de avançar com a extração de todo o lote, a verificação foi baseada na qualidade do RNA. Cada lote foi classificado como bom ou mau com base em malformações esqueléticas. As larvas foram homogeneizadas para extrair o RNA, para posterior síntese do cDNA usado para avaliar a expressão de vários genes relacionados com desenvolvimento esquelético, endócrino, imune e metabólico por qPCR, comparando os piores e melhores lotes em cada espécie dentro de cada idade larval. Os genes usados para análise qPCR foram previamente selecionados com base em pesquisa bibliográfica, onde verificamos quais os potenciais genes importantes durante a ontogenia destas duas espécies e também am genes que já tínhamos "em laboratório" relacionados com os vários processos fisiológicos e daí, 9 genes foram dados como potenciais candidatos para robalo e 12 para dourada com base na comparação de pools de amostra de larva pertencentes a maus lotes com bons lotes. Em geral, a dourada em comparação com o robalo mostrou diferenças significativas em fases mais tardias na ontogenia, na expressão dos genes potenciais, quando comparados os melhores versus os piores lotes. No robalo essas diferenças são detetadas desde o início até ao último estado larval. A análise qPCR identificou 5 genes principais de biomarcadores de qualidade fortemente ligados à ontogenia esquelética em dourada (*gk*, *ostc*, *op*, *on* e *m-cad*) e 2 genes diferentes em robalo (*try* e *apoa1*). Análises adicionais com um número maior de lotes de produção de larvas são necessárias para confirmar a robustez e aplicabilidade dos genes biomarcadores para a fase de incubação da produção nas indústrias de aquacultura. Este trabalho contribuiu para gerar um grande conjunto de dados e, em seguida, treinar o algoritmo num software de estatística para prever a qualidade das larvas com base em um número mínimo de genes. O resultado do trabalho da ferramenta de avaliação de qualidade desenvolvida que será brevemente mencionada aqui, pois foi desenvolvida pelos nossos parceiros da Grécia.

Palavras-chave: aquacultura; desenvolvimento larval; indicadores de qualidade; qualidade de peixe; qPCR; viveiro.

Abbreviations

18S – 18S Ribossomal RNA gene in eukaryotes

ALP – Alkaline phosphatase

CATK – Cathepsin K

cDNA – Complementary DNA

CLIP2 – CAP-Gly domain-containing linker protein 2

Col I – Collagen 1

Col1A1 – Collagen type I alpha 1 chain

Ct – Cycle threshold

ELR – End of larval rearing

FF – First feeding

Fib1a – Fibrillin 1

FL – Flexion

FN1a – Fibronectin 1a

IGF1– Insulin-like growth factor 1

KPIs – Key performance indicators

MM – Metamorphosis

OCN – Osteocalcin

OP – Osteopontin

OPG/RANKL/RANK – System with roles in osteoclast maturation, bone modeling, and bone remodeling

OSN/ON – Osteonectin

qPCR – Quantitative polymerase chain reaction

QTLs – Quantitative trait locus

Runx2 – Runt-related transcription factor 2

TRAP – Tartrate-resistant phosphatase

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CHAPTER I

General Introduction

1.1. Fish ontogeny and prediction of quality

Larvae ontogenesis and juvenile quality are affected by a range of factors during the hatchery phase (Pittman et al., 2013), as will be described later. Furthermore, events occurring early in development have the potential to impact the developmental trajectory and performance of later stages (Herbst et al., 2011). Development of fast, simple, and low-cost methods to predict the quality of larvae and ideally link this to the performance of later stages is a priority for hatcheries. Moreover, the earlier during the production cycle that larval quality indicators can be defined the greater the potential financial savings as poor-performing batches can be eliminated. For example, some external morphological anomalies can be detected early in development and such fish can be removed from cultures or if a high proportion of a batch is malformed the culture can be interrupted. However, if malformations only become evident in juveniles, then this leads to a significant reduction in their sale value despite a similar overhead for their production compared to high quality juveniles (Koumoundouros et al., 2017). Quality descriptors and predictors already applied to fish larvae and related to morphological traits, bone metabolism, nutritional condition, growth potential and immune status are described in Koumoundouros, et al. 2017, but combining morphological characteristics with expression of selected genes, can be a powerful new approach for the early classification of larval quality even before blatant signs become obvious. Understanding fish ontogeny related to growth potential, bone development, immune and nutritional status can help to identify fish quality predictors as described below.

1.1.1. Growth and Endocrine System

Prediction of the growth potential of juvenile fish as early as possible is of major interest to the hatcheries. Knowledge about the endocrine system and the application of hormones particularly to induce reproduction has contributed to a successful and sustainable fish aquaculture industry. Knowledge about the endocrine system has contributed solutions for artificial reproduction, gamete viability, improved growth and through improved diets the means to avoid disease and malformations (Power et al., 2007). During early larvae development and growth, organs are functionally activated, and endocrine factors are involved in this process and their relative importance and role varies across ontogeny (Tanaka et al., 1995).

Hormones linked to development and traits (metamorphic success, stress, growth rate, development of the musculoskeletal system etc) of interest in hatchery stages include thyroid hormones (*THs*), cortisol, growth hormone (*GH*), insulin-like growth factors (*IGF I* and *II*), and prolactin (*PRL*) (Power et al., 2001). Perturbations in normal endocrine levels during larval or juvenile development compromises larval viability and quality, modifies metamorphosis and reduces juvenile viability and performance (Power et al., 2001). Additional information at the level of molecular mechanisms is needed to understand how embryo and larvae ontogeny is related to different endocrine axis in order to identify and implement strategies to improve larval and juvenile quality(Pittman et al., 2013). However, some evidence shows that events happening at the very beginning of larval development can have long term effects on growth. For example, thyroid hormones receptors are expressed in several fish species, in eggs before hatch and in larvae, including gilthead sea bream (Power et al., 2001)and the presence of *IGF* is detected in developing embryos (Reinecke et al., 2005; Rescan, 2005). In European sea bass, muscle growth can be modified by early temperature-dependent imprinting in embryos and larvae controlled most likely by epigenetic mechanisms (Alami-Durante et al., 2007).

1.1.2. Skeletal development

In hatcheries, skeletal abnormalities represent an important KPI (key performance indicator) since they significantly impact fish welfare and also the commercial value of larvae and juveniles. In fish under intensive production their skeletal development can be affected by environmental and nutritional factors and if inadequate, can give rise to skeletal deformities (Helvik, 2009). Skeletal anomalies can be detected as early as the embryonic and early larval stages even before the skeleton is fully developed (Boglione et al., 2013; Kourkouta et al., 2022a). In the European sea bass notochord anomalies in hatched larvae can be lethal (Koumoundouros et al., 2001) and caudal anomalies in post flexion larvae affect swimming with consequences for growth(Boglione et al., 2013b) (Boglione et al., 2013b). In the gilthead sea bream, lordotic morphology appeared at 18dph (Sanatamaría et al., 1994), and lordosis at 60dph (Boglione et al., 2013b).

To understand fish skeletogenesis, a list of potential markers linked to bone anomalies is needed and can be good tools to determine the quality of larvae and subsequent juveniles in fish farms. Helping fish farmers decide which production batches should be reared through to the juvenile stage or having a tool that can classify the production quality can give added value to their production. The lack of validated robust markers and

the lack of equipment and qualified personal is a limitation for the development and validation of quality monitoring tools for commercial fish producers. Another limitation of current approaches is the mismatch between quantitative skeletal markers and the prediction of the likely emergence of skeletal anomalies (Monzón et al., 2017). The status in relation to the use of skeletal morphological indicators is that more research is required, and more robust and predictive markers are necessary for the aquaculture industry.

Genes related to bone metabolism and formation that can be used as potential molecular biomarkers of fish skeletal quality during early development stages are: tartrate-resistant acid phosphatase (*TRAP)* and cathepsin K (*CATK)* (Boglione, 2001); osteoprotegerin/receptor activator NF-Kb/ receptor activator NF-Kb ligand (*OPG/RANKL/RANK* system) (Khosla, 2001; Komuro et al., 2001); Retinoic acid (Harada et al., 1995); runt-related transcription factor 2 *(Runx2)*, alkaline phosphatase (*ALP),* cartilage intermediate layer protein 2 (*CLIP2)* and insulin growth factor 1 (*IGF1)*, osteonectin (*OSN)* and fibronectin 1a (FN1a) (Zapata et al., 2006); collagen 1 (*Col1A1)* (Estêvão et al., 2011); fibrillin 1 (*Fib1a)* at the first stages of bone formation, again *Col1A1, OSN* and osteopontin (*OP)* in regulating matrix production and mineralization and osteocalcin (*OCN)* in laster larval stages (Riera-Heredia et al., 2018).

1.1.3. Immune system

The ontogeny of the fish immune system helps to understand better the development and function of the immune system. Teleost fish are the main model of the present study, and the immune system has a differing level of maturation an response at different larval stages. Knowledge about the immune system can help to solve issues linked to disease in aquaculture, by establishing methods and the best timing for preventative treatments using vaccination (Zapata et al., 2006) and avoiding costly vaccination at early stages when the immune system is insufficiently developed to respond effectively (Vadstein et al., 2013). Sequencing of immune-related genes has contributed to a better understanding of immune system development, although much more knowledge is needed (Vadstein et al., 2013).

Immune protection of the egg, embryo and early larval stages is conferred by the mother during vitellogenesis and oogenesis (Mulero et al., 2007; Swain & Nayak, 2009), this fact is supported by results showing the ontogeny of lysozyme activity in gilthead sea bream eggs and larvae from different broodstocks (Li et al., 2021). Innate immunity to

which lysozyme contributes protects early developmental stages of larvae against microorganism present in the environment.

In the gilthead sea bream and European sea bass, there is delayed development of lymphocytes, and this prevents fish from mounting and effective antibody response until several weeks after hatching (Vadstein et al., 2013) this may explain the susceptibility of early developmental stages to pathogens. Skin is the first immune barrier in fish and is the first organ in contact with whatever is in the surrounding environment (Alvarez-Pellitero, 2008; Esteban & Cerezuela, 2015). Genes related to the skin such as *col1A1* have a different expression between larvae and adults (Campinho et al., 2007) and the expression of different genes isoforms between stages makes them a potential biomarker of quality as they may influence the skins capacity to protect them from pathogens.

1.2. Hatchery production

Modern hatcheries are generally under cover and provide facilities for artificial breeding and larval rearing up until the juvenile stage when fish are transferred to outdoor ponds or sea cages until harvesting (Føre et al., 2018; (Hamre, 2013). High egg quality is a priority for hatcheries since the eggs underpins all the subsequent production stages (Migaud et al., 2013) and for this reason broodstock that are known to produce good quality eggs are selected for breeding (Hamre, 2013). Larval rearing conditions (biotic and abiotic) are adapted to be like the natural conditions in the wild to ensure successful production year-round. Controlling photoperiod/light, salinity, temperature, and water quality are all parameters that fluctuate and so remain a challenge for hatcheries (Føre et al., 2018; Migaud et al., 2013). The advantages of hatcheries are several: 1- hatchery production makes it is possible to extend natural spawning outside of the normal breeding season (FAOb; (Hamre, 2013), by using hormones, photoperiod and temperature to induce spawning (FAOb), 2- they remove dependency on wild-caught juveniles and broodstock (Hamre, 2013; Migaud et al., 2013), since the selected high performing broodstock are maintained long-term (FAOa; FAOb) and are reared from eggs and are supplied to on growing farms (Hamre, 2013), 3- captive broodstock and a closed lifecycle means that traits of interest can be improved by genetic selection (survival rate, growth rate disease resistance, lower age of maturation, etc) and can contribute to improve offspring and the predictability of juvenile performance ((Hamre, 2013).

1.3. Hatchery problems, perspectives and solutions

The hatchery production of aquaculture species is of crucial importance as it supplies the juveniles for grown on and determines the productivity and quality of the commercialized fish. However, the hatchery is high cost and high risk due to losses caused by inadequate nutrition and physical/chemical/microbial conditions (Helvik, 2009), all factors that affect the survival rate and growth of eggs and larvae. Furthermore, the microbiota and inappropriate development may alter immune system development and contribute to an increased susceptibility to diseases and promote skeletal malformations, which leads to poor-quality larvae and poorly performing juveniles, this reveals the importance of the hatchery in determining the quality and performance of subsequent stages and in this way, it affects the sustainability and growth of Mediterranean fish aquaculture (Migaud et al., 2013). The following sections identify knowledge gaps and management strategies to overcome some of the identified challenges:

1.3.1. Egg quality and Survival rate

Egg quality is the first step to guarantee larvae quality and good juvenile performance. European sea bass and gilthead sea bream eggs have a survival rate of < 5-10% (Migaud et al., 2013). Parameters such as survival, hatching rate and behavior are crucial for the performance of larvae and subsequent stages, although they do not explain all performance related problems that can arise after early development. Strategies used in hatcheries to minimize the probability of larval rearing problems, include discard of eggs when parasite infections are detected in water and elimination of poor-quality eggs, identified by their failure to float on the water surface (Moretti et al., 1999).

1.3.2. Larvae quality

Larval survival depends on several external factors such as feeding, water quality (Hamre, 2013), larval density and other abiotic factors (oxygen dissolves, photoperiod, salinity, light, density) (Migaud et al., 2013). In “good” performing batches of larvae their viability is around 80%, and lower levels of viability generally lead to the discard of poorly performing batches. Factors used to assess quality include the shape of larvae, skeletal malformations, growth, rate and behavior (Moretti et al., 1999).

1.3.3. Manipulation of abiotic factors- solutions and strategies

To ensure continuous year-round availability of gametes and to improve their quality strategies include the induction of maturation and spawning of European sea bass (Prat et al., 1999)and gilthead sea bream (Kissil et al., 2001) by reducing the water temperature and by manipulation of the photoperiod (Migaud et al., 2013). Thermal shock and photoperiod manipulation improve weight gain (Pittman et al., 2013); decreasing the salinity can improve survival rate and growth (Pittman et al., 2013); improving water quality by controlling oxygen, temperature, salinity and turbidity reduce the risk of dysbiosis, which negatively affects larval development and juvenile performance (Business Food, 2018). Water quality problems tend to be resolved by UV treatment of water entering the hatchery, since it helps to sterilize the water, avoiding microbial contamination(Helvik, 2009).

1.3.4. Nutrition

Success of larval feeding depends on the development of morphological, physiological, and behavioral traits that allow effective feeding and nutrient assimilation (Rønnestad et al., 2013). Understanding of optimal larval nutrition is still limited by knowledge gaps despite progress in recent years. A major concern with live prey is that it is difficult to predict and ensure appropriate nutritional composition and availability. Optimization of nutrition is still needed to improve larval and juvenile quality (Harada et al., 1995), and it is suggested should start with the broodstock (Migaud et al., 2013). For example, some vitamins are essential for early developmental stages (Fernández et al., 2008) and safeguard skeletal development, other studies report that essential long-chain polyunsaturated fatty acids are related to egg quality and larval quality (Migaud et al., 2013) and digestible carbohydrates (20-30%) affect growth (Pittman et al., 2013).

Diets using microalgae for their antioxidant and immunomodulatory properties can work as immunostimulants in aquaculture and be used as part of a disease control strategy (Carballo et al., 2018, 2019), they can also induce larval programming with long-term effects on growth (Carballo et al., 2020). Furthermore, excess phosphorus or deficiency in diets affects survival and bone calcification in the early development of rainbow (Fernández et al., 2008).

1.3.5. Growth

Other problems in fish production are the significant gaps in knowledge regarding growth potential (Valente et al., 2013). The rearing of larvae and fish at high densities in commercial production systems is associated with a reduction in the survival rate (Valente et al., 2013) and growth potential (Business Food, 2018). Improved understanding about the biology of muscle growth, the genetic basis of flesh quality traits and the influence of environmental factors on growth and product quality can contribute to improve the sustainability and productivity of commercial aquaculture (Valente et al., 2013). It was shown in a study of trout that hybrids had a better growth performance and gastrointestinal tract development compared to the non-hybrid species, this approach can be beneficial for aquaculture of such species (Najafpour et al., 2021).

Growth hormone (*GH*), insulin-like growth factor 1 and 2 (*IGFI* and *IGFII*) are important genes for growth in several terrestrial farmed species. The use of comparative genomics is contributing to demonstrate if these genes also act as candidate growth-regulatory factors in fish, this strategic approach can quickly identify gene candidates that can be applied in aquaculture to improve species performance (Louro et al., 2016).

1.3.6. Diseases

Aquatic environments favor the survival of bacteria outside the host (Pridgeon & Klesius, 2012) and infectious diseases are a problem in hatchery-based intensive production and contribute to unpredictable mortality in early larval stages and reduces the economic viability of hatcheries (Monzón et al., 2017; Zrncic, 2020). European sea bass and gilthead sea bream are highly manipulated in commercial hatcheries as part of the standard production regime, and intensive fish farming can create stress-producing conditions that negatively impact on larval performance and can lead to increased disease susceptibility and sometimes death (FAOa; Gabriel et al., 2011) or reduce growth potential (Mateus et al., 2017). In the hatchery European sea bass larvae have an average survival rate of 85% and Sea bream of 80% and mortality related to infections is 10% for both species (Muniesa et al., 2020). A related problem is linked to authorization for treatments of diseases, particularly considering current problems of bacterial resistance caused by indiscriminate use of antibiotics (FAOa). Complimentary approaches used to decrease and attenuate disease problems include hygiene control combined with drug and probiotic therapies in early larval stages (Monzón et al., 2017). Some evidence suggests that microbe - fish interactions may be important, but this is still relatively little studied

and poorly understood (Montalban-Arques et al., 2015; Zrncic, 2020), although several recent studies have highlighted their importance in hatchery stages of gilthead sea bream and European sea bass (Najafpour et al., 2021, 2022, 2023), for example in Najafpour 2023, it was found that most of the pathogenic bacteria in larval rearing came from rotifer and artemia rather than from water of algae .

1.3.7. Skeletal malformations

A long term and persistent problem in aquaculture is the loss of larvae and fish due to skeletal malformations, these anomalies, and their relationship to rearing conditions still remain largely unknown (Kourkouta et al., 2022a). It is estimated that skeletal malformations account for losses of up to 50 000 000 € per year (Boglione et al., 2013b) for European aquaculture and that the severity of the problem is related to the species and can vary between 30 to 100% (Boglione, 2001). A high incidence of skeletal malformations is a major challenge and a major cause of commercial loss for commercial larval rearing and juvenile performance of gilthead sea bream and European sea bass (Boglione et al., 2013a). Skeletal malformations cause abnormal larvae swimming and body shape (Moretti et al., 1999) and affect feeding, which reduces growth rates and increases the susceptibility to stress and infectious diseases (Boglione et al., 2013a). Furthermore, fish with severe malformations cannot be commercialized as consumers will not purchase them and (Boglione et al., 2013b), they either have to be remove manually, which is costly (Kourkouta et al., 2022a) or are diverted to side streams such as fillets or fish meal that have less added value (Le Vay et al., 2007a; Lijalad & Powell, 2009)**.** Causative factors of bone malformations during larval rearing, include nutrition (Fernández et al., 2008; Georga et al., 2011; Izquierdo et al., 2013; Printzi et al., 2021; Rodgers et al., 2003), temperature (Balon, 1983; Boglione et al., 2013; Campinho et al., 2004; Polo et al., 1991; Riera-Heredia et al., 2018; Sfakianakis et al., 2004) and a range of other abiotic factors (Cobcroft & Battaglene, 2009; Sawada et al., 2018), as well as rearing protocols (Izquierdo et al., 2010; Koumoundouros et al., 2001) and genetic factors (Fragkoulis et al., 2018; Negrín-Báez et al., 2015).

In a study of Sea bream larvae coming from different Mediterranean hatcheries, 10 abiotic variables and 14 nutritional factors were considered as important KPIs for most of the skeletal malformations identified except for the most frequent, gill-cover abnormalities (Kourkouta et al., 2022a), that increases the susceptibility to disease, decreases growth rate and increases the incidence of mortality (Boglione et al., 2013b).

Improvements in rearing conditions of European sea bass and Sea bream including dietary manipulations (Izquierdo et al., 2010; Lewis-McCrea & Lall, 2010) and manipulation of abiotic factors can (Divanach et al., 1997; Georgakopoulou, Sfakianakis, et al., 2007; Polo et al., 1991; Sfakianakis et al., 2006) decrease the prevalence of malformations. However, abnormalities were not totally eliminated indicating other factors also play a role and knowledge about the contribution of genetic and epigenetic factors to skeletal abnormalities is needed (Boglione et al., 2013a). One problem that has limited the progress in relation to defining causative factors of skeletal malformations is the non-standardized description of abnormalities, that limits comparability between studies (Boglione et al., 2013b).

1.4. Genetics and Omics technologies: Context in Biotechnology

Development of methods to screen larvae to detect low performance related to growth potential as soon as possible can contribute to avoid production losses. Currently, hatcheries compare egg quality based on egg floatability, with good eggs floating and poor eggs sinking (Migaud et al., 2013). Recent research has been directed at identifying tools to identify quality markers using selective breeding (Migaud et al., 2013), genetic markers identified when traits of wild and farmed fish are compared (Liu, 2007) identification of genetic markers using Quantitative trait Locus (QTLs), this allows a correlation between a phenotype trait with a location in the genome (Louro et al., 2016). Genomics has been used to identify molecular markers based on their expression profile in animals and can identify candidate genes linked to specific traits (Migaud et al., 2013). The use of genetic and molecular biology to identify gene candidates related to larvae quality and to predict juvenile performance may be one way to overcome the bottlenecks mentioned before.

Candidate gene approaches can be used to establish the link between causative factors of low performance such as nutrition, temperature, water quality, stress, diseases, malformations, and gene expression. Sequencing data may identify genes that can be used for marker assisted selection directed at hatchery traits such as larval survival, disease resistance, and growth performance (Valente et al., 2013). The current technological advances in relation to molecular analysis means that the cost is lower and the speed, and robustness of analysis is higher and is now more accessible to hatcheries (Valente et al., 2013). Genomics and proteomics methodologies are contributing to the development of

successful breeding programs in the Sea bream industry (Power et al., 2010) and genetic selection programs have been used to screen heritability and quantitative loci determining growth, disease resistance and skeletal malformations in Sea bream (Ferosekhan et al., 2022; Riera-Heredia et al., 2019a). QTLs can work as an early step to identify a gene that causes trait variations of interest and is useful for identification of candidate genes that can be used to select for traits linked to fish quality fish and save costs (Louro et al., 2016).

1.4.1. qPCR molecular tool for larvae quality screen

Quantitative PCR (qPCR) is a modern technology for studying gene expression. Detection of PCR products in real-time PCR uses oligonucleotides labelled with fluorescence, which allows detection when specific primers hybridize to a template of interest and polymerization of the nucleotides occurs to generate a complementary sequence. The advantage of real time qPCR is its high sensitivity and large dynamic range and accuracy and for these reasons it has been used for many applications (Pabinger et al., 2009). For example, quality descriptors and predictors for fish larvae related to gamete quality (Guerra et al., 2013) and bone metabolism (Koumoundouros et al., 2017), were identified by using larvae RNA transcripts to quantify specific gene expression with qPCR.

1.5. Fish species and biology, aquaculture history and production

European sea bass and gilthead sea bream are distributed along the Northeast Atlantic coasts, in the Mediterranean Sea and in the Black Sea (Çoban et al., 2020). Both species are carnivorous, and the adults eat primarily Mollusca and crustacea (FAOa; FAOb) (Çoban et al., 2020). These species are eurythermal and euryhaline (FAOa; FAOb) and are found in the open sea, in brackish coastal lagoons and European sea bass are also occasionally found in freshwater rivers (FAOa; FAOb).

European sea bass reproduces once a year from December to March in the Mediterranean Sea and the season can extend into June in the Atlantic Sea. European sea bass eggs are small and pelagic and the adults spawn in water with low salinities near to river mouths, estuaries or in littoral areas. gilthead sea bream is a protandrous hermaphrodite species, that matures first as a male and then inverts sex in its second or third year of maturation (Bauchot, 1981). In aquaculture sex inversion can be controlled using hormones and by social cues. Male Sea bream generally become sexually mature

when they are 2 years old (20-30 cm) while females mature at 2-3 years (33-40 cm), breeding occurs in the open sea (30-50m of deep) at the end of Autumn. Mature female gilthead sea bream spawn between 20,000 to 80,000 eggs per day across four months from October and during the spring the juveniles migrate to warmer coastal waters, as they are very sensitive to low temperatures (lower than 4ºC can be lethal) (FAOb).

The gilthead sea bream and European sea bass are the most economically important species in Mediterranean aquaculture (Polovina et al., 2020) and represent 95% of the fish farmed in the Mediterranean (Lembo et al., 2007). Aquaculture production is advancing very quickly compared to other large food sectors. From 2000 to 2016, global fisheries almost doubled (Çoban et al., 2020; FAOc). The gilthead sea bream and European sea bass are the most consumed aquaculture fish across Europe and internationally (Migaud et al., 2013). The European sea bass was the first non-salmonid species that was produced in aquaculture by France and Italy at the end of the 1970s. Nowadays, Greece, Turkey, Italy, Croatia and Egypt are the biggest European sea bass producers. The principal consumers are from Italy and Spain, and Greece is the main exporter of this species and approximately 70% of their production is exported. Production costs are uneven across the different production stages with on growing accounting for 15-25% of the cost and the hatchery 30% of costs with feeds representing one of the major expenses and contributing approximately 30% to the cost. For example, juvenile production costs 0.30 €/kg and for the marketable fish the cost is about 4.00 €/kg, in Italy (FAOa).

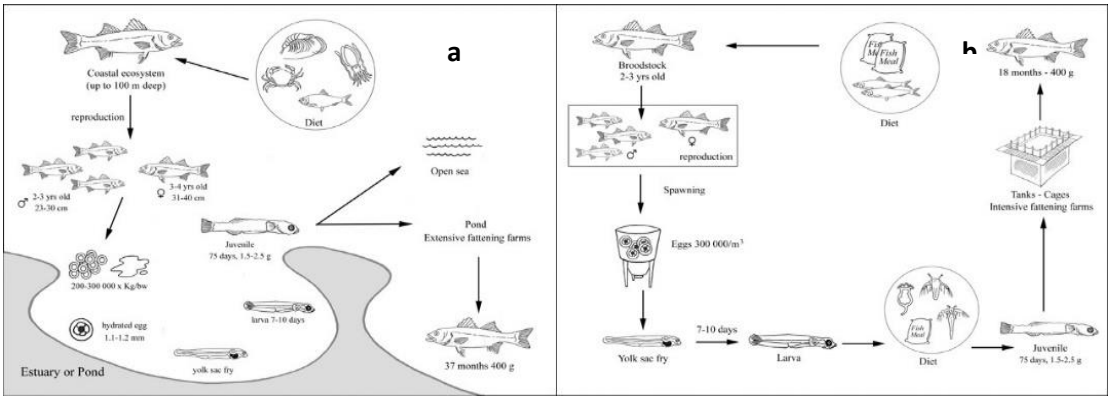

Figure 1.1| *a) Production cycle of European sea bass extensive system b) – production cycle of European sea bass - intensive system (FAOa, 2023).*

The gilthead sea bream was first cultured under extensive conditions in coastal lagoons and saltwater ponds, then in the 1980s intensive rearing systems were developed, along with artificial breeding and large-scale production started in Italy, Spain and Greece. This species adapts very well to both ponds and cages and the high survival rates

of gilthead sea bream meant that the costs of production fell by 60% between 1990 and 2000 and is still decreasing (FAOb). European sea bass and gilthead sea bream are usually

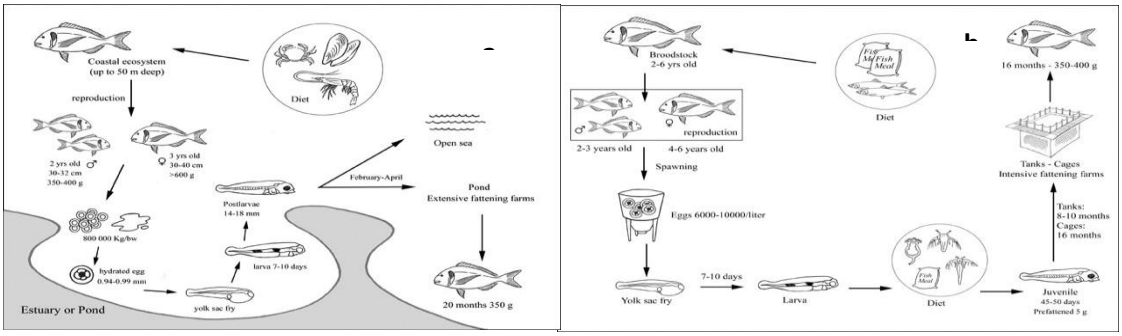

Figure 1.2| *a) Production cycle of gilthead sea bream - extensive system b) Production cycle of gilthead sea bream - intensive system (FAOb, 2023).*

produced in the same infrastructure and on the same farms due to their similar biology (Polovina et al., 2020). In both species, larval rearing up until the juvenile stage may occur in two different production systems (figure 1.1;1.2), before transfer to ponds or sea cages for on growing to the commercial size.

1.5 Objectives

My thesis work was developed in an industry-driven European project called PerformFish, in work package two: "juvenile quality and growth potential". The species studied were the European sea bass and gilthead sea bream and the main aim was to identify factors affecting KPIs in hatcheries. The specific objectives of my work were to contribute to the development of tools to improve and predict larval quality during the hatchery phase by using existing technical and biological knowledge to identify, optimize, and validate robust biomarkers of larval quality, by searching for gene candidates as indicators of quality and by quantifying their expression in batches of larvae classified as the best and poorest from commercial hatcheries. Finally, by comparing the expression of the identified molecular markers between four early developmental stages the discriminatory value of the selected markers for the poorest and best production baches of European sea bass and gilthead sea bream was evaluated to see how soon during larval production differences were detected.

CHAPTER II

Comparative ontogeny of European sea bass (*Dicentrarchus labrax*) and Gilthead sea bream (*Sparus aurata*) using putative biomarkers of larval quality

Soraia Santos*, Andreas Tsipourlianos**, Katerina Moutou**, and DM Power*

*Centre of Marine Science (CCMAR), University of Algarve, Campus of Gambelas, Faro, 8005-139, Portugal

University of Thessaly (UTH),** Biopolis, 41500 Larissa, Greece

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Keywords: aquaculture; commercial hatcheries; development; fish quality; quality indicators; qPCR.

1. Introduction

The European sea bass (*Dicentrarchus labrax*) and gilthead Sea bream (*Sparus aurata*) are two important aquaculture species in the Mediterranean Sea (Polovina et al., 2020, Lembo et al., 2007). The hatchery production of these two aquaculture species is of crucial importance as it supplies the juveniles and determines the productivity and quality of the commercialized fish. However, the hatchery stage is high-cost due to the requirement of the production regime exacerbated by significant production losses that can be caused by inadequate nutrition and changes in the physical/chemical and microbiological conditions in the tank environment (Helvik, 2009), which affects larval survival and growth. Furthermore, changes in water microbiota induced by opportunistic pathogens and inappropriate development of the larvae leads to poor quality larvae and poorly performing juveniles as it can compromise immune system development and function leading to an increased susceptibility to diseases and skeletal malformations all of which affect the sustainability and growth of Mediterranean fish aquaculture (Migaud et al., 2013).

Events occurring early in development have the potential to impact the developmental trajectory and performance of later stages (Herbst et al., 2011). The quality of juvenile fish produced by hatcheries determines the performance of the subsequent grow-on stages and makes this a crucial production stage that impacts several key performance indicators (KPI) such as mortality, survival, deformities, and growth amongst others (performfish.eu). For this reason, the development of fast, simple, and low-cost quality screening methods is a priority for hatcheries so they can determine and predict the quality of larvae and ideally link this to the performance of later stages. Moreover, the earlier during the production cycle that reliable quality indicators can be applied should bring considerable financial savings as poorly performing larval batches can be stopped. Currently since external morphological anomalies can be detected this approach can be used, to manually select and remove malformed larvae from production batches or if there is a high proportion of malformed larvae in a production batch the culture can be stopped. However, if malformations only become evident in juveniles, then this leads to a significant reduction in their sale value despite a similar overhead for their production compared to higher quality juveniles (Koumoundouros et al., 2017). Some quality descriptors and predictors related to morphological traits, bone metabolism, nutritional condition, growth potential and immune status are already used by commercial hatcheries (Koumoundouros, et al. 2017). Here we suggest that combining morphological

characteristics with expression of selected genes, can be a powerful approach for classifying larval quality. Understanding fish ontogeny related to growth potential, bone development, immune and nutritional status can help to identify molecular (genes) quality predictors for fish larvae.

The existing literature clearly suggests that development of indices for the prediction of fish quality of normal ontogeny, growth potential, immune and nutritional condition is possible, by exploiting the rapidly growing knowledge on the developmental mechanisms of early life stages (Koumoundouros, et al. 2017). Sequencing data may identify genes that can be used for marker assisted selection directed at relevant hatchery traits such as larvae survival, disease resistance, and growth performance (Valente et al., 2013). The current technological advances in relation to molecular analysis means that the cost is falling and the speed, and robustness of analysis is higher and is becoming more accessible to hatcheries (Valente et al., 2013). Methodologies in genomics and proteomics are contributing for the success of breeding programs in the gilthead sea bream industry (D. M. Power et al., 2010). Genetic selection programs have been used to screen heritability and quantitative loci determining growth, disease resistance and skeletal malformations in gilthead sea bream (Ferosekhan et al., 2022; Riera-Heredia et al., 2019). PerformFISH is an industry driven European project, with as one of its key aims, to ameliorate poor performance during the first production phases of Mediterranean Marine Fish Farming (MMFF). In workpackage 2, of the project the objective was to identify factors affecting key performance indicators (KPIs) in the hatchery through industry-wide monitoring and meta-analysis, developing tools to improve and predict larval quality during the hatchery stage. By using existing technical and biological knowledge the aim was to identify, optimize, and validate robust biomarkers of larval quality by using them to assess the best and the poorest batches of larvae from different hatcheries. Different larval developmental stages were analyzed to establish how early it might be possible to detect and predict fish performance and, in this way, minimize losses from hatcheries.

Gilthead sea bream and European sea bass larvae from the best and poorest production batches of several commercial hatcheries in the Mediterranean were analysed. The main objectives of the study was to, a) establish a standardized sampling strategy and technical sampling manual, b) to develop a standardized screening approach for selection of potential molecular biomarkers of interest, c) to assess the quality of samples, d) optimize molecular techniques (RNA extraction, cDNA synthesis, primers and qPCR); and d) to

quantify the candidate genes in different ontogenetic stages of larvae and compare gene expression in the best and poorest batches from the different Mediterranean hatcheries.

2. Materials and methods

2.1. Biological material

Gilthead sea bream and European sea bass larvae from different development stages: first feeding (FF), flexion (FL), end of larvae rearing (ELR) and mid metamorphosis (MM) (figure 2.1) fixed in Methanol or RNA later were receive from 4 Mediterranean hatcheries, two located in Greece, one in Italy and one in France. Hatchery names are not mentioned to avoid divulging sensitive commercial information, and they were given the following codes, A, B, C, D, to permit association of hatcheries and analysis outcomes during the study. Across two production years 74 batches of gilthead sea bream and 36 batches of European sea bass were analyzed that were obtained from different times during the year and for differing quality batches.

Table 2.1| *Larvae samples from different development stages received from all hatcheries, in methanol or in RNAlater.*

	GILTHEAD SEA BREAM						EUROPEAN SEA BASS					
	Methanol			RNALater			Methanol			RNALater		
HATCHERY	Tot samples	Tot Batches	Complete Batches (with 4 stages)	Tot samples	Tot Batches	Complete Batches (with 4 stages)	Tot samples	Tot Batches	Complete Batches (with 4 stages)	Tot samples	Tot Batches	Complete Batches (with 4 stages)
A	90	8	7	90	8	7	69	8	5	69	8	5
B	96	20	19	93	20	19	-	-	-	-	-	-
C	115	28	21	96	28	20	23	6	4	22	6	4
D	0	0	0	86	18	14	0	0	0	101	20	19

2.2. Sample handling and quality control

A sampling strategy and methods were developed in collaboration with industry to ensure that critical knowledge held by hatcheries was taken into consideration during the design of the sampling procedure. Specimen stage, sampling approach, sample number and preservation method were considered and validated in preliminary trials, in which trial samples were collected and sent to CCMAR and duration shipping assessed, status of package recorded and then the quality of extracted RNA determined. In relation to the hatchery sampling procedures criteria considered during the design of the standard operating procedures for sampling was a) ease of classifying stage, b) adequacy of sample

size and representation of larvae, c) analytical procedures to be performed, d) the efficiency of the preservation methods, both short- and long-term e) health and safety issues related to the methods used, f) the limitations and logistics of transport and likely detrimental effects of the duration of transport. The work carried out during my thesis contributed to the preparation of the sampling manual and was approved by all the consortium members, including the companies is indicated in the following link http://performfish.eu/wp-content/uploads/2021/02/D2_2_approved.pdf.

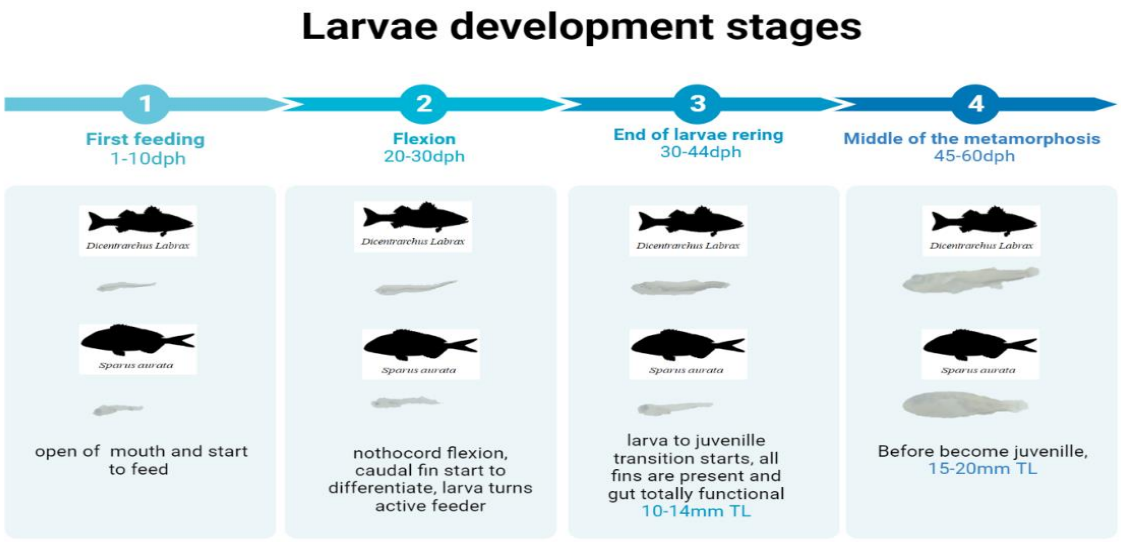

Figure 2.1| *The salient characteristics of the larval development stages of the European sea bass and gilthead sea bream that were sampled and analyzedanalyzed are indicated: 1-First feeding; 2- Flexion; 3-End of larval rearing; 4-Middle of metamorphosis [Power, D et al, 2020]***.**

When the samples from hatcheries arrived at CCMAR they were registered with an arrival number, which was unique for each batch of samples. After verification of the number and stages of packed samples from hatcheries they were transferred to a freezer and kept at -20ºC until quality control procedures were carried out (figure 2).

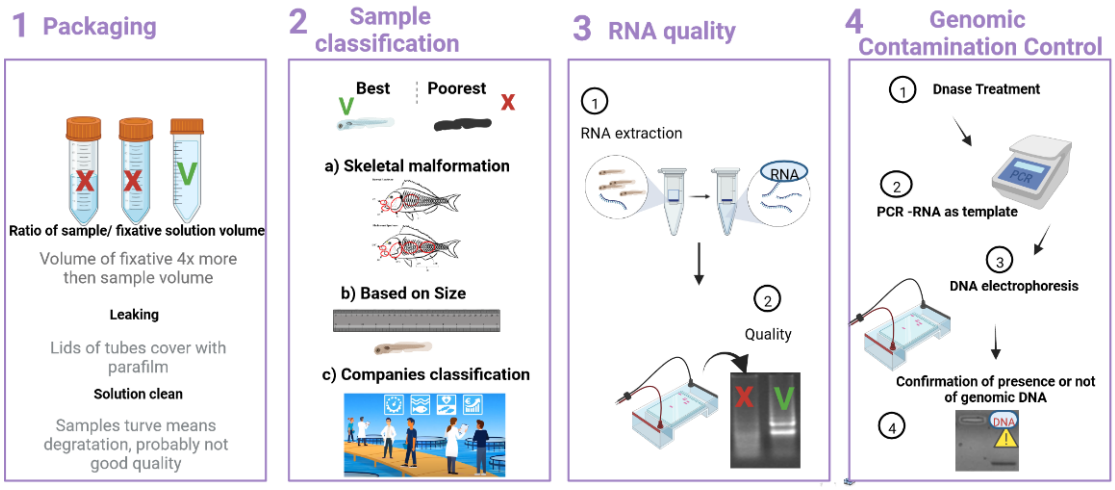

Figure 2.2| ***The main quality control (QC) steps implemented*** *for larvae samples received from the different hatcheries before molecular analysis.*

At the quality control stage, each group of samples from the same batch at each development stage was in individual tubes, a set of criteria was implemented to validate the quality of samples during transport and long-term storage. For example, it was verified that the volume of the solution used for sample preservative had to be at least four times the volume of the sample to ensure that samples and all nucleic acids and proteins were saturated by the fixative solution (figure.2.2). The first control step did not lead to sample discard unless there was clear indication that the samples were damaged or could not be identified due to inadequate labelling (e.g. inadequate volume of reagents). A small selection of samples were selected and total RNA extracted and the quality of the extracted RNA was assessed by PCR. Several individuals were extracted per batch to ensure a representative sample was obtained and if multiple extractions for a given batch yielded poor quality RNA when analysed by spectroscopy and by agarose gel electrophoresis, the samples were discarded.

2.3. Sample classification

The received samples were classified as being from Best and Poor batches according to the three parameters listed below.

a) Skeletal malformations

Sea bream fish larval samples at 40-50dph were stained to shown bone and cartilage following the protocol described by (Walker, M. & Kimmel, C., 2007) for posterior verification of the presence, characteristic and frequency within each production batch from hatcheries of individuals with skeletal abnormalities. The abnormality

incidence was registered by batch and they were classified as having a very high or low incidence of skeletal malformation and on this basis were classified as poor or best batches, respectively (Kourkouta, C et all., 2022). The incidence of skeletal malformations for European sea bass and the classification as good or bad followed the same general procedure as outlined for the gilthead sea bream.

b) Body size

Twenty mid metamorphosis (45-60dph) larvae from each batch were weighed and their length measured, larvae batches were classified as best or poorest batches when the average of the 20 individuals analyzed deviated from the standard size determined by considering all batches measured. The batches with larvae bigger than the determined standard size were classified as good and the batches with a size substantially smaller than the standard size were classified as poor. An average length above 1.7 cm for the gilthead sea bream and 1.5 cm for the European sea bass were considered to be good batches. Batches with values below these thresholds were classified as poor batches. In addition, high size variability of larvae within the same batch were also considered to be a poor batch.

c) Quality assessment by the company/hatchery

Each company/hatchery gave their own batch classification of best and poorest based on their own internal quality assessment parameters before sending the samples to CCMAR for analyses. The basis of the quality estimates of the hatcheries involved in the project, with the exception of one hatchery, was not provided as it was considered to be confidential company information. In the case of the hatchery that provided the basis of their quality classification they indicated that it was based on the incidence of skeletal malformations of larvae at 60dph.

2.4. Total RNA extraction and RNA quality assessment

Larvae samples (n = 6/batch/stage/hatchery) were disrupted with TRK Lysis Buffer from E.Z.N.A® Total RNA Kit I (R6834, Omega) containing β-mercaptoethanol (A1108 PanReac AppliChem) using two iron beads. Tissue homogenization was done using a Mixer Mill MM 400 (Retsch, Germany) at a frequency of 30 Hz, and using three cycles of 30 sec each. Subsequently total RNA was extracted with an E.Z.N.A® Total RNA Kit I (R6834, Omega). For determination of the concentration and quality of the RNA extracted it was quantified by spectrophotometry (NanoDrop 1000 spectrophotometer, Thermo Fisher Scientific, USA) and by agarose gel electrophoresis

on 1% TBE 1X gels. For the preliminary analysis of the samples received from hatcheries the poorest and best batches were selected and total RNA was extracted from two samples of each stage per batch, quantified using a nanodrop 1000 and the integrity was evaluated by electrophoresis of samples on a 1% agarose gel. RNA quality was assessed visually considering the relative intensity of the 28S and 18S ribosomal bands RNA (rRNA) (figure 2.3), a higher intensity of the 28S rRNA band and the absence of appreciable amounts of other rRNA molecules was taken as an indicator that samples were of high quality. Batches in which most samples from all stages gave good results for the preliminary evaluation of RNA quality were identified and subsequently the remaining samples were extracted.

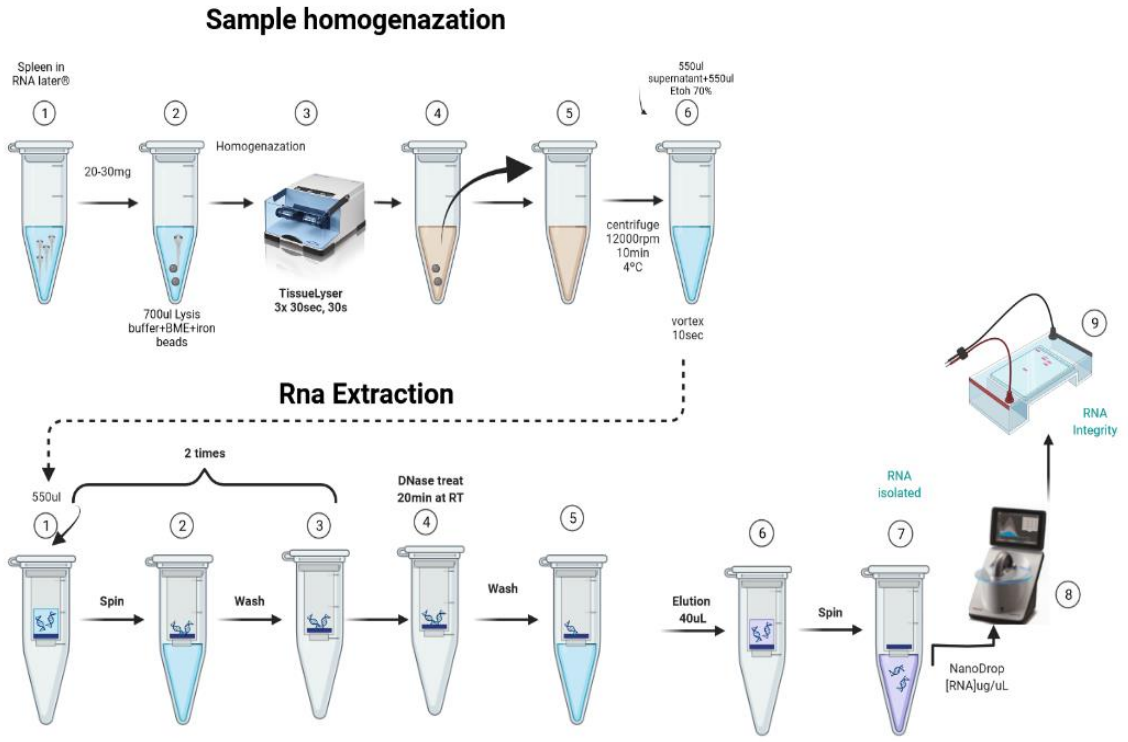

Figure 2.3| *Schematic outline of the workflow for total RNA extraction. An appropriate number of larvae (determined based on the larval size and preliminary analysis) were placed in the lysis buffer and the extraction protocol implemented. The scheme was prepared using BioRender.*

2.5. Elimination of genomic DNA contamination

Contaminating genomic DNA was removed from total RNA extracts using an E.Z.N.A RNase-Free DNase Set I kit (Omega, USA) and following the manufacturer's procedure. In brief, DNAse treatment was performed on the columns used to purify the RNA during the extraction protocol by incubating the column with 1.5ul (20 Kunitz/µL)

of DNase for 15 min at room temperature. To confirm that potential genomic DNA contamination was removed from the extracted total RNA, a PCR using a reference gene 18S rRNA was performed. Since the PCR primers are designed for use with cDNA they should not amplify 18S rRNA and an amplification product should only be obtained with contaminating genomic DNA. The thermocycling conditions used for the PCR were: 2 minutes at 95ºC, followed by 20 cycles of 20s at 95ºC for denaturation, 20 s at 60◦C for primer annealing, and 30 s at 72ºC of extension, followed by a final 5 minutes at 72 ºC for extension.

The presence of a PCR product in the final reaction, determined by agarose gel electrophoresis (as outlined above) was indicative of genomic contamination and the absence of a product indicated that the extracted RNA did not contain contaminating genomic DNA. For samples that were contaminated with genomic DNA, an extra DNase treatment was performed using a Precision DNase kit (Primerdesign Ltd, UK) following the manufacturers protocol. This involved the addition of 0.5 ul* of Precision DNase enzyme plus 1/10 of 10X Precision DNase reaction buffer was added to the RNA samples, and then incubated for 25 minutes at 30 ºC and 5 minutes at 55ºC for DNase enzyme inactivation. The PCR procedure outlined above was repeated to confirm the additional DNase treatment was effective.

2.6. cDNA synthesis

cDNA synthesis was performed with 500 ng of DNase treated tRNA in a 40 ul reaction volume with 200 ng of random hexamers (Jena Biosciences, Germany), 10 U of RiboLock RNase Inhibitor (Nzytech, Portugal) and 0.5 mM dNTPs (Nzytech, Portugal) and 200 U of RevertAid reverse transcriptase (ThermoFisher, USA). The reaction mix was incubated for 10 min at 20 ◦C followed by 50 min at 42 ◦C and the enzyme was inactivated by heating for 5 min at 72 ◦C (figure 3). To confirm the success of the cDNA synthesis, a PCR reaction to amplify the reference gene 18S rRNA was performed, using 1 µl cDNA diluted 1/10 in a reaction mix containing 10mM dNTPs, 10mM of each primer, and 0.2 U of DreamTaq (Thermo Scientific, USA). The PCR thermocycling conditions were 2 minutes at 95ºC, and then 17 cycles of, 20s at 95ºC for denaturation, 20 s at 60◦C of primer annealing and 30 s at 72ºC for extension followed by a final extension cycle at 72 ºC for 5 minutes (figure 2.4).

*Enzyme concentration not supplied; volume of enzyme adapted to our samples after some optimization.

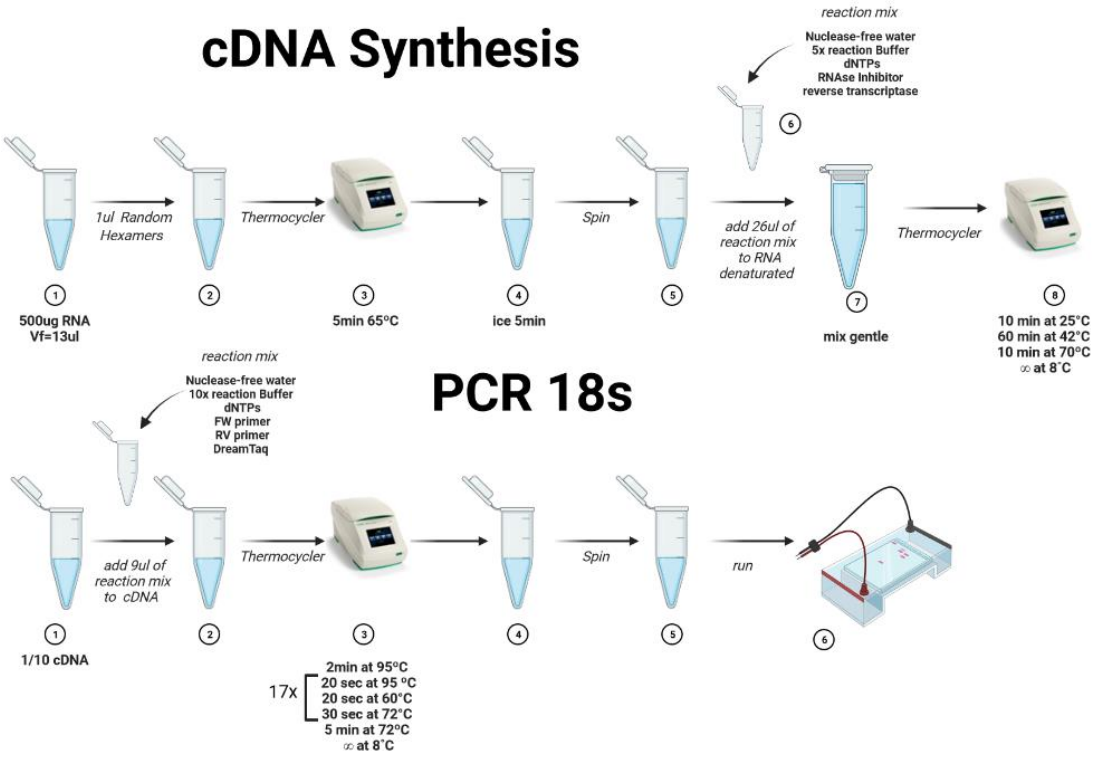

Figure 2.4 | *A schematic showing the main steps for cDNA synthesis (upper figure) and the main steps of the PCR protocol used for all gene amplifications although the figure illustrates the 18S rRNA thermocycle conditions.*

2.7. Candidate gene selection

Step 1: Candidates genes were selected that might have an association with to larval quality and included genes related to bone/skeletal formation, metabolism, growth, immune system, endocrine, and development. The genes selected were identified using, a) exhaustive searches of published articles containing molecular analysis of gilthead Sea bream and the European sea bass, and b) the presence of primer pairs available "in-house" from previous studies related to the immune system, bone development, endocrine regulation, and growth.

Step 2: All primers available in house and identified in articles were initially optimized using reverse transcriptase (RT) PCR and cDNA from a pool of larvae representing equal concentrations of RNA from each developmental stage from several samples and for both species. The aannealing temperature used for each primer pair was the temperature and conditions reported in publications or for primers "in house" the optimized conditions and 35 thermocycles were applied for all PCR reactions. If necessary, some optimization was carried out by assessing several different annealing

temperatures for some primer pairs. Primers that gave a single intense reaction product were selected for the next analytical step in the pipeline. Step 3: Primers selected in step 2 were used for qquantitative Real-Time (qPCR), with several pools of cDNA representing different larval batches and those primer pairs that had a Ct below 29 cycles and gave single reaction products (determined by running a melt curve) and had a high efficiency (95 – 105%) were selected for further analysis. A cut-off of 29 cycles was applied so that if there was variability between good and poor batches of larvae that lead to an increase in the Ct it would still be within the reliable range for quantification by qPCR.

Step 4: Primers that passed the criteria for selection in step 3 were used in qPCR with several cDNA pools produced from best and poorest batches (classification based on size) of larvae at ELR and MM stages. The criteria for selection of genes and primer pairs were the presence of a clear difference in the Ct (> 2 cycles) between the best and poorest batches. Based on the criteria of a differential expression (DE) pattern between good and bad batches of larvae 10 genes were retained for large scale screening. The 10 genes putatively best linked to larval quality were tested on the cDNA prepared from individual samples from a diversity of good and poor quality of production batches ***(supplementary table 4 and 5).***

2.8. Quantitative expression (q-PCR)

In the context of the MSc thesis only some examples of candidate genes that were tested as potential biomarkers of larvae quality are presented. The aim was to present the overall strategy used to screen for and identify promising candidate markers and to create a resource for other scientists working on gilthead Sea bream and the European sea bass For this reason an exhaustive comparison of all batches received from producers is not provided and instead the approach used is exemplified by the two best batches versus the two poorest batches of gilthead sea bream and European sea bass at all developmental stages analyzed (4 stages) from two different hatcheries.

cDNA from n = 4-6 larvae/development stage of best and poorest batches (skeletal malformation classification) were used to test the expression of the candidate genes selected using qPCR in a CFX384 Touch Real-Time PCR Detection System (Bio-Rad, USA) at CCMAR. All qPCR reactions were performed in duplicate with a final reaction volume of 6 µL and contained 2.5 ng of cDNA, 200 nM of the specific primers and the

master mix reagent used was Forget-Me-Not™ EvaGreen® qPCR Master Mix (Biotium, US) or Sso Fast EvaGreen supermix (Bio-Rad Laboratories, USA).

The reference genes were analysed by the University of Thessaly, Greece. The reference genes used on all samples included RPL13 (ribosomal protein L13a) and RPS18 (40S ribosomal protein S18) in the gilthead sea bream and FAU (40S ribosomal protein S30) and also RPL13 for the European sea bass. Primer sequence, amplicon size, amplicon melting temperature, qPCR efficiency, and accession number are listed for all the genes amplified using the selected and optimized primers are indicated in *supplementary table 2*. The qPCR conditions such as master mix reagent used, qPCR detection system, thermocycling condition and melting curve obtained are indicated in *supplementary table 3*. All reactions gave a single amplification product as revealed by a single peak in the melting curve and primer specificity was confirmed by sequencing the PCR products. The controls used for each qPCR assay included a no-template control, a cDNA synthesis control (RNA without reverse transcriptase) and an internal control that was run in all qPCR assay plates (a pool of all cDNA sample for each species).

mRNA expression levels were calculated as fold change by normalizing each target gene by dividing the detected copy number by the reference gene using in the gilthead sea bream RPS18, which had a low variability between all samples and RPL13 that had low variability in the European sea bass. The efficiency of the primers and the quality of the qPCR reactions were confirmed using a standard curve prepared from serial dilutions (1:10) of the purified amplicon obtained for each of the candidate genes analyzed. All amplicons were sequenced to confirm the specificity of the qPCR amplification.

2.9. Statistical analysis

Statistical analysis of the results and graphical outputs were performed using the program Sigmaplot (12.3 software version). Data in the study is presented as the mean ± standard error of the mean (SEM). To compare the relationship between age and quality (best and poorest) and compare best and poorest batches within each age group a two-way analysis of variance (two-way ANOVA) was applied, followed by Tukey test. The cut-off for statistical significance was set at $p < 0.05$ and higher significance was taken at $p < 0.001$.

2.10. Meta-analysis

From all the larval cDNA samples prepared, 50% of the volume was sent to our partners in Greece for qPCR analysis with other selection of genes more related with muscle, growth and protein metabolism. This was to permit a much greater number of candidate genes to be analyzed following the same screening approach as outlined above. The intention was to generate a large dataset and then train machine learning algorithms to predict larval quality based on a minimal gene number (not part of the approach for this smaller dataset). The outcome of the collaborative work (part reported in this thesis and another part conducted in Greece) will present the quality assessment tool developed and is only briefly mentioned here (figure 2.5).

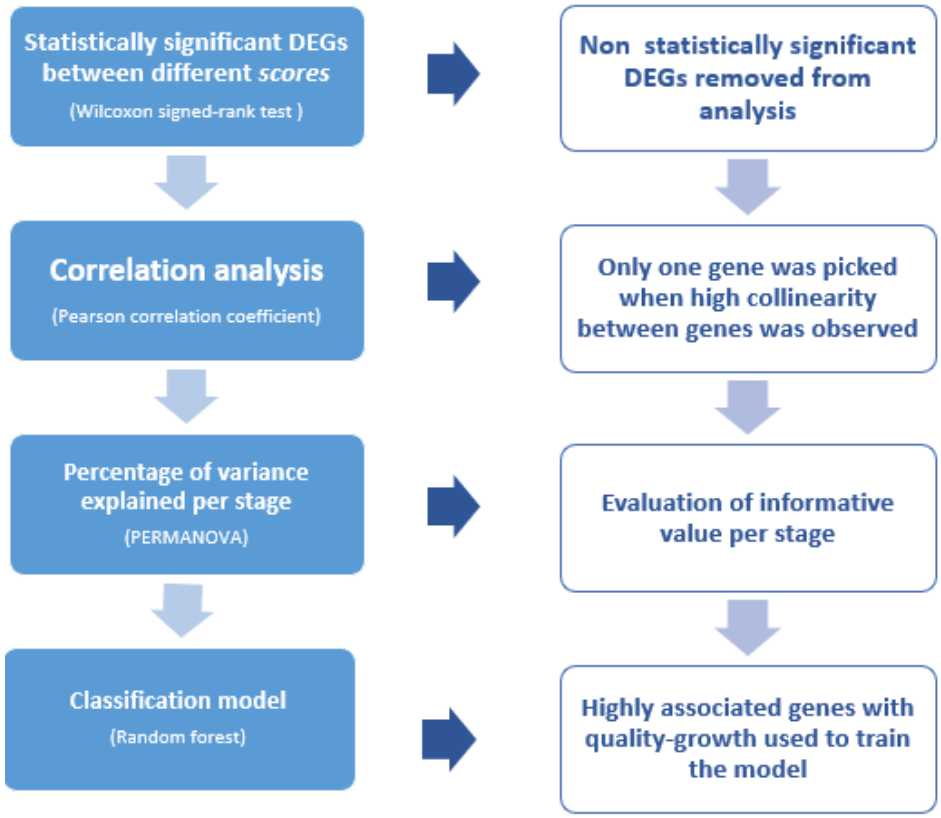

Figure 2.5| Approach of a set statistical analysis for classification of larvae quality model by **Tsipourlianos, A** (supervision, Prof K Moutou, University of Thessaly, Greece)**.**

3.Results

3.1. Sampling Manual "Performfish worpackage 2".

Based on the work performed to establish optimal and standardised protocols for sampling, molecular biology and related procedures an "e-book for quality indicators and a photo-atlas" was prepared and is available on the PerformFISH website

(http://performfish.eu/). The main contribution made to the e-book are reported in this thesis and included the optimization of protocols and procedures for sample collection, preservation, sample shipping procedures including the labelling of sample tubes and the qPCRs analysis procedures. This online manual includes all the methodology used to identify the potential genes candidates for juvenille quality which are discribed stepwise in simple validated protocols at a level suitable for hatchery technicians and other people working in aquaculture.

The sampling guide, includes the number of larvae samples per developmental stage analysed, anaesthetization, preservation and transport and was validated by the hatcheries who participated in the sampling and sent the samples that were used for this work. The guide and protocols are available for public consultation with the view of making the methods accesible for project consortium members project but also for other interested stakeholders that want to follow up the work or establish standard practice.

Figure 2.6| *a) E-book: quality indicators and photoatlas, available on the performfish.eu website, submitted in 2022, November. b) optimized KPI quality markers, available in website http://performfish.eu/wp-content/uploads/2021/02/D2_2_approved.pdf.*

3.2. Larvae batch classification

The good and poor samples received from each hatchery were classified by the hatcheries using their own classification system although the batch classification and the respective criterion were not provided except for one hatchery. For this reason, the weight and length of 20 larvae at mid-metamorphosis in each batch of gilthead sea bream and European sea bass received were measured so batches could be classified as best and poor

(Table 2). The batches classified with this approach were used to develop and optimize the PCR primers.

The batch classification was also defined using the incidence of skeletal malformations and batches classified with this approach compared to the quality defined based on size (Table 2.1; 2.2). If batches had coincident classifications, best and poor, using size and incidence of skeletal malformation they were analyzed using qPCR with the selected genes (see section 3.5 of the results). The batches with a coincident match between the two classification methods are indicated in bold in table 2.1 and 2.2. From the 74 gilthead sea bream batches received, 44 batches were classified and for the European sea bass 23 of the 36 batches received were classified as best and poor based on both weight and malformation classifications. The batches received that were not given a "best" or "poor" classification because they were of intermediate quality were not analyzed. Taking into consideration time and money constraints only the top 2 best and 2 poorest batches/hatchery/species based on the skeletal quality scoring were used.

Table 2.2 | *The gilthead sea bream batches that were classified based on size (SIZE) and incidence of skeletal malformations (SK.MF) by the participating hatcheries (A, B, C and D).*

Gilthead sea bream												
Hatchery A			**Hatchery B**			**Hatchery C**				**Hatchery D**		
Batch	**SIZE**	**SK.MF**	**Batch**	**SIZE**	**SK.MF**	**Batch**	**SIZE**	**SK.MF**	**hatchery**	**Batch**	**SIZE**	**SK.MF**
A1	POOR	-	B1	POOR	-	C1	POOR	-	-	D1	BEST	-
A2	**POOR**	**POOR**	B2	POOR	-	C2	POOR	-	POOR	D2	BEST	-
A3	POOR	BEST	B3	BEST	-	C3	POOR	-	-	D3	POOR	-
A4	**BEST**	**BEST**	B4	**BEST**	**BEST**	C4	CTRL	BEST	-	D4	**BEST**	**BEST**
A5	BEST	-	B5	-	BEST	C5	BEST	-	-	D5	**POOR**	**POOR**
A6	BEST	-	B6	-	POOR	C6	BEST	-	-	D6	NO SAMPLE	BEST
A7	-	POOR	B7	-	POOR	C7	BEST	-	-	D7	**POOR**	**POOR**
A8	-		B8	-	-	C8	BEST	-	POORES T	D8	BEST	-
			B9	-	-	C9	-	BEST	-	D9	BEST	-
			B10	-	-	C10	-	**POOR**	**POOR**	D10	BEST	-
						C11	-	-	BEST	D11	BEST	-
						C12	-	-	BEST	D12	BEST	-
						C13	-	-	BEST			
						C14	-	POOR	-			

Table 2.3 | The European sea bass batches that were classified based on size (SIZE) and incidence of skeletal malformations (SK.MF) by the participating hatcheries (A, B, C and D).

Dicentrarchus labrax								
Hatchery A			**Hatchery C**			**Hatchery D**		
Batch	**SIZE**	**SK.MF**	**Batch**	**SIZE**	**SK.MF**	**Batch**	**SIZE**	**SK.MF**
A1	POOR	BEST	C1	BEST	POOR	D1	POOR	BEST
A2	**POOR**	**POOR**	C2	BEST	POOR	D2	BEST	POOR
A3	BEST	-	C3	POOR	-	D3	BEST	-
A4	BEST	-	C4	POOR	-	D4	BEST	-
A5	BEST	-	C5	-	BEST	D5	**POOR**	**POOR**
A6	BEST	-	C6	-	BEST	D6	POOR	-
A7	BEST	-				D7	POOR	-
A8	-	POOR				D8	-	BEST
A9	-	-						

3.3. RNA quality, genomic DNA elimination and cDNA synthesis

Some of the samples received in RNAlater were of inadequate quality, and so could not be used for analysis and so instead total RNA was extracted from equivalent samples fixed in methanol. A comparison of samples prepared from samples stored with the two approaches did not yield extracts of total RNA that were appreciably different in the quality or quantity of total RNA (figure 2.7A). Verification of the extracted total RNA from the larvae using PCR for 18S rRNA revealed the samples that did not contain genomic DNA contamination (figure 2.7B). The cDNA synthesis of all samples was verified by non-saturating PCR to confirm the comparability of amplicon size and quantity in all samples analyzed (figure 2.7C). Agarose gel electrophoresis of PCR products was used to compare the cDNA of all samples. Anomalous samples were identified by an absence of product, discrepant amplicon quantities compared to other samples (as assessed by product intensity) or by possessing a product of a different size. Anomalous samples were reevaluated and excluded from the analysis if confirmed to be outlier samples.

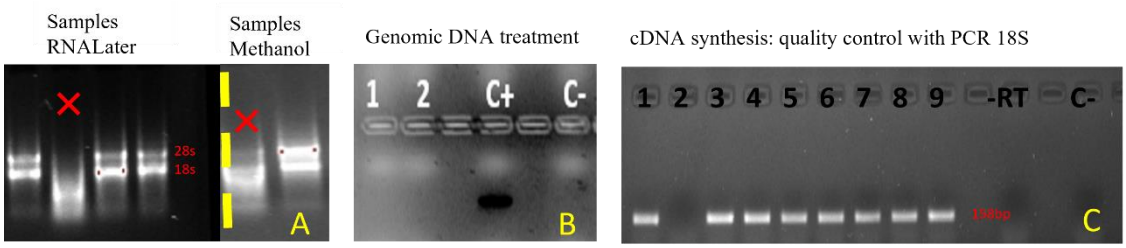

Figure 2.7| *A) Agarose gel (1% TAE1x buffer) electrophoresis of total RNA. Four samples of total RNA extracted from larvae stored in RNAlater are shown on the left-hand side of the image and on the right-hand side of the image 2 samples of total RNA extracted from larvae stored in methanol are shown. Note that the good quality total RNA extracts have two clear bands corresponding to the main ribosomal RNA bands, 28S rRNA and 18S rRNA. Red crosses identify poor quality samples. B) Agarose gel electrophoresis (2%, TAE 1x buffer) of the reaction products of PCR for 18S rRNA with total RNA extracted from the larval samples (1 and 2). The absence of a PCR product confirmed the efficiency of the DNase treatment to eliminate contaminating genomic DNA. C+ is a positive control and illustrates a cDNA sample with an amplicon of the expected size (158bp size) for 18S rRNA and C- is the negative control with no template to confirm the absence of contamination caused by technical problems. C) Agarose gel electrophoresis (2% TAE1x buffer) of cDNA from larval samples (1-9) used for PCR amplification of 18S rRNA. Note samples 1, 3-9 all have a single amplicon of the expected size (158bp) and -RT is the cDNA synthesis control in which the enzyme reverse transcriptase was omitted and C- is the non-template negative control. 18S rRNA was not amplified in sample 2 and the sample was tested again and if no product was obtained was excluded from the analysis.*

3.4. Primer screen for potential genes as biomarkers of larvae quality

For the European sea bass a total of seventy-nine genes were tested by PCR, 29 were for bone-related pathway genes, 27 were for endocrine-related genes, 15 were for growth-related genes, for the full list see *figure 2.8*. After applying the devised screening strategy (see supplementary tables 4 and 5 for details) nine genes were selected as the best putative candidate biomarkers for analysis. For gilthead sea bream sixty-nine genes were initially selected for analysis using PCR, 20 were for bone-related genes, 16 were for growth related genes and 9 were for immune genes, for the full list see *figure 2.8*. Twelve genes with an amplification threshold < 30, a single reaction product, and notable differences in expression level between good and poor larvae were selected as the best putative candidate biomarkers for use in PCR.

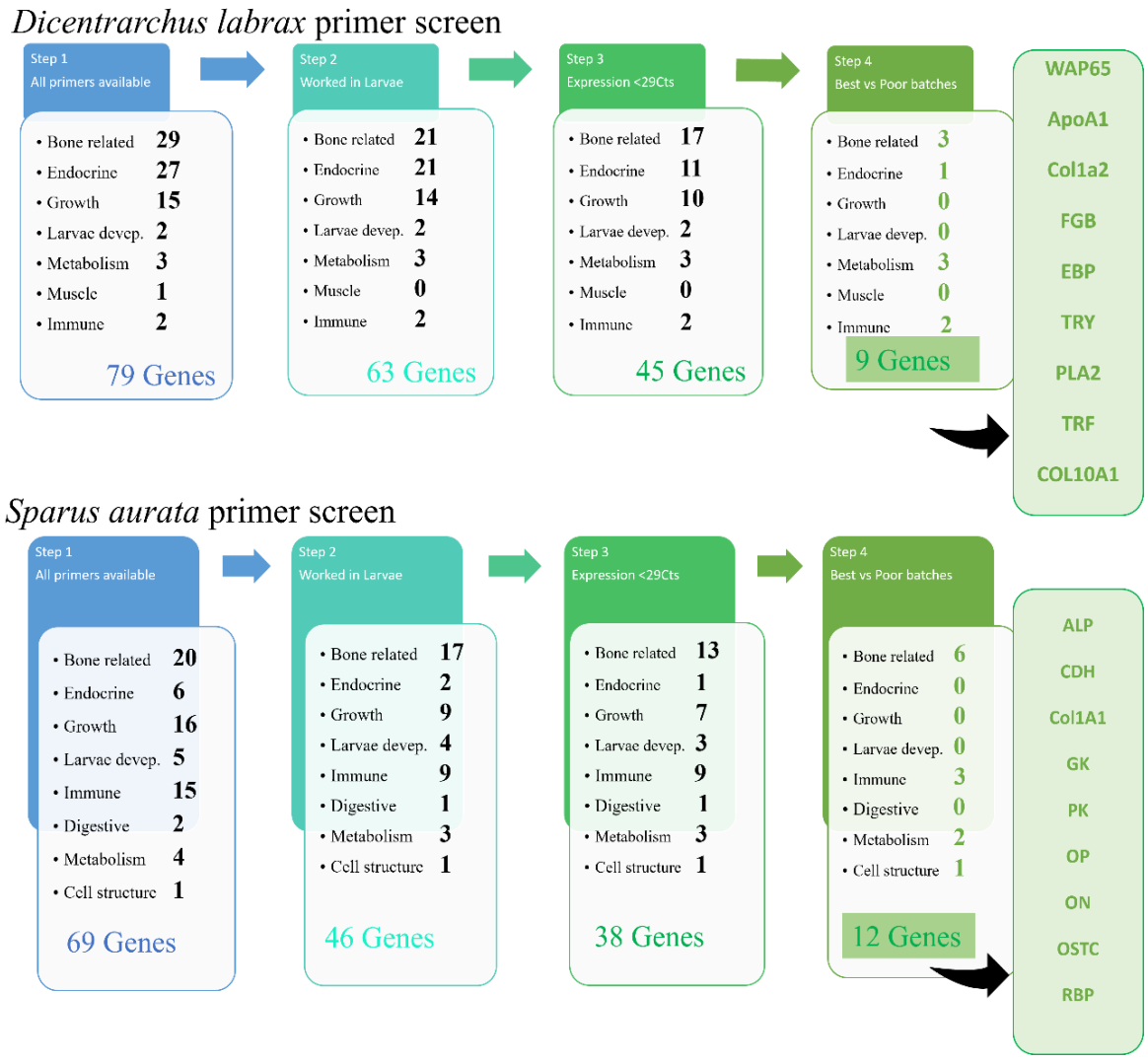

Figure 2.8| *The workflow for primer selection of the main quality related biomarker genes for European sea bass and Gilthead Sea bream. An overview of the main steps are presented, and reveal the starting number of primers per category and the reduction in their number as they pass through the main screening steps until the final potential candidate genes of larvae quality.*

3.5 qPCR expression analysis

After the application of the nine genes selected as potential quality candidate genes in qPCR for the European sea bass, only eight passed the quality criteria for qPCR, namely amplification of a single product, a specific PCR reaction and good efficiency (>90%). Since the qPCR developed for col1a2 was poor efficiency despite testing alternative conditions it was removed from the list of candidate quality markers. In the European sea bass application of one-way ANOVA to all the qPCR results obtained with the 8 candidate quality biomarkers revealed that the expression of all genes in most of the larval stage were significantly different ($p<0.001$) between hatcheries (table 2.4).

Table 2.4| *One-way-ANOVA analysis using SigmaPlot 12.3 version for expression of putative candidate genes associated with quality in* ***European sea bass****. Statistical analysis reveals differences in gene expression among hatcheries within each stage. Significant differences are indicated by small asterisk: * for* $p < 0.05$ *and ** for* $p < 0.001$*; n = 6 larvae/batch.*

DL	**FF**	**FL**	**ELR**	**MM**
WAP65	**	-	**	**
TRF	**	*	**	**
COL10A1	**	**	-	**
TRY	**	**	**	-
APOA1	**	**	**	**
EBP	**	**	**	**
FGB	**	**	**	**
PLA2	**	**	**	**

Two-way ANOVA was then applied to identify differences between the stages of ontogeny analyzed in the European sea bass and to establish if they were linked to the quality of the batches analyzed (figure 2.9). All the genes analyzed except for *ebp and fgb*, revealed an interaction between quality and the ontogeny of the different stages (table 2.5).

At first feeding (± 8 dph) five of the nine candidate genes for European sea bass had a different expression in the poorest batches compared to the best batches and included: *wap65*, *col101a* ($p < 0.001$) and *trf*, *apoa1* and *pla2* ($p < 0.05$). For the flexion larval stage (± 25 dph) only four genes were significantly different between the good and bad batches from the two hatcheries analyzed and included: *trf*, *try* and *apo1a1* ($p < 0.001$) and *col10a1* ($p < 0.05$). The expression of the genes *trf*, *try* and *apo1a1* were also significantly different and related to quality in the ELR stage (± 35 dph). Finally at metamorphosis (± 50 dph) only the *try* gene ($p < 0.001$) and *col10a1* ($p < 0.05$) were significantly different. Regarding the statistical analysis of *trf*, *col101a*, *try* and *apoa1*, when gene expression among ages were compared, showed divergence in the results for the poorest batches and the best batches when quality is separated. Considering candidate genes between ontogenetic stages and disregarding quality no differences between ontogeny stages when quality is not taking in account. *Ebp* and *pla2* gene expression are different when all stages are together, and quality compared.

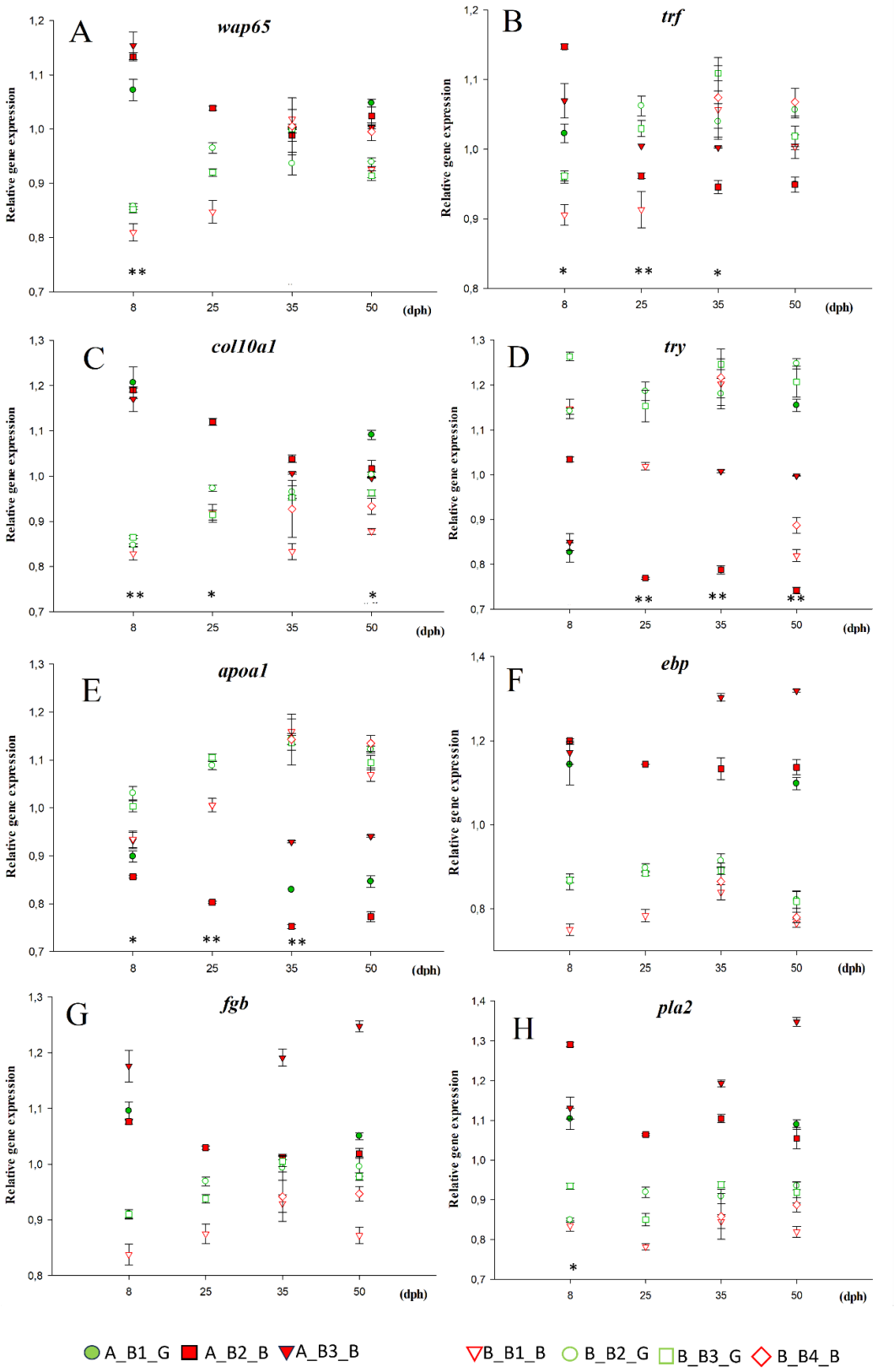

Figure 2.9| *Quantitative Real-Time PCR (qPCR) results of candidate genes transcripts associated with larvae quality for the different development stages of larvae analyzed (FF- first feeding at 8 dph; FL-flexion at 25 dph; ELR- end of larvae rearing at 35 dph and MM- middle of metamorphosis at 50 dph) in the* ***European sea bass.*** *The graphs show the results obtained for the genes selected,* ***(A)*** *wap65,* ***(B)*** *trf,* ***(C)*** *Col10A1,* ***(D)*** *try,* ***(E)*** *apoa1,* ***(F)*** *ebp,* ***(G)*** *fgb, and* ***(H)***

*pla2. The relative abundance of the gene transcripts was determined by qPCR and the results normalized using rps13. The different symbols in graphs means different batches within each company (B1-B3/B4), the green color correspond to best batches (G) and the red color corresponds to poorest batches(B), filled symbols belong to company A and unfilled symbols belongs to company B. The results for the different larvae stages are presented using a scatter plot with each point representing the average of a batch of larvae. Significant differences in gene transcript abundance was established by comparison of the expression of the best and poorest batches of cultured larvae from two different sites/hatchery, within each age. Groups that were significantly different are indicated by an asterisk: * for p < 0.05 and ** for p < 0.001; Two- way ANOVA; n = 6 larvae/batch.*

Table 2.5| *Two-way-ANOVA analysis using SigmaPlot 12.3 version for expression of putative candidate genes associated with quality in* ***European sea bass****. A- statistical analysis when the two factors (stage of larvae development and good & poor batch quality) are not interacting.* ***B*** *and* ***C*** *statistical analysis when the two factors, age and quality are dependent.* ***B*** *indicates the differences among ages in good or bad-quality batches.* ***C*** *indicates good and poor batches within each stage. Significant differences are indicated by * for p < 0.05 and ** for p < 0.001, n = 6 larval samples/batch.*

European sea bass genes	**A**		**B**		**C**			
	Stage	**good&poor**	**good**	**poor**	**FF**	**FL**	**ELR**	**MM**
WAP65	-	-	-	-	**	-	-	-
TRF	-	-	FF vs FL ** FF vs ELR** ELR vs MM*	FF vs FL **	*	**	*	-
COL10A1	-	-	-	FF vs MM** FF vs ELR**	**	*	-	*
TRY	-	-	-	FF vs FL** FF vs MM** ELR vs MM** FL vs ELR **	*	**	**	**
APOA1	-	-	FF vs ELR** MM vs ELR **	-	*	**	**	-
EBP	NO	YES	-	-	-	-	-	-
FGB	-	-	-	-	-	-	-	-
PLA2	NO	YES	-	-	*	-	-	-

For the gilthead sea bream gene screening for the potential quality-related candidate genes, the nine genes initially selected, passed the criterion for qPCR amplification being of high efficiency, generating a single product that was confirmed to be the target gene by sequencing. One-way ANOVA analysis with all the qPCR results obtained with the 9 candidate quality biomarkers revealed that the expression of most of the genes in most of the larval stage were significantly different ($p<0.05$) between hatcheries at the flexion stage (25dph), *col1a1*, *op* and *on* were also at first stage (table 2.6)

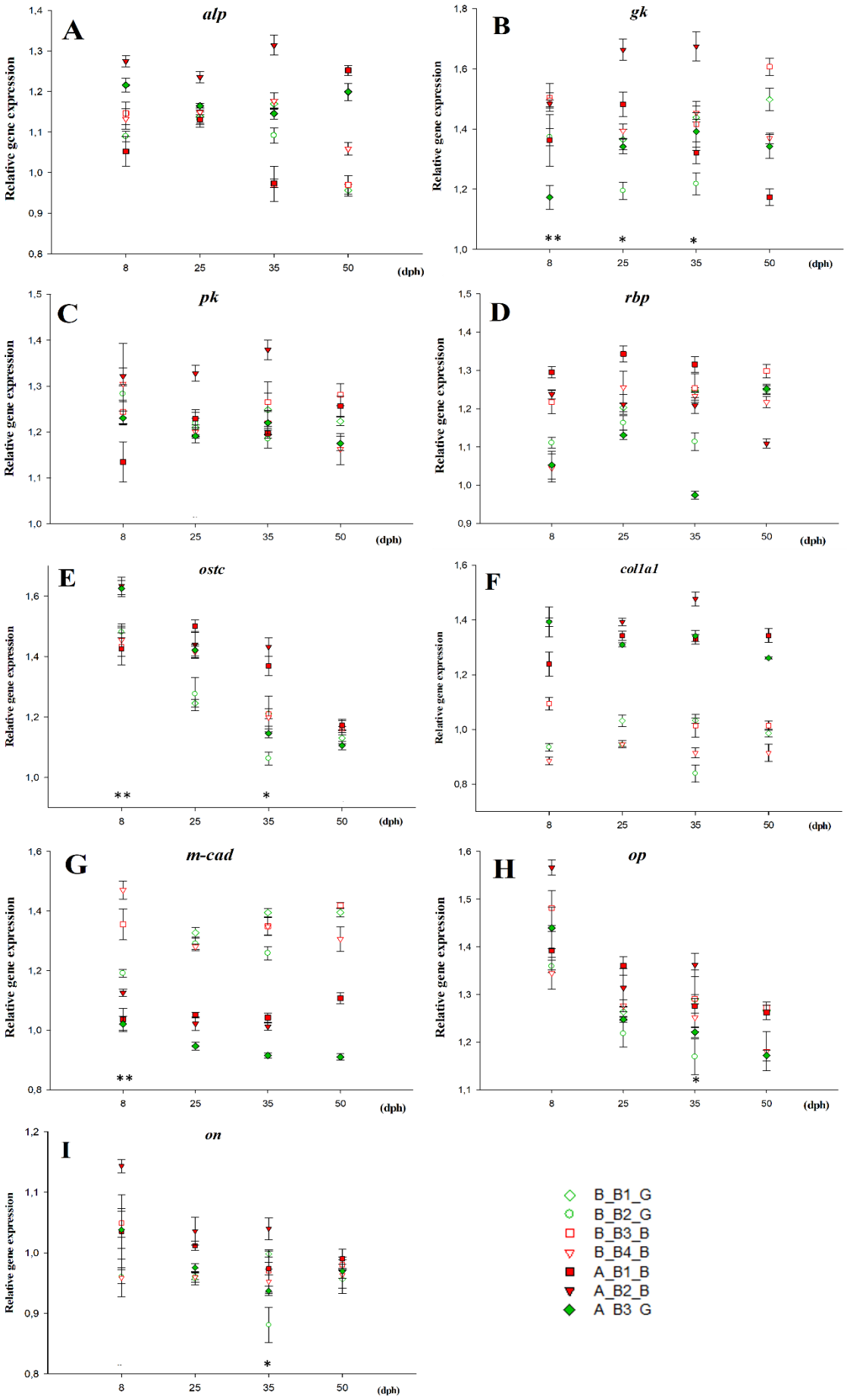
A
alp
B
gk
C
pk
D
rbp
E
ostc
F
col1a1
G
m-cad
H
op
I
on
Relative gene expression
Relative gene expresison
(dph)
8
25
35
50
**
*
B_B1_G
B_B2_G
B_B3_B
B_B4_B
A_B1_B
A_B2_B
A_B3_G

Figure 2.10| *Quantitative Real-Time PCR (qPCR) analysis in the different development stages of larvae analyzed (FF- first feeding at 8 dph; FL-flexion at 25 dph ; ELR- end of larvae rearing at 35 dph and MM- middle of metamorphosis at 50 dph) in* ***gilthead sea bream*** *of candidate gene transcripts associated with larvae quality: quality* ***(A)*** *alp, (****B****) gk,* ***(C)*** *pk,* ***(D)*** *rbp,* ***(E)*** *ostc,* ***(F)*** *col1a1,* ***(G)*** *m-cad,* ***(H)*** *op and* ***(I)*** *on. The relative abundance was determined by qPCR and the results normalized using* rps13. *The different symbols on graphs represent different batches within each company (B1-B3/B4), green color correspond to best batches (G) and red color corresponds to poorest batches (B), filled symbols represent company A and empty symbols represent company B. Significant differences in gene transcripts were established by comparison with the expression of the best and poorest batches of cultured larvae from two different sites/hatcheries, for each age analysed. Significant differences are indicated by * for* $p < 0.05$ *and ** for* $p < 0.001$*; Two- way ANOVA; n = 6 larvae samples/batch.*

Table 2.6| *One-way-ANOVA analysis using SigmaPlot 12.3 version for expression of putative candidate genes associated with quality in* ***gilthead sea bream****. Statistical analysis indicates the difference in gene expression among hatcheries within each stage. Significant differences are indicated by * for* $p < 0.05$ *and ** for* $p < 0.001$*; n = 6 larvae samples/batch.*

SA	***FF***	***FL***	***ELR***	***MM***
alp	-	*	-	**
gk	-	**	*	**
pk	-	*	-	-
rbp	-	*	*	-
ostc	-	*	**	-
m-cad	-	*	-	*
col1a1	*	-	-	-
op	*	*	-	-
on	*	**	-	-

The most significant differences ($p < 0.001$) between the good and poor-quality batches were identified for the genes *gk, ostc* and *m-cad* at the first feeding stage using two-way ANOVA (figure 2.10). At 35 dph, the expression of the genes *gk*, *ostc*, *op* and *on* were significantly different ($p < 0.05$) between good and bad batches. Expression of *alp, pk col1a1* and *m-cad* genes demonstrated no interaction between quality and the stage of ontogeny ($p < 0.05$). At the last stage of larval development before becoming juveniles, none of the genes revealed any differences between the poorest and best batches (table 2.7).

Two-way ANOVA revealed that *alp* and *pk* was significantly different between developmental stage irrespective of quality but *ostc* and *op* showed differences between developments stages within poor larvae batches and within best batches when quality was analyzed independently (table 2.7).

Table 2.7| *Two-way-ANOVA analysis using SigmaPlot 12.3 version for expression of putative candidate genes associated with quality in gilthead sea bream.* ***A*** *statistical analysis when the two factors (stage of larvae development and good & poor batch quality) do not interact.* ***B*** *and* ***C*** *statistical analysis when the two factors, age and quality are dependent.* ***B*** *indicates the differences among ages in good or bad quality batches.* ***C*** *indicates significantly different batches classified as good and poor within each stage. Significant differences are indicated by * for* $p < 0.05$ *and ** for* $p < 0.001$*; n = 6 larvae samples/batch.*

Gilthead sea bream Genes	**A**		**B**		**C**			
	stage	**good&poor**	**good**	**poor**	**FF (8dph)**	**FL (25dph)**	**ELR (35dph)**	**MM (50dph)**
ALP	FF vs MM* FL vs MM *	YES	-	-	-	-	-	-
GK	-	-	-	FF vs FL *	**	*	*	-
PK	FF vs FL *	NO	-	-	-	-	-	-
RBP	-	-	-	-	-	-	-	-
OSTC	-	-	FF vs FL** FF vs ELR** FF vs MM** FL vs MM** FL vs ELR **	FF vs ELR** FF vs MM* FL vs MM**	**	-	*	-
M-CAD			-	FF vs FL** FL vs ELR *	**	-	-	-
COL1A1	-	-	-	-	-	-	-	-
OP	-	-	FF vs FL** FF vs ELR** FF vs MM**	FF vs FL** FF vs ELR** FF vs MM**	-	-	*	-
ON	-	-	-	-	-	-	*	-

3.6 Meta-analysis

After analysis of significantly different DEGs (differential expressed genes) between good versus poor larval batches it was observed that the highest number of DEGs were at mid-metamorphosis, only one gene did not differ at any stage (*alp*) in the gilthead sea bream. In European sea bass, all genes differed at least in one stage, the highest number of DEGs was observed at first feeding, and expression of the *ebp* gene discriminated between the good and poor-quality larvae in all developmental stages (table 2.8).

Using random forest analysis (work done by the University of Thessaly, UTH) and including all the genes identified and analyzed by CCMAR and another group of genes identified and analyzed by UTH (n = 15 -20) a core gene set composed of 5 genes was obtained that when used to analyze larval quality predicted the quality of larvae with

an accuracy of ≥ 0.8, that is an 80% probability of separating good from bad quality batches of gilthead sea bream or European sea bass using qPCR gene expression analysis. The core larval quality gene markers identified were different for the two species but also for the species stage, as observed in our smaller scale analysis.

In summary, the genes identified in the present thesis permit identification of good and bad quality larvae for gilthead sea bream and European sea bass and also provide insight into gene expression patterns associated with specific physiological processes during development. More specifically for the European sea bass a panel of genes was identified that discriminate between the quality in European sea bass in each developmental stage analyzed. In the case of the gilthead sea bream marker genes that discriminate between the quality were only found for the MM stage for the other genes screened by our partner from Greece that genes were related to muscle development, growth and protein metabolism (confidential data).

Table 2.8| *Statistical analysis using Wilcoxon signed-rank test, comparison between good and bad gilthead sea bream (SA) and European sea bass (DL) larval samples from the four developmental stages classified based on skeletal malformation.*

SA	*FF*	*FL*	*ELR*	*MM*	*DL*	*FF*	*FL*	*ELR*	*MM*
Alp	-	-	-	-	*wap65*	**	-	*	-
Gk	-	*	-	-	*Trf*	***	-	-	-
Pk	-	-	*	-	*col10a1*	-	-	**	-
Rbp	-	-	-	*	*Try*	***	-	-	*
Ostc	-	*	-	-	*apoa1*	***	*	-	-
m-cad	-	**	-	-	*Ebp*	***	*	**	**
col1a1	*	-	*	*	*Fgb*	***	-	*	-
Op	**	-	-	-	*pla2*	**	-	***	***
On	*	-	-	-					

4. Discussion

The results of this work produced a lot of the data to be applied in the final quality tool which will predict the performance of the future juveniles. This tool is based on expression pattern of some genetic biomarkers related to crucial physiological functions in very early larval development stages of gilthead sea bream and European sea bass. A lot of information was collected and organized with the aim of making it accessible to all companies and the scientific community with the view of moving towards more economic and sustainable hatcheries and aquaculture production in the Mediterranean. In the following discussion I will briefly consider the main outcomes of my study.

4.1 Sampling manual

The sampling manual developed was helpful to standardize sample collection and handling by the multiple hatcheries that participated in the study. The aim was to minimize sample variability caused by technical issues and after preliminary adjustments and trials the objective was achieved in collaboration with the hatcheries. In addition, the sampling manual facilitated and promoted the involvement of hatcheries since it gave a clear outline of the objectives and guidelines of the researchers' needs. Furthermore, since many of the technical staff in the hatcheries did not have experience in sampling or laboratory-based analysis, the sampling manual provided an overview of the importance of sampling and a detailed protocol for optimal sample collection and mailing since it was not logistically possible to visit all collaborators. The sampling manual was a co-creative process as the hatcheries were involved in the initial development and optimization of sampling and sample preservation. Similarly, to make it possible to link sample quality with production conditions an extensive spreadsheet was prepared for recording the zootechnical parameters of each production site. Specific problems encountered with the sampling that were solved during the project was the way hatcheries classified the development stage of larvae. For example, for the same developmental stage we received larval samples that differed significantly in dph and those that were very discrepant in size or stage were excluded from the analysis. The manual was a good vehicle for the development of new collaborations and to create channels for communication and contribute measures to improve larval welfare.

4.2. Classification of larvae

The classification of larvae was initially established by the companies that sent samples from their production batches classified as best or poorest based on bone malformations since this is one of the biggest problems in hatcheries (Boglione, 2001). However, since skeletal malformations are generally only detectable at later developmental stages (Koumoundouros et al., 2017), they cannot be used as a monitoring tool for the identification of production batch quality. Malformations have a negative impact for several reasons, they are a fish welfare issue and cause significant economic losses since deformed fish have less value and hatcheries have to remove them manually from production batches at the early juvenile stage, which is a costly procedure (Boglione, 2001; Boglione et al., 2003, 2009; Cahu et al., 2003; Castro et al., 2008; Matsuoka, M., 2003; Georgakopoulou, Angelopoulou, et al., 2007; Hilomen-Garcia, 1997; Koumoundouros et al., 1997; Lall & Lewis-McCrea, 2007; Le Vay et al., 2007b; Powell et al., 2009)

One difficulty of working with commercial companies is their unwillingness to share internal data and so only one of the collaborating hatcheries provided a description of their quality classification approach. Quality is defined by the relationship between the number of good post larvae (about 60 dph) in a production batch as a percentage of the number of larvae stocked in the rearing tanks (1 dph). For example, 250,000 good quality post larvae (60 dph), could come from 1 million stocked larvae, and have a survival rate of 50% and a malformation rage of 50% so of the 500,000 live larvae half of them (250,000) would be deformed and would be discarded. Alternatively, 300,000 live larvae could correspond to a batch with 30% survival and 17% incidence of malformation, which would again give 250,000 good quality larvae. So, although the initial idea was to work with samples quality based on the hatchery classification cross referenced with the malformation index, the lack of information from hatcheries, and the potential variability arising from the hatcheries classification approach meant that the use of malformations screening cross referenced with growth gave a good approach for establishing good and bad quality batches for screening of potential quality biomarkers. Subsequently after preliminary primer selection using the good and bad samples we opted to use quality as defined by malformation rate as it is more directly related to quality than growth.

4.3. *Screening for candidate genes of larvae quality*

Related to the screening of candidate genes, the approach taken was to select a large suite of genes either available *in house* or from the literature and associated with physiological processes of production relevance (Supplementary information table 1 and 2). We found that it is four times more data available about gilthead sea bream than European sea bass, with our work we found that they can behave so differently, so we hope we are providing here new information to starting point of new studies about European sea bass that is needed. The primers for candidate biomarkers that were selected for further analysis came from the results of qPCR and the application of the defined criteria (see methods and results). Interestingly, although from the preliminary screening different candidate gene biomarkers were selected for European sea bass and gilthead sea bream the physiological processes in which they were involved were the same. For example, since malformations were the main factor for quality classification many genes identified that were significantly different between good and poor samples were related to bone (*ebp, col10a1, alp, col1a1, op, on, ostc*) and others were related to metabolism of lipids and carbohydrates (*pk, gk, pla2* and *apoA1*).

The enrichment of genes for lipidic or carbohydrates metabolism in the good and poor-quality larvae is interesting and may be related to the higher energy requirements of malformed larvae that may have to swim more or are less able to swim and capture prey. Linked to the metabolic-related biomarkers identified, it has previously been shown that fish larvae fed with inappropriate lipids in their diet have an increased incidence of malformations of the spinal column and fin rays (Cahu et al., 2003). In contrast optimal levels of vitamin A can reduce skeletal malformation in gilthead sea bass (Mazurais et al., 2008) although high levels of Vitamin A and PUFA can cause malformations in early larval development (Villeneuve et al., 2006) and the importance of these dietary factors may contribute to explain the difference between good and poor quality larvae. The results of the present thesis demonstrate that the approach used may be promising for selection of other biomarkers related to other quality parameters (growth etc) or other characteristics of interest. The involvement of multiple hatcheries and large sample numbers means that hatchery specific modifications are avoided and only strong trait specific modifications that change in all samples are selected as biomarkers.

4.4. *Gene expression: hatcheries/ontogeny/quality*

The gene expression of the selected candidate genes was selected to discriminate between poor and good quality production batches of larvae, and revealed what markers are modified early in development, during development and their variability within and between hatcheries. From the 500 samples analyzed per species, from the four collaborating companies, the results are presented for only a small selection of production batches (two good and two bad quality) and for only some candidate genes to illustrate the differentially expressed genes (DEGs) identified between the best and poorest batches for each candidate gene in all development stages. In overall study, several candidate biomarkers were identified in all stages for the European sea bass but fewer for the gilthead sea bream presents much more individual variability, in graphs it is possible to see that the error bars are big (figure 2.10).

Robustness with only one gene is not possible to see the state of ontogeny. The state of ontogeny is useful to determine if the development of fish is contributing for the delay or not of the expression of each candidate gene. In sea bream genes such as *ostc*, *op*, and *on* the expression seems to decrease along the age but with no statistical differences (figure 2.10), this are also observed in a study with the expression of *on* but from 1dph until 15dph and also decrease again from 24dph until 85dph (Estêvão et al., 2005), so the maximum level of *on* seems to reach between 25-35dph that correspond to the beginning of ossification (Faustino & Power, 1999). Abnormalities look to increase the expression of bone related genes, and the other genes in general, as we can see in the graphs in all most cases red symbols which koumoundouros hare the bad samples are more expressed than best samples. Make us to think why these genes especially the one related to bone formation are more expressed in bad samples than in good samples as it was demonstrated in sea bream juveniles the opposite, when we look to literature, we found out those bone related genes are downregulated in presence of skeletal anomalies (Riera-Heredia et al., 2019b).

Also we tried to compare our results with what we have in literature, for example in European sea bass, in a study where they monitored the expression of several genes during larval development from 7-43dph it was found that col1a1 was higher at 31dph and trypsin (try) between 7-17dph (Darias et al., 2008), in other study with European sea bass, when we tried to compare with our results it is difficult to reach conclusions, as we are

looking for samples that; first they are produce at large scale, second, they belong to different sites, 3rd we are looking at different time sampling point.

a) *Hatcheries*

In European sea bass all genes showed difference levels of expression between the two hatcheries in most of the larval stages, this was expected since larvae coming from different production sites were obtained from genetically different broodstock and were maintained under different management regimes (Koumoundouros et al., 2017). The differences in the conditions of rearing change the expression of the candidate genes presumably due to their involvement in physiological functions that were developing and so were susceptible to alterations. In the case of the gilthead sea bream the divergence between the two production sites was not so evident, but the expression level of all the candidate genes had a divergent expression in at least one or two stages. The European sea bass and gilthead sea bream were both produced by the same two hatcheries, and so a possible explanation for why the gilthead sea bream was less divergent between the two sites was because of the very high variability of gene expression encountered within individual hatcheries suggesting this species may be genetically more diverse. In fact, recent studies on the genetics of gilthead sea bream (Gkagkavouzis, 2021), revealed differences in a genome-wide investigation between wild population compared to several locals of gilthead sea bream hatcheries in Greece, and from their results it is possible to see also differences between the hatcheries and also between the wild different local that were correspond to different countries in Mediterranean. Also in (Kourkouta et al., 2022b), revealed genetic diversity and significant genetic distances among hatcheries. This can answer to our variability of samples coming from our two different hatcheries. In fact, when we were looking for a reference gene was difficult to find a good one that were stable, because even for a housekeeping gene the variability was notable.

One of the reasons that can contributed for the difficulties of having more homogeneous samples especially in the case of sea bream is how hatcheries classified the development stages, is something based on morphological aspects and behavior that can be slightly variable between companies, so we don't have sure if they are correct or not, also we saw that the days post hatch for each development stage is variable even within same company, but between hatcheries is noticeable so meantime rich differences of 10dph.

Taking into consideration the variability in gene expression between different samples (composed of several individuals) in European sea bass and gilthead sea bream due to their genetic background and production conditions, is this a problem for the identification of gene biomarkers? In fact, identifying genes that are significantly different in the larvae irrespective of company or broodstock reveals core genes that are common robust biomarkers associated with quality. By conducting a large-scale study it was possible to identify "reference genes of quality" since their expression was more strongly linked to quality than to origin or individual variability. To further validate the quality of the biomarkers identified we propose "blind" testing on new samples for which quality is not provided to the technician and in this way the discriminatory power of the biomarkers could be consolidated.

b) Genetics and function

Even if the gene biomarkers are not subsequently useful for the quality benchmarking tool developed, they can provide insight into larvae and organ/tissue development. The genes selected in the study are related with the endocrine system and growth (Tanaka et al., 1995; Power et al., 2001), skeletal formation (Boglione et al., 2013; Kourkouta et al., 2022; Koumoundouros et al., 2001; Zapata et al., 2006; Estêvão et al., 2011; Riera-Heredia et al., 2018), the immune system, and metabolism, that are known to be important during early fish ontogeny also showed previously in this study in the introduction (1.1). Several genes belong to the same pathways and have previously been proposed as potential predictors of larval quality they well summarized by koumoundouros et al., 2017, also included some of our candidate genes such as *col1a, col10a1, ostc, on* and *op,* but to identify the levels of these proteins or the respective gene expression requires expensive equipment and lack of trained people on hatcheries, they are not applied yet at industrial level to identify for this case bone malformation is larvae or juvenile, using molecular analysis. We tried to compare the results from our study using samples obtained from industrial scale production with previous studies using small scale experiments, but we could not find points of comparability. For example , other studies from where I based my choice of primers for the candidates genes, for example; most of them were done in aim to see differences between diets and thermo-challenges, to see what affects during early larval stage of European sea bass (Vagner et al., 2007), diets in gilthead sea bream juveniles (Caballero-Solares et al., 2015), a lot about skeletal malformation in gilthead sea bream but in juvenile(Riera-Heredia et al., 2018, 2019) when

is possible to detect most of the abnormalities. So, we conclude no study was ever done with the scope of our study where the expression of genes related to crucial function at early fish development and fish performance were compared between the best batches and the poorest batches in large scale production from several hatcheries from different countries of the Mediterranean. This can contribute as a starting point of further investigation in aim to improve the KPIs in hatcheries and maybe prevent some malformation for example.

The fact that our study was transversal and used samples from large scale industrial production from several companies from different regions around the Mediterranean Sea it is more likely to identify useful biomarkers that are applicable to industrial production. For the preceding reasons the identified biomarkers are likely to have a broad applicability in industry. Although the quality classification in our study was based on bone malformations the genes tested covered the skeletal system but also the endocrine system, immune and digestive metabolism, which were probably indirectly be affected due to the interline between the systems (Moretti et al., 1999; Boglione et al., 2013a).

c) Ontogeny

The genes related to bone formation such as *ostc* and *op* in case of gilthead sea bream are different between ontogenetic stages when good and bad can be identified based on skeleton malformations (table 2.7), but also in early stages when bone is forming (Riera-Heredia et al., 2019b). In European sea bass the genes *trf, col10a1, try* and *apoa1* also were differential expressed between different developmental stages, and *col10a1*, *try* had a different expression between developmental stages of different quality (good and poor) (table 2.5). It is interesting that *col1a1*, which is important in bone formation and has been associated with malformations (Riera-Heredia et al., 2019b) is significantly different in poor quality larvae at different stages and this is presumably linked to anomalous bone development in these larvae. Similarly, trypsin (*try*), which is differentially expressed during ontogeny and is associated with successful metamorphosis and stomach formation in raibow trout (Najafpour et al., 2021). Considering the quality classification only the poor larvae presented significant differences in *try* expression, and this was present from early to late developmental stages, indicating that a "poor"

developmental trajectory occurs early in development. In the case of *apoa1*, that has metabolic functions (Sahoo, et al., 2017), significant differences occurred across ontogeny but when quality was considered only the good larvae were significantly different between developmental stages suggesting metabolism is adjusted across ontogeny and contributes to well performing larvae.

d) Quality biomarkers

European sea bass showed significant quality-related biomarkers for five of the eight candidate genes tested (table 2.5). In very early stages putative quality-linked gene biomarkers that were DE between stages included, *wap65* and *col101a* for FF, *trf*, *try* and *apoa1* for FL, *try* and *apoa1* for ELR and finally *try* for MM. In contrast, in the gilthead sea bream only 3 genes were potential quality-linked gene biomarkers and included: *gk*, *ostc* and *m-cad (*table 2.7*)*. In the case of the gilthead sea bream the differences in gene expression of the putative quality-linked gene biomarkers were detected in very early development, around 7 dph, permitting early intervention and corrective measures including elimination of poorly performing production batches. Nonetheless, of note is that although the absolute level of expression of the putative quality linked biomarkers was different between hatcheries the pattern of expression was constant e.g. in poorly performing batches presented the same general pattern e.g. high or low in given stages. Now it would be interesting to collect more data from a greater number of hatcheries to further consolidate the developed tool. It should be noted that a validated quality biomarker tool now exists for European sea bass and was developed by integrating the data presented in the present thesis (and other data not reported) with the data from Greek colleagues who developed predictive models using random tree ML (briefly described below but not the core objective of this thesis).

4.5 *Meta-analysis*

All the qPCR analysis presented in the thesis and additional analysis on all received batches of fish larvae from the four collaborating hatcheries and not reported here were used with similar data from Greece to train a machine learning (ML) algorithm and to identify the minimal gene set that could discriminate good from bad batches irrespective of origin, broodstock, management practices or other parameters. More than 500 data points were used to identify the model that could relate gene expression pattern to quality and five genes were identified that could identify good and bad production

batches with more than 80% prediction accuracy. A multi-tier statistical analysis was used to determine the ontogenetic pattern of expression of each gene, to identify the degree of coordinated expression in different developmental stages and finally to identify those genes or combinations of them that differentiated between good from bad batches.

5. Conclusion and future work

Our screening had as its main objective to identify genes for inclusion in the ML model that would ultimately generate a cost-effective and robust tool for early discrimination of good and poor larval quality. Nonetheless, the genes identified and analyzed in the present thesis are useful in their own right as they identify processes that "go wrong" or "are essential" during ontogeny and cause/or are associated with the performance of production batches of gilthead sea bream and European sea bass. In this way the genes contribute to the understanding of the origin of the problem that causes abnormalities or differences in larval quality during the hatchery stage.

This is the first large scale study involving so many hatcheries in Europe and at a production level as far as we are aware, and so the insights coming from the study can form the basis of industry relevant experiments. Such experiments can be designed to establish parameters and measures that can be implemented to improve management practices that can minimize variation in larval batches produced in hatcheries. This is possible since core genes linked with quality have been identified that are applicable across all the participating hatcheries.

The results of the present study were established using a classification of quality based on skeletal malformation incidence and so the genes identified, and their expression profiles identify candidate genes for future studies of factors contributing to skeletal deformities in the European sea bass and gilthead sea bream but also other important aquaculture species.

In summary, the outcome of the present thesis was part of a much bigger strategy to establish a blueprint and pipeline for establishing quality markers for the hatchery stage of aquaculture species. This blueprint outlines an approach for commercially relevant research into quality that can be used for studies with a similar aim for other aquaculture species.

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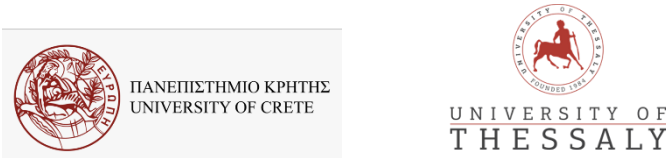
Collaboration:



Bibliography

Alami-Durante, H., Olive, N., & Rouel, M. (2007). Early thermal history significantly affects the seasonal hyperplastic process occurring in the myotomal white muscle of *Dicentrarchus labrax* juveniles. *Cell and Tissue Research*, *327*(3), 553–570. https://doi.org/10.1007/s00441-006-0321-2

Alvarez-Pellitero, P. (2008). Fish immunity and parasite infections: from innate immunity to immunoprophylactic prospects. *Veterinary Immunology and Immunopathology 126* (3-4) 171–198. https://doi.org/10.1016/j.vetimm.2008.07.013

Balon, E. K. (1983). Epigenetic mechanisms: reflections on evolutionary processes. *Canadian Journal of Fisheries and Aquatic Sciences*, *40*(11), 2045–2058. https://doi.org/10.1139/f83-236

Bauchot, M. (1981). Sparidae. FAO species identification sheets for fishery purposes. *Eastern Central Atlantic, 4*.

Boglione, C. (2001). Skeletal descriptors and quality assessment in larvae and post-larvae of wild-caught and hatchery-reared/gilthead sea bream *Sparus aurata L*. 175. *Aquaculture , 192*, 1–22. https://doi.org/10.1016/S0044-8486(00)00446-4

Boglione, C., Costa, C., Di Dato, P., Ferzini, G., Scardi, M., & Cataudella, S. (2003). Skeletal quality assessment of reared and wild sharpsnout sea bream and pandora juveniles. *Aquaculture*, *227*(1–4), 373–394. https://doi.org/10.1016/S0044-8486(03)00515-5

Boglione, C., Gisbert, E., Gavaia, P., Witten, P. E., Moren, M., Fontagné, S., & Koumoundouros, G. (2013). Skeletal anomalies in reared European fish larvae and juveniles. Part 2: Main typologies, occurrences and causative factors. *Reviews in Aquaculture*, *5*(1). https://doi.org/10.1111/raq.12016

Boglione, C., Marino, G., Giganti, M., Longobardi, A., De Marzi, P., & Cataudella, S. (2009). Skeletal anomalies in dusky grouper *Epinephelus marginatus* (Lowe 1834) juveniles reared with different methodologies and larval densities. *Aquaculture*, *291*(1–2), 48–60. https://doi.org/10.1016/j.aquaculture.2009.02.041

Business, F. (2018). Improving the welfare of European sea bass and gilthead sea bream. http://hyperphysics.phy-astr.gsu.edu/hbase/Tables/Soundv.html

Caballero-Solares, A., Viegas, I., Salgado, M. C., Siles, A. M., Sáez, A., Metón, I., Baanante, I. V., & Fernández, F. (2015). Diets supplemented with glutamate or glutamine improve protein retention and modulate gene expression of key enzymes of hepatic metabolism in gilthead seabream (*Sparus aurata*) juveniles. *Aquaculture*, *444*, 79–87. https://doi.org/10.1016/j.aquaculture.2015.03.025

Cahu, C., Infante, J. Z., & Takeuchi, T. (2003). Nutritional components affecting skeletal development in fish larvae. *Aquaculture*, *227*(1–4), 245–258. https://doi.org/10.1016/S0044-8486(03)00507-6

Cahu, C. L., Infante, J. L. Z., & Barbosa, V. (2003). Effect of dietary phospholipid level and phospholipid:neutral lipid value on the development of sea bass *(Dicentrarchus labrax*) larvae fed a compound diet . *British Journal of Nutrition*, *90*(1), 21–28. https://doi.org/10.1079/bjn2003880

Campinho, M. A., Moutou, K. A., & Power, D. (2004). Temperature sensitivity of skeletal ontogeny in *Oreochromis mossambicus*. *Journal of Fish Biology*, *65*(4), 1003–1025. https://doi.org/http://dx.doi.org/ 10.1111/j.1095-8649.2004.00505.x

Campinho, M. A., Silva, N., Sweeney, G. E., & Power, D. M. (2007). Molecular, cellular and histological changes in skin from a larval to an adult phenotype during bony fish metamorphosis. *Cell and Tissue Research*, *327*(2), 267–284. https://doi.org/10.1007/s00441-006-0262-9

Carballo, C., Chronopoulou, E. G., Letsiou, S., Maya, C., Labrou, N. E., Infante, C., Power, D. M., & Manchado, M. (2018). Antioxidant capacity and immunomodulatory effects of a chrysolaminarin-enriched extract in Senegalese sole. *Fish and Shellfish Immunology*, *82*, 1–8. https://doi.org/10.1016/j.fsi.2018.07.052

Carballo, C., Mateus, A. P., Maya, C., Mantecón, L., Power, D. M., & Manchado, M. (2020). Microalgal extracts induce larval programming and modify growth and the immune response to bioactive treatments and LCDV in Senegalese sole post-larvae. *Fish and Shellfish Immunology*, *106*, 263–272. https://doi.org/10.1016/j.fsi.2020.07.020

Carballo, C., Pinto, P. I. S., Mateus, A. P., Berbel, C., Guerreiro, C. C., Martinez-Blanch, J. F., Codoñer, F. M., Mantecon, L., Power, D. M., & Manchado, M. (2019). Yeast β-glucans and microalgal extracts modulate the immune response and gut microbiome in Senegalese sole (*Solea senegalensis*). *Fish and Shellfish Immunology*, *92*, 31–39. https://doi.org/10.1016/j.fsi.2019.05.044

Castro, J., Pino-Querido, A., Hermida, M., Chavarrías, D., Romero, R., García-Cortés, L. A., Toro, M. A., & Martínez, P. (2008). Heritability of skeleton abnormalities (lordosis, lack of operculum) in gilthead seabream (*Sparus aurata*) supported by microsatellite family data. *Aquaculture*, *279*(1–4), 18–22. https://doi.org/10.1016/j.aquaculture.2008.04.023

Çoban, D., Demircan, M. D., & Tosun, D. D. (2020). Marine aquaculture in turkey: advancements and management. *Editors*. www.tudav.org

Cobcroft, J. M., & Battaglene, S. C. (2009). Jaw malformation in striped trumpeter *Latris lineata* larvae linked to walling behaviour and tank colour. *Aquaculture*, *289*(3–4), 274–282. https://doi.org/10.1016/j.aquaculture.2008.12.018

Darias, M. J., Zambonino-Infante, J. L., Hugot, K., Cahu, C. L., & Mazurais, D. (2008). Gene expression patterns during the larval development of european sea bass (*Dicentrarchus Labrax*) by microarray analysis. *Marine Biotechnology*, *10*(4), 416–428. https://doi.org/10.1007/s10126-007-9078-1

Divanach, P., Papandroulakis, N., Anastasiadis, P., Koumoundouros, G., & Kentouri, M. (1997). Effect of water currents on the development of skeletal deformities in sea bass (Dicentrarchus labtax L.) with functional swimbladder during postlarval and nursery phase. *Aquaculture*, *156*(1–2), 145–155. https://doi.org/10.1016/S0044-8486(97)00072-0

Esteban, M. Á., & Cerezuela, R. (2015). Fish mucosal immunity: Skin. In *Mucosal Health in Aquaculture* (pp. 67–92). https://doi.org/10.1016/B978-0-12-417186-2.00004-2

Estêvão, M. D., Redruello, B., Canario, A. V. M., & Power, D. M. (2005). Ontogeny of osteonectin expression in embryos and larvae of sea bream (*Sparus auratus*). *General and Comparative Endocrinology*, *142*(1-2), 155–162. https://doi.org/10.1016/j.ygcen.2004.11.018

Estêvão, M. D., Silva, N., Redruello, B., Costa, R., Gregório, S., Canário, A. V. M., & Power, D. M. (2011). Cellular morphology and markers of cartilage and bone in the marine teleost Sparus auratus. *Cell and Tissue Research*, *343*(3), 619–635. https://doi.org/10.1007/s00441-010-1109-y

FAOa. (2023, January). Cultured Aquatic Species Information Programme - *Dicentrarchus labrax* (Linnaeus, 1758*)*. FAO. https://www.fao.org/fishery/en/aqspecies/bss

FAOb. (2023). Cultured Aquatic Species Information Programme - *Sparus aurata (*Linnaeus, 1758*).* FAO. https://www.fao.org/fishery/en/aqspecies/sbg

FAOc. (2018). FAO. The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals, Rome. https://www.fao.org/documents/card/en/c/i9540en

Faustino, M., & Power, D. M. (1999). Development of the pectoral, pelvic, dorsal and anal fins in cultured sea bream. *Journal of Fish Biology*, *54*(5), 1094–1110. https://doi.org/10.1111/j.1095-8649.1999.tb00860.x

Fernández, I., Hontoria, F., Ortiz-Delgado, J. B., Kotzamanis, Y., Estévez, A., Zambonino-Infante, J. L., & Gisbert, E. (2008). Larval performance and skeletal deformities in farmed gilthead sea bream (*Sparus aurata)* fed with graded levels of Vitamin A enriched rotifers (*Brachionus plicatilis*). *Aquaculture*, *283*(1–4), 102–115. https://doi.org/10.1016/j.aquaculture.2008.06.037

Ferosekhan, S., Sarih, S., Afonso, J. M., Zamorano, M. J., Fontanillas, R., Izquierdo, M., Kaushik, S., & Montero, D. (2022). Selection for high growth improves reproductive performance of gilthead seabream *Sparus aurata* under mass spawning conditions, regardless of the dietary lipid source. *Animal Reproduction Science*, *241*. https://doi.org/10.1016/j.anireprosci.2022.106989

Føre, M., Frank, K., Norton, T., Svendsen, E., Alfredsen, J. A., Dempster, T., Eguiraun, H., Watson, W., Stahl, A., Sunde, L. M., Schellewald, C., Skøien, K. R., Alver, M. O., & Berckmans, D. (2018). Precision fish farming: A new framework to improve production in aquaculture. In *Biosystems Engineering* 173, 176–193. https://doi.org/10.1016/j.biosystemseng.2017.10.014

Fragkoulis, S., Batargias, C., Kolios, P., & Koumoundouros, G. (2018). Genetic parameters of the upper-jaw abnormalities in Gilthead seabream *Sparus aurata*. *Aquaculture*, *497*, 226–233. https://doi.org/10.1016/j.aquaculture.2018.07.071

Gabriel, U. U., Gabriel, U., & Akinrotimi, A. (2011). Management of stress in fish for sustainable aquaculture. *Researcher,* 3 (4), 28-38.

Georga, I., Glynatsi, N., Baltzois, A., Karamanos, D., Mazurais, D., Darias, M. J., Cahu, C. L., Zambonino-Infante, J. L., & Koumoundouros, G. (2011). Effect of vitamin A on the skeletal morphogenesis of European sea bass, *Dicentrarchus labrax* (Linnaeus, 1758). *Aquaculture Research*, *42*(5), 684–692. https://doi.org/10.1111/j.1365-2109.2010.02676.x

Georgakopoulou, E., Angelopoulou, A., Kaspiris, P., Divanach, P., & Koumoundouros, G. (2007). Temperature effects on cranial deformities in European sea bass, *Dicentrarchus labrax (L.). Journal of Applied Ichthyology*, *23*(1), 99–103. https://doi.org/10.1111/j.1439-0426.2006.00810.x

Georgakopoulou, E., Sfakianakis, D. G., Kouttouki, S., Divanach, P., Kentouri, M., & Koumoundouros, G. (2007). The influence of temperature during early life on phenotypic expression at later ontogenetic stages in sea bass. *Journal of Fish Biology*, *70*(1), 278–291. https://doi.org/10.1111/j.1095-8649.2007.01305.x

Gkagkavouzis, K. et al. (2021). Investigating Genetic Diversity and Genomic Signatures of Hatchery-Induced Evolution in Gilthead Seabream *(Sparus aurata*) Populations. *Diversity*, 13(11), 563. https://doi.org/10.3390/d13110563

Guerra, S. M., Valcarce, D. G., Cabrita, E., & Robles, V. (2013). Analysis of transcripts in gilthead seabream sperm and zebrafish testicular cells: MRNA profile as a predictor of gamete quality. *Aquaculture*, *406–407*, 28–33. https://doi.org/10.1016/j.aquaculture.2013.04.032

Harada, H., Miki, R., Masushige, S., & Kato, S. (1995). Gene expression of retinoic acid receptors, retinoid-X receptors, and cellular retinol-binding protein I in bone and its regulation by vitamin A. *Endocrinology*, *136*(12), 5329–5335. https://doi.org/10.1210/endo.136.12.7588278

Hamre, K., Yúfera, M., Rønnestad, I., Boglione, C., Conceição, L. E. C., & Izquierdo, M. Fish larval nutrition and feed formulation: knowledge gaps and bottlenecks for advances in larval rearing. *Reviews in Aquaculture, 5*(1) 26-58. https://doi.org/10.1111/j.1753-5131.2012.01086.x

Helvik, J. V. (2009). Large-scale Programmes Aquaculture-An Industry in Growth The fish larva: a transitional life form, the foundation for aquaculture and fisheries Report from a working group on research on early life stages of fish. *Book.* www.forskningsradet.no/publikasjoner

Herbst, T., Sichelstiel, A., Schär, C., Yadava, K., Bürki, K., Cahenzli, J., McCoy, K., Marsland, B. J., & Harris, N. L. (2011). Dysregulation of Allergic Airway Inflammation in the Absence of Microbial Colonization. *American Journal of Respiratory and Critical Care Medicine*, *184*(2), 198–205. https://doi.org/10.1164/rccm.201010-1574OC

Hilomen-Garcia, G. V. (1997). Morphological abnormalities in hatchery-bred milkfish (Chanos chanos Forsskal) fry and juveniles. *Aquaculture*, *152*(1–4), 155–166. https://doi.org/10.1016/S0044-8486(96)01518-9

Izquierdo, M. S., Scolamacchia, M., Betancor, M., Roo, J., Caballero, M. J., Terova, G., & Witten, P. E. (2013). Effects of dietary DHA and α-tocopherol on bone development, early mineralisation and oxidative stress in *Sparus aurata* (Linnaeus, 1758) larvae. *British Journal of Nutrition*, *109*(10), 1796–1805. https://doi.org/10.1017/S0007114512003935

Izquierdo, M. S., Socorro, J., & Roo, J. (2010). Studies on the appearance of skeletal anomalies in red porgy: effect of culture intensiveness, feeding habits and nutritional quality of live preys. *Journal of Applied Ichthyology*, *26*(2), 320–326. https://doi.org/10.1111/j.1439-0426.2010.01429.x

Khosla, S. (2001). Minireview: The OPG/RANKL/RANK System. *Endocrinology*, *142*(12), 5050–5055. https://doi.org/10.1210/endo.142.12.8536

Kissil, G. W., Lupatsch, I., Elizur, A., & Zohar, Y. (2001). Long photoperiod delayed spawning and increased somatic growth in gilthead seabream *(Sparus aurata*). *Aquaculture*, *200*(3–4), 363–379. https://doi.org/10.1016/S0044-8486(01)00527-0

Komuro, H., Olee, T., Kühn, K., Quach, J., Brinson, D. C., Shikhman, A., Valbracht, J., Creighton-Achermann, L., & Lotz, M. (2001). The osteoprotegerin/receptor activator of nuclear factor κB/receptor activator of nuclear factor κB ligand system in cartilage. *Arthritis and Rheumatism*, *44*(12), 2768–2776. https://doi.org/10.1002/1529-0131(200112)44:12<2768::AID-ART464>3.0.CO;2-I

Koumoundouros, G., Divanach, P., & Kentouri, M. (2001). The effect of rearing conditions on development of saddleback syndrome and caudal fin deformities in *Dentex dentex (L.)*. *Aquaculture*, *200*(3–4), 285–304. https://doi.org/10.1016/S0044-8486(01)00552-X

Koumoundouros, G., Gisbert, E., Fernandez, I., & Cabrita, E. (2017). Quality descriptors and predictors in farmed marine fish larvae and juveniles. *Success Factors for Fish Larval Production*, *December 2018*. http://ebookcentral.proquest.com/lib/nord/detail.action?docID=5216978.

Koumoundouros, G., Monzón, I. F., Galindo-Villegas, J., Koumoundouros, G., Gisbert, E., Fernandez, I., Cabrita, E., & Conceição, L. E. C. (2017). Quality Descriptors and Predictors in Farmed Marine Fish Larvae and Juveniles Chapter 10 Quality Descriptors and Predictors in Farmed Marine Fish Larvae and Juveniles. *Executive Summary Background*. http://ebookcentral.proquest.com/lib/nord/detail.action?docID=5216978.

Koumoundouros, G., Oran, G., Divanach, P., Stefanakis, S., & Kentouri, M. (1997). The opercular complex deformity in intensive gilthead sea bream (*Sparus aurata L.*) larviculture. Moment of apparition and description. In *Aquaculture* (Vol. 156, Issues 1–2, pp. 165–177). Elsevier B.V. https://doi.org/10.1016/S0044-8486(97)89294-0

Kourkouta, C., Tsipourlianos, A., Power, D. M., Moutou, K. A., & Koumoundouros, G. (2022a). Variability of key-performance-indicators in commercial gilthead seabream hatcheries. *Scientific Reports*, *12*(1). https://doi.org/10.1038/s41598-022-23008-z

Kourkouta, C., Tsipourlianos, A., Power, D. M., Moutou, K. A., & Koumoundouros, G. (2022b). Variability of key-performance-indicators in commercial gilthead seabream hatcheries. *Scientific Reports*, *12*(1). https://doi.org/10.1038/s41598-022-23008-z

Lall, S. P., & Lewis-McCrea, L. M. (2007). Role of nutrients in skeletal metabolism and pathology in fish - An overview. In *Aquaculture* (Vol. 267, Issues 1–4, pp. 3–19). Elsevier. https://doi.org/10.1016/j.aquaculture.2007.02.053

Le Vay, L., Carvalho, G. R., Quinitio, E. T., Lebata, J. H., Ut, V. N., & Fushimi, H. (2007a). Quality of hatchery-reared juveniles for marine fisheries stock enhancement. In *Aquaculture* 268 (1-4) 169–180. https://doi.org/10.1016/j.aquaculture.2007.04.041

Lembo, G., Carbonara, P., Scolamacchia, M., Spedicato, M. T., & McKinley, R. S. (2007). Use of muscle activity indices as a relative measure of well-being in cultured sea bass *Dicentrarchus labrax* (Linnaeus, 1758). In *Developments in Fish Telemetry* (pp. 271–280). Springer Netherlands. https://doi.org/10.1007/978-1-4020-6237-7_25

Lewis-McCrea, L. M., & Lall, S. P. (2010). Effects of phosphorus and vitamin C deficiency, vitamin A toxicity, and lipid peroxidation on skeletal abnormalities in Atlantic halibut (*Hippoglossus hippoglossus*). *Journal of Applied Ichthyology*, *26*(2), 334–343. https://doi.org/10.1111/j.1439-0426.2010.01431.x

Li, L., Cardoso, J. C. R., Félix, R. C., Mateus, A. P., Canário, A. V. M., & Power, D. M. (2021). Fish lysozyme gene family evolution and divergent function in early

development. *Developmental and Comparative Immunology*, *114*. https://doi.org/10.1016/j.dci.2020.103772

Lijalad, M., & Powell, M. D. (2009). Effects of lower jaw deformity on swimming performance and recovery from exhaustive exercise in triploid and diploid Atlantic salmon *Salmo salar L*. *Aquaculture*, *290*(1–2), 145–154. https://doi.org/10.1016/j.aquaculture.2009.01.039

Liu, Z. (2007). Amplified Fragment Length Polymorphism (AFLP). *Aquaculture Genome Technologies, 4.*

Louro, B., Kuhl, H., Tine, M., de Koning, D. J., Batargias, C., Volckaert, F. A. M., Reinhardt, R., Canario, A. V. M., & Power, D. M. (2016). Characterization and refinement of growth related quantitative trait loci in European sea bass (*Dicentrarchus labrax*) using a comparative approach. *Aquaculture*, *455*, 8–21. https://doi.org/10.1016/j.aquaculture.2016.01.004

Mateus, A. P., Costa, R., Gisbert, E., Pinto, P. I. S., Andree, K. B., Estévez, A., & Power, D. M. (2017). Thermal imprinting modifies bone homeostasis in cold-challenged sea bream (*Sparus aurata*). *Journal of Experimental Biology*, *220*(19), 3442–3454. https://doi.org/10.1242/jeb.156174

Mazurais, D., Darias, M. J., Gouillou-Coustans, M. F., Le Gall, M. M., Huelvan, C., Desbruyères, E., Quazuguel, P., Cahu, C., & Zambonino-Infante, J. L. (2008). Dietary vitamin mix levels influence the ossification process in European sea bass (*Dicentrarchus labrax*) larvae. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, *294*(2). https://doi.org/10.1152/ajpregu.00659.2007

Migaud, H., Bell, G., Cabrita, E., Mcandrew, B., Davie, A., Bobe, J., Herráez, M. P., & Carrillo, M. (2013). Gamete quality and broodstock management in temperate fish. In *Reviews in Aquaculture* 5 (1) 194-223. https://doi.org/10.1111/raq.12025

Montalban-Arques, A., De Schryver, P., Bossier, P., Gorkiewicz, G., Mulero, V., Gatlin, D. M., & Galindo-Villegas, J. (2015). Selective manipulation of the gut microbiota improves immune status in vertebrates. In *Frontiers in Immunology* 6, 512. https://doi.org/10.3389/fimmu.2015.00512

Moretti, A., Aquatrade, S., Castelraimondo, S., Mario, I., Fernandez-Criado, P., Maricoltura, G. C., Rosignano, D., Srl, S., Solvay, R., & Guidastri, R. (1999). *Food and Agriculture Organization of the United Nations Rome*.

Mulero, I., García-Ayala, A., Meseguer, J., & Mulero, V. (2007). Maternal transfer of immunity and ontogeny of autologous immunocompetence of fish: A minireview. *Aquaculture*, *268*(1-4), 244–250. https://doi.org/10.1016/j.aquaculture.2007.04.046

Muniesa, A., Basurco, B., Aguilera, C., Furones, D., Reverté, C., Sanjuan-Vilaplana, A., Jansen, M. D., Brun, E., & Tavornpanich, S. (2020). Mapping the knowledge of the main diseases affecting sea bass and sea bream in Mediterranean. In *Transboundary and Emerging Diseases* 67 (3), 1089–1100. https://doi.org/10.1111/tbed.13482

Najafpour, B., Dorafshan, S., Paykan Heyrati, F., Canario, A. V. M., & Power, D. M. (2021). Comparative ontogeny of the digestive tract of Oncorhynchus mykiss ♀ x Salmo trutta caspius ♂ triploid hybrids to their parental species. *Aquaculture Nutrition*, *27*(2), 427–438. https://doi.org/10.1111/anu.13196

Negrín-Báez, D., Navarro, A., Lee-Montero, I., Soula, M., Afonso, J. M., & Zamorano, M. J. (2015). Inheritance of skeletal deformities in gilthead seabream (Sparus aurata) –lack of operculum, lordosis, vertebral fusion and LSK complex. *Journal of Animal Science*, *93*(1), 53–61. https://doi.org/10.2527/jas.2014-7968

Pabinger, S., Thallinger, G. G., Snajder, R., Eichhorn, H., Rader, R., & Trajanoski, Z. (2009). QPCR: Application for real-time PCR data management and analysis. *BMC Bioinformatics*, *10*, 268. https://doi.org/10.1186/1471-2105-10-268

Pittman, K., Yúfera, M., Pavlidis, M., Geffen, A. J., Koven, W., Ribeiro, L., Zambonino-Infante, J. L., & Tandler, A. (2013). Fantastically plastic: Fish larvae equipped for a new world. *Reviews in Aquaculture*, *5*(1). https://doi.org/10.1111/raq.12034

Polo, A., Yúfera, M., & Pascual, E. (1991). Effects of temperature on egg and larval development of Sparus aurata L. *Aquaculture*, *92*(C), 367–375. https://doi.org/10.1016/0044-8486(91)90042-6

Polovina, E. S., Kourkouni, E., Tsigenopoulos, C. S., Sanchez-Jerez, P., & Ladoukakis, E. D. (2020). Genetic structuring in farmed and wild Gilthead seabream and European seabass in the Mediterranean Sea: Implementations for detection of escapees. *Aquatic Living Resources*, *33*. https://doi.org/10.1051/alr/2020007

Powell, M. D., Jones, M. A., & Lijalad, M. (2009). Effects of skeletal deformities on swimming performance and recovery from exhaustive exercise in triploid Atlantic salmon. *Diseases of Aquatic Organisms*, *85*(1), 59–66. https://doi.org/10.3354/dao02056

Power, D., Llewellyn, L., Faustino, M., Nowell, M., Bjornsson, Bt., Einarsdottir, I., Canario, A., & Sweeney, G. (2001). Thyroid hormones in growth and development of fish. In *Comparative Biochemistry and Physiology PartC*, 130.

Power, D. M., Guerreiro, P. M., Hubbard, P. C., Huertas, M., Morgado, I. M., & Fuentes, J. (2010). Endocrinology applied to aquaculture of finfish.

Power, D. M., Louro, B., Houston, R., Anjos, L., & Cardoso, J. C. R. (2010). Genomic-proteomic research in Sparidae and its application to genetic improvement Chapter contents. http://www.fao.org/fishery/countrysector/naso

Prat, F., Zanuy, S., Bromage, N., & Carrillo, M. (1999). Effects of constant short and long photoperiod regimes on the spawning performance and sex steroid levels of female and male sea bass. *Journal of Fish Biology*, *54*(1), 125–137. https://doi.org/10.1111/j.1095-8649.1999.tb00617.x

Pridgeon, J. W., & Klesius, P. H. (2012). Major bacterial diseases in aquaculture and their vaccine development. In *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*, 7. https://doi.org/10.1079/PAVSNNR20127048

Printzi, A., Kourkouta, C., Fragkoulis, S., Dimitriadi, A., Geladakis, G., Orfanakis, M., Mazurais, D., Zambonino-Infante, J., & Koumoundouros, G. (2021). Balancing between *Artemia* and microdiet usage for normal skeletal development in zebrafish (*Danio rerio*). *Journal of Fish Diseases*, *44*(11), 1689–1696. https://doi.org/10.1111/jfd.13487

Reinecke, M., Björnsson, B. T., Dickhoff, W. W., McCormick, S. D., Navarro, I., Power, D. M., & Gutiérrez, J. (2005). Growth hormone and insulin-like growth factors in fish: Where we are and where to go. *General and Comparative Endocrinology*, *142*(1-2), 20–24. https://doi.org/10.1016/j.ygcen.2005.01.016

Rescan, P. Y. (2005). Muscle growth patterns and regulation during fish ontogeny. *General and Comparative Endocrinology*, *142*(1-2), 111–116. https://doi.org/10.1016/j.ygcen.2004.12.016

Riera-Heredia, N., Martins, R., Mateus, A. P., Costa, R. A., Gisbert, E., Navarro, I., Gutiérrez, J., Power, D. M., & Capilla, E. (2018). Temperature responsiveness of gilthead sea bream bone; an in vitro and in vivo approach. *Scientific Reports*, *8*(1). https://doi.org/10.1038/s41598-018-29570-9

Riera-Heredia, N., Vélez, E. J., Gutiérrez, J., Navarro, I., & Capilla, E. (2019a). Gene expression analyses in malformed skeletal structures of gilthead sea bream (*Sparus aurata*). *Journal of Fish Diseases*, *42*(8), 1169–1180. https://doi.org/10.1111/jfd.13019

Riera-Heredia, N., Vélez, E. J., Gutiérrez, J., Navarro, I., & Capilla, E. (2019b). Gene expression analyses in malformed skeletal structures of gilthead sea bream (*Sparus aurata*). *Journal of Fish Diseases*, *42*(8), 1169–1180. https://doi.org/10.1111/jfd.13019

Rodgers, B. D., Weber, G. M., Kelley, K. M., & Levine, M. A. (2003). Prolonged fasting and cortisol reduce myostatin mRNA levels in tilapia larvae; short-term fasting elevates. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, *284*(5 53-5). https://doi.org/10.1152/ajpregu.00644.2002

Rønnestad, I., Yúfera, M., Ueberschär, B., Ribeiro, L., Sæle, Ø., & Boglione, C. (2013). Feeding behaviour and digestive physiology in larval fish: Current knowledge, and gaps and bottlenecks in research. *Reviews in Aquaculture*, *5*(1). https://doi.org/10.1111/raq.12010

Sanatamaría, J. A., Andrades, J. A., Herráez, P., Fernández-Llebrez, P., & Becerra, J. (1994). Perinotochordal connective sheet of gilthead sea bream larvae (*Sparus aurata* , L.) affected by axial malformations: An histochemical and immunocytochemical study. *The Anatomical Record*, *240*(2), 248–254. https://doi.org/10.1002/ar.1092400212

Sawada, Y., Honryo, T., Agawa, Y., & Kurata, M. (2018). Teratogenic effects of isolated and combined short-term hypercapnia and hypoxia on red sea bream (*Pagrus major*) embryos. *Aquaculture Research*, *49*(9), 3176–3186. https://doi.org/10.1111/are.13781

Sfakianakis, D. G., Georgakopoulou, E., Papadakis, I. E., Divanach, P., Kentouri, M., & Koumoundouros, G. (2006). Environmental determinants of haemal lordosis in European sea bass, *Dicentrarchus labrax* (Linnaeus, 1758). *Aquaculture*, *254*(1–4), 54–64. https://doi.org/10.1016/j.aquaculture.2005.10.028

Sfakianakis, D. G., Koumoundouros, G., Divanach, P., & Kentouri, M. (2004). Osteological development of the vertebral column and of the fins in *Pagellus erythrinus* (L. 1758). Temperature effect on the developmental plasticity and morpho-anatomical abnormalities. *Aquaculture*, *232*(1–4), 407–424. https://doi.org/10.1016/j.aquaculture.2003.08.014

Swain, P., & Nayak, S. K. (2009). Role of maternally derived immunity in fish. In *Fish and Shellfish Immunology*, 27(2), 89–99. https://doi.org/10.1016/j.fsi.2009.04.008

Tanaka, M., Tanangonan, J. B., Tagawa, M., de Jesus, E. G., Nishida, H., Isaka, M., Kimura, R., & Hirano, T. (1995). Development of the pituitary, thyroid and interrenal glands and applications of endocrinology to the improved rearing of marine fish larvae. *Aquaculture*, *135*(1–3), 111–126. https://doi.org/10.1016/0044-8486(95)01019-X

Vadstein, O., Bergh, Ø., Gatesoupe, F. J., Galindo-Villegas, J., Mulero, V., Picchietti, S., Scapigliati, G., Makridis, P., Olsen, Y., Dierckens, K., Defoirdt, T., Boon, N., De Schryver, P., & Bossier, P. (2013). Microbiology and immunology of fish larvae. *Reviews in Aquaculture*, *5*(1). https://doi.org/10.1111/j.1753-5131.2012.01082.x

Vagner, M., Robin, J. H., Zambonino Infante, J. L., & Person-Le Ruyet, J. (2007). Combined effects of dietary HUFA level and temperature on sea bass (*Dicentrarchus labrax*) larvae development. *Aquaculture*, *266*(1–4), 179–190. https://doi.org/10.1016/j.aquaculture.2007.02.040

Valente, L. M. P., Moutou, K. A., Conceição, L. E. C., Engrola, S., Fernandes, J. M. O., & Johnston, I. A. (2013). What determines growth potential and juvenile quality of farmed fish species? *Reviews in Aquaculture*, *5*(1). https://doi.org/10.1111/raq.12020

Villeneuve, L. A. N., Gisbert, E., Moriceau, J., Cahu, C. L., & Infante, J. L. Z. (2006). Intake of high levels of vitamin A and polyunsaturated fatty acids during different developmental periods modifies the expression of morphogenesis genes in European sea bass (*Dicentrarchus labrax)*. *British Journal of Nutrition*, *95*(4), 677–687. https://doi.org/10.1079/bjn20051668

Zapata, A., Diez, B., Cejalvo, T., Gutiérrez-De Frías, C., & Cortés, A. (2006). Ontogeny of the immune system of fish. *Fish and Shellfish Immunology*, *20*(2), 126–136. https://doi.org/10.1016/j.fsi.2004.09.005

Zrncic, S. (2020). Diagnostic Manual for the main pa-thogens in European seabass and Gilthead seabream aquaculture OPTIONS méditerranéennes SERIES B*: Studies and Research*. www.iamz.ciheam.org

Annex 1: Supplementary Information

***Table 2**- Literature used for the screening of the candidate genes for larval quality in European sea bass.*

Specie	File name	Stage	Process	Treat	Tissue/Organ	Gene
European sea bass	Azeredo, R, et al_2015	juvenille	immune system	diets	spleen, gut	GATM, SMOX, CD33, CCR3, AMD1 IL-10, IL-20
European sea bass	Azeredo, R, et al_2017	juvenille	immune system	prebiotics diets	gut	MMP9, TCR, IL10, ARG1, ARG2, CCR3, GATM, SMOX
European sea bass	Babbucci, M, et al_2016	larvae, juveniles	skeletal development	-	lower jaws, cranial regions	Zic2, Sobp
European sea bass	Blázquez, M et al_2009	eggs, larvae, juvenilles	ontogenesis, dif sexual	temperature	-	cyp19a1a, cyp11b, arb, era, erb1 and erb2
European sea bass	Bogevik.A.S et al_2014	120g	fish performance /sex maturation	diets	gonad , brain	Cyp11a, Cyp19a, Cyp19b, FSHβ, LHβ, GnRH, Kiss1, Kiss2, Kissr4
European sea bass	Calduch-Giner, J_2016	adult 200g	instestine	intestine	gut	transcriptome
European sea bass	Crespel, A et al_2017	larvae	skeletal development, poluition	poluition co2	skeletal	ostc, CA2 and CA4 Slc9a1, Slc9a3, Slc4a4, Slc4a1
European sea bass	Ferraresso, S et al_2010	-	skeletal development	-	skeletal	check table in article
European sea bass	Ferreira. M et al_2014	200–300g	immune system	-	primary hepatocytes	CYP1A and ABCC1 and ABCC2
European sea bass	Gonzalez-rovira, A et al_2009	...	lack of genes	diets	brain	Δ6 FAD
European sea bass	Guardiola, FA, et al_2016	50g	immune system	diets	head-kidney and anterior gut	rbl ,sod , il-1b and hep genes, lyz
European sea bass	Herrera-Perez, P et al_2011	juvenille 40-100g	physiological and behavioural processes	-	pineal organ	Melatonin Opsin
European sea bass	Kaitetzidou.E, et al_2015	Egg, larvae	fish development	-	eggs and larvae	Transcriptome sequencing, miRNAs
European sea bass	Mitter, K et al_aug2009, Mitter, K et al_nov2009	embryos/larvae	diets immune system /ontogenesis	-	all body	Ef-1a and L13a , Fau and L13a
European sea bass	Montero.D, et al 2015	larvae	Stress	diets	whole larvae	ARA, C-FOS , STAR, CYP11b, HSP70, GR, HS90, SOD, CAT, GPX ARA ,CAT ,COX , CYP11β , DHA , EPA , GH , GLC ,GPX GR , HSP , HPI , IGF , LC-PUFA , PIn , PGE , SOD , StAR
European sea bass	Pozo.A et al_2012	400g	Circadian rhythm		whole brain	cry1 and 2
European sea bass	Pozo.A et al_jan2012	100g	Daily rhythms	diurnal/nocturnal	Brain, liver and mid-intestine	per1 and β-actin
European sea bass	Sarropoulou.E_2009	-	immune system	A:V. Anguillarum and B: nodovirus ifection	liver, spleen, head kidney, gill, peritoneal exudates and intestine brain	cDNA libraries
European sea bass	Sarropoulou.E_2010	Embryo, larvae	immune system	V. anguillarum, with Nodavirus and control samples	Embryonic and larval samples	Wap65-1 and Wap65-2
European sea bass	Schaeck, M_2016	larvae	reference genes	-	intestine	ef1a, rpl13a, rps18 and faua
European sea bass	Seemann.F et al 2016	larvae juvenille	immune system	E2	head kidney	Era, Erb1, Erb2 . IL-1β, IL-6, TNF-α and TGF-β
European sea bass	Torre.C.D et al_2012	juvenille	immune system	Crude oil and mesocosm exposure	liver samples	cyp1a
European sea bass	Vagner.M, et al 2007	larvae	Growth performances, skeletal development, larvae development	effects of dietary	lipase gene, Δ6D gene ,PLA2, PPAR α and PPAR β gene	HUFA, high unsaturated fatty acids
European sea bass	Viegas.I et al_2013	218g	diets	effects of dietary	blood	G6Pase
European sea bass	Viegas.I et al_2014	-	DIETS	Refeeding Food deprivation	liver samples	PK , mALT, cALT; G6PDH, 6PGDH
European sea bass	Zarski et al_2017	eggs	egg developmental	-	eggs	rnf213, irf7, usp5, plec, cenpf
European sea bass	Darias et al 2008	larvae	ontogeny	Larval Development	larvae	check table in article

***Table 2**- Literature used for the screening of the candidate genes for larval quality in gilthead sea bream.*

Specie	File name	Stage	Process	Treat	Tissue/Organ	Gene
Gilthead sea bream	Abalos.M, et al 2008	juvenile	growth	Dietary bioaccumulation	liver	AhR:, Actin, CYP4501A1
Gilthead sea bream	Acerete.L, et al 2007	65g	imune system	lipopoly ccharide (LPS) treatment.	several tissues	IL-1β, TNF-α, Mx protein, cathepsin D PPAR-γ
Gilthead sea bream	Alves.R.N, et al 2010	376g	stress	repeated handling and crowding at high stocking densit	-	several genes, consult in article
Gilthead sea bream	Astola.A , et al 2004	-	Genomic Structure and Functional Analysis	-	blood	somatolactin ,SL Gene
Gilthead sea bream	Awad. E, et al 2015	8g	Immune system	dietary administration of fenugreek	head kidney and liver	MHC1, CSF-1R, IL-8, and IgM)
Gilthead sea bream	Bahi.A , et al 2017	13.32g	Immune system	dietary administration of fenugreek+probiotics	head-kidney	igm, tcr-b, csfr1 and bd)
Gilthead sea bream	Balmaceda-Aguilera et al _2012	100–150 g	salinity acclimation	environmental linities	gills, kidney, liver and brain	GLUT, GLUT1
Gilthead sea bream	Bermejo-Nogales.A, et al 2014	2 year	Antioxidant activity	Feed restriction	several tissues	UCP1, UCP2, UCP1–3, Cox4a, ACTB
Gilthead sea bream	Cabalero-Solares.A, et al 2015	juvenilles, 3,5g	fish metabolism	diets	blood and liver	GK, PFK-1, PK, FBPase-1, G6PDH, ACC1, cALTI, Cast, GDH
Gilthead sea bream	Calduch-Giner.J.A, et al 2013	-	Transcriptome	-	liver, gills, brain, intestine, head kidney, adipose tissue	several genes, consult in article
Gilthead sea bream	Campinho_GCEgoitrogens_2012	50–60 g	goitrogenic effect.	MMI, PTU and TU VIA DIETS	whole pituitaries, liver and kidney	D1, D2, TSHb, TTR, TRb
Gilthead sea bream	Carnevali.O, et al 2005	700G	Reprodution	hormones	liver	IGF-I, IGF-II
Gilthead sea bream	Castellana.B, et al 2008	150g	imune system	no	several tissues	sbIL-6
Gilthead sea bream	Castillo-Briceno.P, et al 2009	800g	imune system	colagen	head kidney	il1b, tnfa, cox2 ccl4 tgfb1 il6 il1r2 mmp9 mmp13 timp2a timp2b
Gilthead sea bream	Castillo-Briceno.P, et al 2010	150g	imune system	virus	several tissues	mmp2 mmp9 mmp13 timp2a timp2b col1a1 itgb1a
Gilthead sea bream	Castillo-Briceno.P, et al 2011	...	imune system, inflamation	lesion	caudal fin fibroblasts	GFOGER and GLOGEN promote , I, COL-II and COL-III and for COL-II-51
Gilthead sea bream	Cerezuela.Rebeca, et al 2012.1	100g	imune system	diets microalgae	and blood, head-kidney (HK) and digestive tract	EF-1a IgMH TCR-b MHCIa MHCIIa CSF-1R b-def
Gilthead sea bream	Cerezuela.Rebeca, et al 2012.2	50g	imune system, disiease resistance	bacteria	nd blood and head-kidney (HK	IgMH EF-1a TCRb MHCIa MHCIIa CSF-1R b-def
Gilthead sea bream	Codina.M et al 2008	…	Muscle	-	muscle and liver	GFP
Gilthead sea bream	Conceição.N et al 2008	...	Functional analysis of promoter	-	...	MGP
Gilthead sea bream	Cordero et al_2016	92g	imune system	bacteria	skin	(il1b, tnfa, il6, il7, il8, il15, il18, il10 and tgfb
Gilthead sea bream	Cordero, Guardiola et al _2015	42g	imune system	bacteria	blood, head-kidney (HK)	ef1a il1b bd mhcIIa ighm tcrb
Gilthead sea bream	Cordero, Morcillo et al _2016	100g	imune system	bacteria	skin	il1b, tnfa, il6, il7, il8, il15, il18, il10 and tgfb
Gilthead sea bream	Costas.B, et al 2014	112g	imune system	purified antinutrients	blood	casp1 il18 il10 csfr hep def Ig ef1a
Gilthead sea bream	Cuesta.A, et al 2003	125g	imune system	-	head–kidney, thymus, spleen, blood and peritoneal exudate	mFasL,
Gilthead sea bream	Cuesta.A, et al 2005	125g	imune system	cytotoxic cells	several tissues	NCCRP-1
Gilthead sea bream	Cuesta.A, et al 2006	125g	imune system	-	several tissues	several genes, consult in article
Gilthead sea bream	Cuesta.A, et al 2008.1	100-150g	imune system	imunoestimulation	Serum and leucocytes from the head-kidney (HKLs) and peritoneal exudate (PELs)	several genes, consult in article
Gilthead sea bream	Cuesta.A, et al 2008.2	108g	imune system	pesticides	head-kidney (HK),	several genes, consult in article

Gilthead sea bream	Cuesta.A, et al 2008.3	198g	imune system	melatonin	leucocyte from HK	several genes, consult in article
Gilthead sea bream	Cuesta.A, et al 2008.4	50-100g	imune system	ODN	leucocyte from HK	several genes, consult in article
Gilthead sea bream	Cuesta.A, et al 2008.5	adult	imune system	bacteria	Brain, skin, liver, gut, gills, head-kidney (HK), spleen thymus	hep, csf1r, igmh
Gilthead sea bream	Cuesta.A, et al 2010	adult	imune system	virus	Head kidney leukocytes	CCL4, CK
Gilthead sea bream	Cuesta.A, et al 2011	150-250g	imune system	bacteria	several tissues	BD
Gilthead sea bream	Feidantis.K, et al 2009	300g	stress	temperatures	heart and skeletal muscle	MAPK
Gilthead sea bream	Fernandez.I, et al 2014	...	skeletal development	retinoid acid	cell lines	several genes, consult in article
Gilthead sea bream	Fernandez-Trujillo.M.A, et al 2011	...	imune system	(IPNV) virus	plasmidss	VP1, VP5, Mx 1, 2, 3
Gilthead sea bream	Fernandez-Trujillo.M.A, et al 2011.2	60g	imune system	VIRUS	brain, head kidney, liver and spleen	Mx GENES
Gilthead sea bream	Fonseca.V.G, et al 2007	....	mineralization	VNNV infection	osteoblast-like cell line	several genes, consult in article
Gilthead sea bream	Fonseca.V.G, et al 2011		mineralization	-	-	S100-like protein
Gilthead sea bream	Funkenstein, B, et al 2000	80g	Reprodution	E2	LIVER	VTG, TTR
Gilthead sea bream	Mohammed et al_2016	larvae/post larvae	growth	linity exposures	all body	prl, adcyap1, and igf1
Gilthead sea bream	Pellizzari et al_2013	juvenille	imune system	photobacteriosis	head kidney	ADEN DEAM, ANN 3, ARG II, BACTIPP, C-X-C, CHEM, IL 10, MMP9, NEPHR, ORN DEC, SOCS3.
Gilthead sea bream	Tiago, et al 2014	-	skeletal development	-	cells line of branchial arches	BMPR2
Gilthead sea bream	Viegas_et_al_2012	-	skeletal development	-	skeletal cell line	RPL27a, bACT, COL1A1, OP, TNAP, ON, RUNX2 COX2, BMP2, MGP
Gilthead sea bream	Garcia-Castilho, J.et al 2002	150g	imune system	Phylogeny	HK	TNFα, β -actin F, IL-1
Gilthead sea bream	Garcia-Fernandez.C, et al 2011	adult	imune system	Characterization	EYE	TF GENE
Gilthead sea bream	Gonzales.N.E, et al 2017	200g	imune system	histamine	BLOOD	il1b, il10, il4/il13, hrh
Gilthead sea bream	Gonzalez-Mariscal.J.A, et al 2014	-	imune system	Mx PROTEIN	fin clips	Mx PROTEIN
Gilthead sea bream	Gonzalez-Stegmaier.R, et al 2015	-	imune system	Peptide synthesis		Flagelin
Gilthead sea bream	Guardiola et al_2017	13,3g	antioxidant response	fenugreek diet	muscle	ef1a, sod, cat, gr
Gilthead sea bream	Guardiola.F.A, et al 2011	100g	imune system	f 2-deoxy-D-glucose	blood, HK leucocytes	CSF-1R, NCCRP-1, Hep, TCR-b, IgMH, MHC-IIa C3, IL-1b
Gilthead sea bream	Guardiola.F.A, et al 2012	65g	imune system	oxytetracycline, antibiotic	head-kidney and gut	Ef-1α, MHC-Iiα, C3, IL-1β, COX-2
Gilthead sea bream	Guardiola.F.A, et al 2013	104g	imune system	arsenic	leucocyte from HK	igM
Gilthead sea bream	Guardiola.F.A, et al 2014	-	imune system	Deltamethrin	Head-kidney (HK) and liver	EF1a, SOD, CAT, GR, CYP1A1, HSP70
Gilthead sea bream	Hamed.S, et al 2017	12g	imune system	pollutrents	Head kidney, liver and skin	several genes, consult in article
Gilthead sea bream	Hampel.M, et al 2017	-	Transcriptome	human pharmaceuticals	brain	
Gilthead sea bream	Hernandez-cruz.C.M, et al 2015	larvae	Immune system	DHA	Larvae	several genes, consult in article
Gilthead sea bream	Herrero-Turrion,M.J, et al 2003.1	embryos, larvae, juveniles, adults	ontogenic development	grow hormone	embryos, larvae	several genes, consult in article

Gilthead sea bream	Herrero-Turrion,M.J, et al 2003.2	embryos , larvae	development	expresssion PROLACTIN	embryos 30dpf, larvae 1-13d	several genes, consult in article
Gilthead sea bream	Izquierdo.M.S, et al 2008	larvae	grow performance	dietery lipids	larvae	D6 desaturase, b-actin:
Gilthead sea bream	Izquierdo.M.S, et al 2015	larvae	Spawning and egg quality	dietary,	larvae	Gen Δ6 desaturase
Gilthead sea bream	Kaitetzidou.E, et al 2012	47g	Skeletal muscle .imune system	Lipopolysaccharide	white and red muscles, as well as spleen	several genes, consult in article
Gilthead sea bream	Lopez-Castejon.G, et al 2008	-	imune system	-	head-kidney leukocytes	caspase-1
Gilthead sea bream	Maccatrozzo, L., et al 2002	1-year-old seabream	Skeletal muscle	Characterization of MSTN	skeletal muscle	MSTN, GDF-8
Gilthead sea bream	Malandrakis, et al 2014	63g	dna damage	STRESS	liver	GPx
Gilthead sea bream	Malandrakis.E.E , et al 2016	93g	dna damage	STRESS	liver	P53f, GR, Gck , IG, Nr3c
Gilthead sea bream	Martins.D.A, et al 2010	larvae	growth	Lecitin diets	Larvae	GH, POMC, CB1 receptor COX-2, PKC, GR, HSL, FBPase
Gilthead sea bream	Martins.D.A, et al 2011	larvae	stress response	diets	larvae	b-Actin, StAR, GR, PPARa, PLA2, COX-2 5-LOX, HSL, PEPCK
Gilthead sea bream	Martos-Sitcha, et al_2014	213g	stress response	stressors	plasma	CRH
Gilthead sea bream	Mata-Sotres.J.A et al 2015	larvae	larval development	photoperiodo, food intake	larvae	clock, cry1, bmal1, per3, actb
Gilthead sea bream	Mata-Sotres.J.A et al 2016.1	larvae	ontogeny, digestive enzymes	photoperiodo, food intake	gut	try, ctrb, cel1b, pla2, amy2a, actb
Gilthead sea bream	Mata-Sotres.J.A et al 2016.2	larvae	ontogeny, digestive enzymes	food deprived	larvae	try, cel, amy2a, ctrb, pla2, actb
Gilthead sea bream	Meton,I., et al 2003	19g	strarvations	refeeding	liver	PFK-1,+E74:I75 PK, G6P-DH
Gilthead sea bream	Mohammed-Geba.K , et al 2015	150-180g	endocrine	salinities changes	hypothalamus and liver	PACAP, PSS-I, GH, PRL, IGF-I
Gilthead sea bream	Munoz.A.L, et al 2012	30-40g	imune system	virous	brain and head-kidney	tgfb1, ptgs2, tnfa, il1b
Gilthead sea bream	Munoz.I, et al 2013	-	imune system	Molecular Characterization TLR5	tissues or cell pellets	sbTLR5s,
Gilthead sea bream	Munoz.I, et al 2014	300g	imune system	Molecular Characterization TLR22	Head–kidney leucocytes	sbTLR22,
Gilthead sea bream	Murelo.I, et al 2008	larvae	imune system	vacination	larvae	several genes, consult in article
Gilthead sea bream	P.Pinto, et al _2006.1	359g	Reprodution	estrogen	blood ans testis	several genes, consult in article
Gilthead sea bream	P.Pinto, et al _2006.2	3kg, female and 400g male	Reprodution	Molecular Characterization Erbb	pituitary	Erbb, Erba, Era
Gilthead sea bream	Panserat, S., et al 2000.1	150G	HOMEOSTASIS	Molecular Characterization GK	liver, muscle, heart, kidney, brain	GK
Gilthead sea bream	Panserat, S., et al 2000.2	150G	HOMEOSTASIS	dietary carbohydrates	liver, muscle	GK
Gilthead sea bream	Pelegrin,P., et al 2004	150g	imune system	secretion of IL b1	HK	IL-1b
Gilthead sea bream	Perera.E and Yufera.M 2017	larvae	digestive system , imune system	soya bean meal	larvae	several genes, consult in article
Gilthead sea bream	Perez-sanches.J, et al 2015	26g	imune system	prebiotics	anterior and posterior intestine	several genes, consult in article
Gilthead sea bream	Perez-Sanchez.J, et al 2013	150g	imune system	parasites	in, gills, oesophagus, stomach, anterior (AI), middle (MI) and posterior (PI) intestine	several genes, consult in article
Gilthead sea bream	Pinto_Gene_2012	50g imature , 3kg female, 450g male	Reprodution	Molecular Characterization Era	liver, testis, ovary	ERa
Gilthead sea bream	Pirone.A, et al 2008	adullt	neuropeptide	localization	brain, eye	NPY

Gilthead sea bream	Rafael,M.S, et al 2006	Larvae, juvenile and adult fish culture	Skeletal development	Molecular Characterization	CELL CULTURE	BMP-2
Gilthead sea bream	Rafael.M.S, et al 2012	Eggs, larvae, juvenile and adult fish	Muscle DEVELOPMENT	gene expression	all body	FHL2
Gilthead sea bream	Reyes-Becerril.M, et al 2011	84g	imune system	bacteria infection	hk	several genes, consult in article
Gilthead sea bream	Reyes-Becerril.M, et al 2012	80g	imune system	yeast	m skin, intestine, liver and HK tissue,	several genes, consult in article
Gilthead sea bream	Reyes-Becerril.M, et al 2013	80g	imune system	probiotics	head-kidney (HK) and intestine	several genes, consult in article
Gilthead sea bream	Richard.N, et al 2016	87g	nutricional	thermal stress	liver	BHMT, GNMT, DMGDH, GRHPR, EF1 and 1:10 dilution for H-FABP and HGRP),
Gilthead sea bream	Rocha.F, et al 2006	-	imune system	CSF-1R	GILLS	CSF-1R
Gilthead sea bream	Rocha.F, et al 2016	larvae	Glucose metabolism	diets	LARVAE	several genes, consult in article
Gilthead sea bream	Rosa.J, et al 2014	-	Skeletal development	ETS1		Ets1
Gilthead sea bream	Rosa.J, et al 2016	-	Skeletal development	-	CELL CULTURE	bhmt3 , bhmt4, bhmt5, bhmt7
Gilthead sea bream	Ruggeri.B, et al 2007	-	Reprodution	-	BRAIN AND GONADS	CB1
Gilthead sea bream	Saleh.R et al 2014	larvae	Skeletal development	selenium	larvae	several genes, consult in article
Gilthead sea bream	Saleh.R et al 2015.1	larvae	Skeletal development	krill phospholipids	larave	several genes, consult in article
Gilthead sea bream	Saleh.R et al 2015.2	larvae	Skeletal development	polar lipids	larave 60mg	CAT, SOD Glutathione peroxidase , Osteocalcin Osteopontin, Osteonectin, RUNX2, ALP MGP2, BMP4, b-actin
Gilthead sea bream	Sarropoulou,E., et al 2005	-	early development, stress response	-	Embryonic and larval , kidney	microaaray, candidate genes for stress response
Gilthead sea bream	Seiliez,I., et al 2003	0,5G	nutricional	DIETS	all body	D6-Desaturase
Gilthead sea bream	Sepulcre.M.P, et al 2007	150G	imune system	PAMPS	CELLS pellets	IL-1, TNF, COX-2, TLR5, TLR9, Actin
Gilthead sea bream	Sepulcre.M.P, et al 2011	150G	imune system	-	CELLS pellets	il1b, il6, ptgs2, tlr5s, tlr5m, tlr9, cyba, ncf4 lyz, gcsfr, ighm, ight, mcsfr tcrb, rps18
Gilthead sea bream	Tan, X , et al 2002	embryos and adults	Muscle DEVELOPMENT	-	24-hpf embryos, fast and slow muscles from adult	MyoD1 and MyoD2
Gilthead sea bream	Teles.M, et al 2012	108g	stress	cortisol	several tissues	several genes, consult in article
Gilthead sea bream	ten-tsao wong, et al 2004	-	Reprodution	-	oocytes	several genes, consult in article
Gilthead sea bream	Tiago.D.M, et al 2011	-	Skeletal development	vanadate		genes diferencialy expressed with treat (papper)
Gilthead sea bream	Vera.L.M, et al 2013	adults	circadian system	ligth and feed	brain and livers	(Bmal1, Clock) and negative (Per2, Cry1
Gilthead sea bream	Vieira2012	284g	Skeletal development	RELOXIFENE	dentary and vertebra	Osteonectin,Runx2,Fibronectin 1a,CILP2,IGF1 ALP,TRAP,Era,Erba,Erbb,18S,RPS18,Beta-Actin
Gilthead sea bream	Yufera.M et al 2012	larvae	Transcriptome	-	larvae	Selected transcripts with a relevant role in the post-embryonic development (table)
Gilthead sea bream	Yufera.M et al 2014	1g	circadian system	feeding frequency	-	QPEP-F, QPP-F, QACTB-F
Gilthead sea bream	Zapater.C, et al 2013	-	eggs phenotype	-	ovaries	aqp1ab
Gilthead sea bream	Zilberman-Peled.B, et al 2004	adults	circadian system	characterization of AANAT	pineal glands and retinas	sbAANAT1 and sbAANAT2
Gilthead sea bream	Ziv.T, et al 2008	adults	development	proteomics oocytes	oocytes	vitellogenin 1-7

Table 3- *List of the primers used for gene quality candidate genes for expression analysis by quantitative Real-Time PCR. The specie, pathway, protein, gene codes, accession numbers, primer sequences, amplicon sizes (bp), annealing temperatures (Ta, ◦C), qPCR efficiencies (%) and R2 are indicated for each gene.*

Species	Patway	Protein	Gene code	Acession nº	FW primer	RV primer	Ta,ºC	Amplicon (bp)	qPCR eff %	R^2
Dicentrarchus labrax	**Immune system**	Warm-temperature-acclimation-related-65kDa-proteinorhemopexin	**WAP65**	BK006868.1	ATCAAACTCAATGCCTTCACACC	AGCACTCGCCCTCACTAATGG	**58**	97	90,3	1,00
Dicentrarchus labrax	**Lipidic metabolism**	ApolipoproteinA1	**ApoA1**	EU909391.1	CTGGAGAGCCTGAGAGCAATGG	TGTTGATGTTCTGAGCCTGGTTGT	**68**	93	90,3	1,00
Dicentrarchus labrax	**Bone related**	Collagen 1α2	**Col1a2**	CX660451	TCGCCCAGAAGAACTGGTACAGAA	CGTTGTAGGTAAACTCAGTACCACCG	**62**	93	100,6	1,00
Dicentrarchus labrax	**Endocrine system**	Fibrinogenbetachain(fgb)	**FGB**	DLAgn_00214270	ACCGTCCGCTATCAAGAGG	CTTCTCCTGTGCCTGTGGTC	**62**	131	98,6	0,98
Dicentrarchus labrax	**Bone related**	3-beta-hydroxysteroid-Delta(8)	**EBP**	DLAgn_00130480	CCACCTATGTTGCCAATGACC	AACCAGCCCTCAATGACACC	**62**	196	95,5	0,98
Dicentrarchus labrax	**Protein metabolism**	Trypsin	**TRY**	AJ006882	CTCCCTGGTCAACGAGAACT	ACCCTGATGTTGTGCTCTCC	**62**	90	111,5	0,99
Dicentrarchus labrax	**Lipidic metabolism**	high unsaturated fatty acids (HUFA)-Phospholipase A2	**PLA2**	AJ132762.1	TCCTGTGTGTGATGCCTGAT	TCTCGTCGCAGTTGTAGTCG	**62**	212	101,1	1,00
Dicentrarchus labrax	**Immune system**	Transferin(trf)	**TRF**	FJ197144.1	ACACTGCTGGACTGAACAACTACGA	GGATTTCTTCCCGCTGAGGT	**60**	146	103,2	0,99
Dicentrarchus labrax	**Bone related**	Collagen, type X, alpha 1	**COL10A1**	DLAgn_00023820	TGGGAATGAGTGAGGTTATGG	GGATGCTGTAGGCAAAATAGT	**60**	192	105,5	0,98
Dicentrarchus labrax	Ribosome	no description	**FAU**	ENSDLAG00005000664	CTTCGTGAATGTTGTGCC	ACTGATGGATGGTGATGA	**49**	103	102,9	-
Dicentrarchus labrax	Ribosome	ribosomal protein L13a	**RPL13**	ENSDLAG00005000624	GAAGGCATCAACATCTCC	CTCTGAAGTGGTAAGGTC	**48**	109	102,0	
Sparus aurata	**Bone related**	Alkaline phosphatase	**ALP**	AY266359	CTGCCGTCCGTTCCCAGTGTA	CTCATTGTCGGAGTACCAGTC	**57**	176	108,9	0,99
Sparus aurata	**Cells strcuture**	Cadherin	**CDH**	XM_030414763.1	GGCCTTCGTGGGTAGATAGG	TTTAACGTCGACCTGCTGTG	**60**	149	108,0	0,98
Sparus aurata	**Bone related**	Collagenase	**Col1A1**	DQ324363	GAGATGGCGGTGATGTGGCGGAGTC	GCCTGGTTTGGCTGGATGAAGAGGG	**68**	214	104,3	0,99

Sparus aurata	**Metabolism**	Glucokinase	**GK**	AF169368	TGTGTCAGCTCTCAACTCGACC	AGGATCTGCTCTACCATGTGGAT	**60**	89	92,8	0,91
Sparus aurata	**Metabolism**	Pyruvate kinase	**PK**	KF857579	CAAAGTGGAAAGCCGGCAAGGG	GTCGCCCCTGGCAACCATAAC	**60**	82*	109,4	0,99
Sparus aurata	**Bone related**	Osteopontin	**OP**	AY651247	AAAACCCAGGAGATAAACTCAAGACAACCCA	AGAACCGTGGCAAAGAGCAGAACGAA	**68**	190	94,0	0,99
Sparus aurata	**Bone related**	Osteonectin	**ON**	AY239014	AGGAGGAGGTCATCGTGGAAGAGCC	GTGGTGGTTCAGGCAGGGATTCTCA	**68**	170	100,0	1,00
Sparus aurata	**Bone related**	Osteocalcin	**OSTC**	AF048703.1	TCCGCAGTGGTGAGACAGAAG	CGGTCCGTAGTAGGCCGTGTAG	**64**	150	103,9	0,99
Sparus aurata	**Immune system**	Retinol-binding protein I, cellular	**RBP**	P82980	TCCGCACCATAACCACCTTCAAG	CCAGCCTCGTCCTTCCTTCTCC	**60**	168	117,6	0,91
Sparus aurata	Ribosome	ribosomal protein L13a-HK	**RPL13**	ENSSAUG00010003114	TCTGGAGGACTGTCAGGGGCATGC	AGACGCACAATCTTAAGAGCAG	**65-56**	148	101,4	-
Sparus aurata	Ribosome	40S ribosomal protein S18	**RPS18**	ENSSAUG00010000811	AGGGTGTTGGCAGACGTTAC	GAGGACCTGGCTGTATTTGC	**56**	197	103,0	-

***Table 4**- qPCR conditions: master mix solution, qPCR detection system, thermocycle program, melting curve and cDNA quantity are indicated for each gene.*

Species	Gene code	Master mix	qPCR machine	Thermo Cycle	Melting curve	Cdna (ng)
Dicentrarchus labrax	**WAP65**	Forget-Me-Not™ EvaGreen® qPCR Master Mix, Biotium, USA	CFX384 C1000 Touch™ Real-Time PCR Detection System (Bio-Rad, USA)	95ºC- 30``+95ºC- 5``+TA - 30`` , 40 cycles	60ºC- 10``+ 95ºC- 5``	2,5
Dicentrarchus labrax	**ApoA1**	Forget-Me-Not™ EvaGreen® qPCR Master Mix, Biotium, USA	CFX384 C1000 Touch™ Real-Time PCR Detection System (Bio-Rad, USA)	95ºC- 30``+95ºC- 5``+TA - 30`` , 40 cycles	60ºC- 10``+ 95ºC- 5``	2,5
Dicentrarchus labrax	**Col1a2**	Forget-Me-Not™ EvaGreen® qPCR Master Mix, Biotium, USA	CFX384 C1000 Touch™ Real-Time PCR Detection System (Bio-Rad, USA)	95ºC- 30``+95ºC- 5``+TA - 30`` , 40 cycles	60ºC- 10``+ 95ºC- 5``	2,5
Dicentrarchus labrax	**FGB**	Forget-Me-Not™ EvaGreen® qPCR Master Mix, Biotium, USA	CFX384 C1000 Touch™ Real-Time PCR Detection System (Bio-Rad, USA)	95ºC- 30``+95ºC- 5``+TA - 30`` , 40 cycles	60ºC- 10``+ 95ºC- 5``	2,5
Dicentrarchus labrax	**EBP**	Forget-Me-Not™ EvaGreen® qPCR Master Mix, Biotium, USA	CFX384 C1000 Touch™ Real-Time PCR Detection System (Bio-Rad, USA)	95ºC- 30``+95ºC- 5``+TA - 30`` , 40 cycles	60ºC- 10``+ 95ºC- 5``	2,5
Dicentrarchus labrax	**TRY**	Forget-Me-Not™ EvaGreen® qPCR Master Mix, Biotium, USA	CFX384 C1000 Touch™ Real-Time PCR Detection System (Bio-Rad, USA)	95ºC- 30``+95ºC- 5``+TA - 30`` , 40 cycles	60ºC- 10``+ 95ºC- 5``	2,5
Dicentrarchus labrax	**PLA2**	Forget-Me-Not™ EvaGreen® qPCR Master Mix, Biotium, USA	CFX384 C1000 Touch™ Real-Time PCR Detection System (Bio-Rad, USA)	95ºC- 30``+95ºC- 5``+TA - 30`` , 40 cycles	60ºC- 10``+ 95ºC- 5``	2,5

Dicentrarchus labrax	**TRF**	Forget-Me-Not™ EvaGreen® qPCR Master Mix, Biotium, USA	CFX384 C1000 Touch™ Real-Time PCR Detection System (Bio-Rad, USA)	95ºC- 30``+95ºC- 5``+TA -30`` , 40 cycles	60ºC- 10``+ 95ºC- 5``	2,5
Dicentrarchus labrax	**COL10 A1**	Forget-Me-Not™ EvaGreen® qPCR Master Mix, Biotium, USA	CFX384 C1000 Touch™ Real-Time PCR Detection System (Bio-Rad, USA)	95ºC- 30``+95ºC- 5``+TA -30`` , 40 cycles	60ºC- 10``+ 95ºC- 5``	2,5
Dicentrarchus labrax	**FAU**	KAPA SYBR® FAST qPCR Master Mix (2X)	StepOne™ Real-Time PCR System (Thermo)	95ºC- 5`+95ºC- 20``+TA -20`` , 40 cycles	95ºC- 15``+ 50ºC- 1`+ 95ºC- 15``	2,5
Dicentrarchus labrax	**RPL13**	KAPA SYBR® FAST qPCR Master Mix (2X)	StepOne™ Real-Time PCR System (Thermo)	95ºC- 5`+95ºC- 20``+TA -20`` , 40 cycles	95ºC- 15``+ 50ºC- 1`+ 95ºC- 15``	2,5
Gilthead Sea bream	**ALP**	**Sso Fast** EvaGreen supermix, Bio-Rad Laboratories, USA	CFX384 C1000 Touch™ Real-Time PCR Detection System (Bio-Rad, USA)	95ºC- 30``+95ºC- 5``+TA -30`` , 40 cycles	60ºC- 10``+ 95ºC- 5``	2,5
Gilthead Sea bream	**CDH**	**Forget-Me-Not**™ EvaGreen® qPCR Master Mix, Biotium, USA	CFX384 C1000 Touch™ Real-Time PCR Detection System (Bio-Rad, USA)	95ºC- 30``+95ºC- 5``+TA -30`` , 40 cycles	60ºC- 10``+ 95ºC- 5``	2,5
Gilthead Sea bream	**Col1A1**	**Forget-Me-Not**™ EvaGreen® qPCR Master Mix, Biotium, USA	CFX384 C1000 Touch™ Real-Time PCR Detection System (Bio-Rad, USA)	95ºC- 30``+95ºC- 5``+TA -30`` , 40 cycles	60ºC- 10``+ 95ºC- 5``	2,5
Gilthead Sea bream	**GK**	**Sso Fast** EvaGreen supermix, Bio-Rad Laboratories, USA	CFX384 C1000 Touch™ Real-Time PCR Detection System (Bio-Rad, USA)	95ºC- 30``+95ºC- 5``+TA -30`` , 40 cycles	60ºC- 10``+ 95ºC- 5``	2,5
Gilthead Sea bream	**PK**	**Sso Fast** EvaGreen supermix, Bio-Rad Laboratories, USA	CFX384 C1000 Touch™ Real-Time PCR Detection System (Bio-Rad, USA)	95ºC- 30``+95ºC- 5``+TA -30`` , 40 cycles	60ºC- 10``+ 95ºC- 5``	2,5
Gilthead Sea bream	**OP**	**Forget-Me-Not**™ EvaGreen® qPCR Master Mix, Biotium, USA	CFX384 C1000 Touch™ Real-Time PCR Detection System (Bio-Rad, USA)	95ºC- 30``+95ºC- 5``+TA -30`` , 40 cycles	60ºC- 10``+ 95ºC- 5``	2,5
Gilthead Sea bream	**ON**	**Forget-Me-Not**™ EvaGreen® qPCR Master Mix, Biotium, USA	CFX384 C1000 Touch™ Real-Time PCR Detection System (Bio-Rad, USA)	95ºC- 30``+95ºC- 5``+TA -30`` , 40 cycles	60ºC- 10``+ 95ºC- 5``	2,5
Gilthead Sea bream	**OSTC**	**Sso Fast** EvaGreen supermix, Bio-Rad Laboratories, USA	CFX384 C1000 Touch™ Real-Time PCR Detection System (Bio-Rad, USA)	95ºC- 30``+95ºC- 5``+TA -30`` , 40 cycles	60ºC- 10``+ 95ºC- 5``	2,5
Gilthead Sea bream	**RBP**	**Sso Fast** EvaGreen supermix, Bio-Rad Laboratories, USA	CFX384 C1000 Touch™ Real-Time PCR Detection System (Bio-Rad, USA)	95ºC- 30``+95ºC- 5``+TA -30`` , 40 cycles	60ºC- 10``+ 95ºC- 5``	2,5
Sparus aurata	**RPL13**	KAPA SYBR® FAST qPCR Master Mix (2X)	StepOne™ Real-Time PCR System (Thermo)	95ºC- 5`+95ºC- 20``+TA -20`` , 40 cycles	95ºC- 15``+ 50ºC- 1`+ 95ºC- 15``	2,5
Sparus aurata	**RPS18**	KAPA SYBR® FAST qPCR Master Mix (2X)	StepOne™ Real-Time PCR System (Thermo)	95ºC- 5`+95ºC- 20``+TA -20`` , 40 cycles	95ºC- 15``+ 50ºC- 1`+ 95ºC- 15``	2,5

***Table 5**- Primer screen strategy for dicentrachus labrax specie, all steps from all genes available until the step before final gene selection which is showed in table 1.*

Step 1 -all primers available			**Step 2**-Worked in larvae (big pool mix of all stages) and <35cycles			**Step 3**- qPCR Cdna big pool <29cycles			**Step 4**- Best vs Poor of ***Dicentrachus labrax***																			
									DL_ELR									DL_MM												
IN-HOUSE	ACAN	Bone related	IN-HOUSE	ACAN	Bone related	IN-HOUSE	ACAN	Bone related	**Primer sel**	**Patway**	**Gene**	**Comp any**	**G&B DIF**	**+ expressed**	**> 1**	**<29 cts**	**Primer sel**	**Patway**	**Gene**	**Comp any**	**G&B DIF**	**+ expressed**	**> 1**	**<29 cts**
IN-HOUSE	ALP	Bone related	IN-HOUSE	ALP	Bone related	IN-HOUSE	ALP	Bone related																	
IN-HOUSE	ALS	Bone related	IN-HOUSE	ALS	Bone related	IN-HOUSE	ApoA1	lipidic metabolism	IN-HOUSE	Lipidic metabolism	ApoA1	1	1,1	BAD		22,4	IN-HOUSE	lipidic metabolism	ApoA1	AND1	-0,5	GOOD		21,8
IN-HOUSE	ApoA1	lipidic metabolism	IN-HOUSE	ApoA1	lipidic metabolism	IN-HOUSE	CAC1b	Bone related				2	0,9	BAD						AND2	-0,3	BAD		
IN-HOUSE	ARA	Endocrine	IN-HOUSE	ARA	Endocrine	IN-HOUSE	CALCR	Bone related	IN-HOUSE	Endocrine system	CILP2	1	0,6	BAD		26,2				PAN	0,8	BAD		
IN-HOUSE	ARB	Endocrine	IN-HOUSE	AromB	Endocrine	IN-HOUSE	CATK	Bone related				2	0,2	BAD			IN-HOUSE	Endocrine system	CH25H	AND1	1,7	BAD		28,5
IN-HOUSE	AromA	Endocrine	IN-HOUSE	CAC1b	Bone related	IN-HOUSE	CH25H	Endocrine	IN-HOUSE	Bone related	Col10a1	1	0,7	BAD		27,6				AND2	0,9	BAD		
IN-HOUSE	AromB	Endocrine	IN-HOUSE	CALCR	Bone related	IN-HOUSE	CILP2	Endocrine				2	1,6	BAD						PAN	1,4	BAD		
IN-HOUSE	Tect	Endocrine	IN-HOUSE	CATK	Bone related	IN-HOUSE	Col10a1	Bone related	IN-HOUSE	Bone related	Col2a1	1	0,2	BAD		27,2	IN-HOUSE	Endocrine system	CILP2	AND1	0,1	BAD		25,6
IN-HOUSE	CAC1a	Bone related	IN-HOUSE	CH25H	Endocrine	IN-HOUSE	Col2a1_2	Bone related				2	1,7	BAD						AND2	-0,6	GOOD		
IN-HOUSE	CAC1b	Bone related	IN-HOUSE	ChgL	Endocrine	IN-HOUSE	Dio1_2	Endocrine	IN-HOUSE	Endocrine system	EBP	1	-2,4	GOOD		28,6				PAN	0,5	BAD		
IN-HOUSE	CALCR	Bone related	IN-HOUSE	CILP2	Endocrine	IN-HOUSE	Dio2	Endocrine				2	0,7	BAD						AND1	-1,4	GOOD		28,0
IN-HOUSE	CATK	Bone related	IN-HOUSE	Col10a1	Bone related	IN-HOUSE	EBP	Endocrine	IN-HOUSE	Endocrine system	FGB	1	-0,0	GOOD		26,2	IN-HOUSE	Bone related	Col10a1	AND2	1,6	BAD		
IN-HOUSE	CH25H	Endocrine	IN-HOUSE	Col2a1_2	Bone related	IN-HOUSE	FGB	Endocrine				2	0,8	BAD						PAN	-3,2	GOOD		
IN-HOUSE	ChgL	Endocrine	IN-HOUSE	Dio1_2	Endocrine	IN-HOUSE	GR1	Endocrine	IN-HOUSE	Endocrine system	GR1	1	0,2	BAD		28,9				AND1	0,1	BAD		27,3
IN-HOUSE	CILP2	Endocrine	IN-HOUSE	Dio2	Endocrine	IN-HOUSE	H3P	Growth				2	1,1	BAD			IN-HOUSE	Bone related	Col2a1_2	AND2	0,4	BAD		
IN-HOUSE	Col10a1	Bone related	IN-HOUSE	Dio3	Endocrine	IN-HOUSE	IGFBP3a2	Growth	IN-HOUSE	Growth	H3P	1	1,0	BAD		28,8				PAN	0,8	BAD		
IN-HOUSE	Col2a1_1	Bone related	IN-HOUSE	EBP	Endocrine	IN-HOUSE	IGF-II	Growth				2	1,5	BAD						AND1	1,2	BAD		28,2
IN-HOUSE	Col2a1_2	Bone related	IN-HOUSE	ERba*	Endocrine	IN-HOUSE	MCT10	Growth	IN-HOUSE	Growth	IGF-II	1	0,6	BAD		27,4	IN-HOUSE	Endocrine system	EBP	AND2	-1,4	GOOD		
IN-HOUSE	Dio1_1	Endocrine	IN-HOUSE	ERbb	Endocrine	IN-HOUSE	MCT8	Growth				2	1,0	BAD						PAN	2,4	BAD		
IN-HOUSE	Dio1_2	Endocrine	IN-HOUSE	FGA	Endocrine	IN-HOUSE	Mim1	Bone related	IN-HOUSE	Bone related	MyoG	1	0,8	BAD		28,1	IN-HOUSE	Endocrine system	FGB	AND1	1,1	BAD		25,6
IN-HOUSE	Dio2	Endocrine	IN-HOUSE	FGB	Endocrine	IN-HOUSE	MMP9	Bone related				2	1,5	BAD						AND2	0,9	BAD		

IN-HOUSE	Dio3	Endocrine	IN-HOUSE	GH	Growth	IN-HOUSE	MR	Endocrine	IN-HOUSE	Growth	POMCa	1	0,8	GOOD		27,9					PAN	0,8	BAD							
IN-HOUSE	EBP	Endocrine	IN-HOUSE	GR1	Endocrine	IN-HOUSE	MyoG	Bone related				2	0,2	GOOD			IN-HOUSE	Endocrine system	GR1	AND1	0,6	BAD		28,4						
IN-HOUSE	ERaadpt	Endocrine	IN-HOUSE	H3P	Growth	IN-HOUSE	OSX	Bone related	IN-HOUSE	Endocrine system	TRa	1	-0,2	GOOD		27,7				AND2	0,4	BAD							
IN-HOUSE	ERba*	Endocrine	IN-HOUSE	IGFBP3a2	Growth	IN-HOUSE	POMCa	Growth				2	0,8	BAD						PAN	1,5	BAD							
IN-HOUSE	ERbb	Endocrine	IN-HOUSE	IGF-II	Growth	IN-HOUSE	POMCb	Growth	IN-HOUSE	Immune	Trf	1	-0,1	GOOD		25,9				AND1	-0,4	GOOD		27,7					
IN-HOUSE	FGA	Endocrine	IN-HOUSE	MCT10	Growth	IN-HOUSE	PRLR	Endocrine				2	1,0	BAD			IN-HOUSE	Growth	H3P	AND2	0,5	BAD							
IN-HOUSE	FGB	Endocrine	IN-HOUSE	MCT8	Growth	IN-HOUSE	TRa	Endocrine	IN-HOUSE	Immune	WAP65	1	0,6	BAD		27,1				PAN	0,1	BAD							
IN-HOUSE	gcgrc	NOT TESTED	IN-HOUSE	Mim1	Bone related	IN-HOUSE	TRAP	Bone related				2	1,1	BAD						AND1	0,9	BAD		27,0					
IN-HOUSE	gcrpr	NOT TESTED	IN-HOUSE	Mim2	Bone related	IN-HOUSE	TRb	Endocrine	Literature	metabolism	PLA2	AND2	0,1	BAD		26,1	IN-HOUSE	Growth	IGF-II	AND2	0,5	BAD							
IN-HOUSE	GH	Growth	IN-HOUSE	MMP9	Bone related	IN-HOUSE	TRF	Immune				PAN	2,0	BAD						PAN	0,9	BAD							
IN-HOUSE	gip1	NOT TESTED	IN-HOUSE	MR	Endocrine	IN-HOUSE	WAP65	Immune	Literature	Bone related	CA4	AND2	-0,2	GOOD		26,7				AND1	-0,9	GOOD		28,8					
IN-HOUSE	glp2r	NOT TESTED	IN-HOUSE	myf6	Bone related	Literature	Δ6D	Growth				PAN	1,2	BAD			IN-HOUSE	Bone related	Mim1	AND2	1,1	BAD							
IN-HOUSE	GPER	NOT TESTED	IN-HOUSE	MyoG	Bone related	Literature	PLA2	metabolism	Literature	larvae development	AMY2	AND2	0,2	BAD		29,9				PAN	0,8	BAD							
IN-HOUSE	GPERl	NOT TESTED	IN-HOUSE	OPG	Bone related	Literature	PPARα	Growth				PAN	1,1	BAD						AND1	0,2	BAD		27,6					
IN-HOUSE	GR1	Endocrine	IN-HOUSE	OSX	Bone related	Literature	PPARβ	Growth	Literature	metabolism	TRY	AND2	0,3	BAD		19,9	IN-HOUSE	Growth	POMCa	AND2	0,1	BAD							
IN-HOUSE	GR2	Endocrine	IN-HOUSE	POMCa	Growth	Literature	CA4	Bone related				PAN	1,6	BAD						PAN	0,3	BAD							
IN-HOUSE	H3P	Growth	IN-HOUSE	POMCb	Growth	Literature	Slc9a1	Bone related	Literature	larvae development	col1a2	AND2	1,4	BAD		22,0				AND1	0,9	BAD		27,7					
IN-HOUSE	IGFBP3a2	Growth	IN-HOUSE	PRL	Endocrine	Literature	Slc9a3	Bone related				PAN	1,5	BAD			IN-HOUSE	Endocrine system	TRa	AND2	-0,2	GOOD							
IN-HOUSE	IGF-II	Growth	IN-HOUSE	PRLR	Endocrine	Literature	Slc4a4	Bone related												PAN	1,0	BAD							
IN-HOUSE	JunB	NOT TESTED	IN-HOUSE	RANKL	Bone related	Literature	Slc4a1	Bone related												AND1	-0,1	GOOD		24,3					
IN-HOUSE	JunBA	NOT TESTED	IN-HOUSE	TPO	Growth	Literature	AMY2	larvae development									IN-HOUSE	immune	Trf	AND2	-0,2	GOOD							
IN-HOUSE	JunD	NOT TESTED	IN-HOUSE	TRa	Endocrine	Literature	TRY	larvae development												PAN	-0,4	GOOD							
IN-HOUSE	KLP4	NOT TESTED	IN-HOUSE	TRAP	Bone related	Literature	col1a2	larvae development												AND1	0,4	BAD		25,9					
IN-HOUSE	MCT10	Growth	IN-HOUSE	TRb	Endocrine												IN-HOUSE	immune	WAP65	AND2	0,9	BAD							
IN-HOUSE	MCT8	Growth	IN-HOUSE	TRF	Immune															PAN	0,9	BAD							
IN-HOUSE	Mim1 (OSTG)	Bone related	IN-HOUSE	TRH	Endocrine												Literature	metabolism	PLA2	AND1	-0,0			26,0					

IN-HOUSE	Mim2 (OSTG)	Bone related
IN-HOUSE	MMP13	Bone related
IN-HOUSE	MMP9	Bone related
IN-HOUSE	MR	Endocrine
IN-HOUSE	myf6	Bone related
IN-HOUSE	MyoG	Bone related
IN-HOUSE	Nkx2.1	NOT TESTED
IN-HOUSE	OPG	Bone related
IN-HOUSE	OSN	Bone related
IN-HOUSE	OSX	Bone related
IN-HOUSE	Pax7	Muscle
IN-HOUSE	POMCa	Growth
IN-HOUSE	POMCb	Growth
IN-HOUSE	PRL	Endocrine
IN-HOUSE	PRLR	Endocrine
IN-HOUSE	progcga	NOT TESTED
IN-HOUSE	progcgb	NOT TESTED
IN-HOUSE	RANK	Bone related
IN-HOUSE	RANKL	Bone related
IN-HOUSE	SCTR	Bone related
IN-HOUSE	TPO	Growth
IN-HOUSE	TRa	Endocrine
IN-HOUSE	TRF	Immune
IN-HOUSE	TRAP	Bone related
IN-HOUSE	TRb	Endocrine
IN-HOUSE	TRH	Endocrine
IN-HOUSE	TSHbeta	Growth
IN-HOUSE	TSHbeta	Growth
IN-HOUSE	TSH-R	Growth
IN-HOUSE	VtgII	Endocrine
IN-HOUSE	WAP65	Immune
Literature	Δ6D	Growth
Literature	PLA2	metabolism
Literature	PPARα	Growth
Literature	PPARβ	Growth
Literature	CA4	Bone related
Literature	Slc9a1	Bone related
Literature	Slc9a3	Bone related
Literature	Slc4a4	Bone related
Literature	Slc4a1	Bone related
Literature	AMY2	larvae development
Literature	TRY	metabolism
Literature	col1a2	larvae development

			AND2	**1,7**	**BAD**		
			PAN	**-0,2**	GOOD		
Literature	Bone related	CA4	AND1	**-0,5**	GOOD		27,3
			AND2	**0,0**			
			PAN	**-0,3**	GOOD		
Literature	Bone related	Slc4a1	AND1	**-0,5**	GOOD		27,8
			AND2	**0,4**	**BAD**		
			PAN	**-0,5**	GOOD		
Literature	metabolism	TRY	AND1	**0,0**			18,7
			AND2	**0,9**	**BAD**		
			PAN	**-0,1**	GOOD		
Literature	Larvae development	col1a2	AND1	**-1,0**	GOOD		21,3
			AND2	**0,8**	BAD		
			PAN	**0,4**	BAD		

IN-HOUSE	**TSH-R**	Growth
IN-HOUSE	**VtgII**	Endocrine
IN-HOUSE	**WAP65**	Immune
Literature	**Δ6D**	Growth
Literature	**LPL**	Growth
Literature	**PLA2**	metabolism
Literature	**PPARα**	Growth
Literature	**PPARβ**	Growth
Literature	**CA2**	Bone related
Literature	**CA4**	Bone related
Literature	**Slc9a1**	Bone related
Literature	**Slc9a3**	Bone related
Literature	**Slc4a4**	Bone related
Literature	**Slc4a1**	Bone related
Literature	**AMY2**	larvae development
Literature	**TRY**	metabolism
Literature	**col1a2**	larvae development

***Tabela 6**-Primer screen strategy for sparus aurata specie, all steps from all genes available until the step before final gene selection witch is showed in table 1.*

Step 1 -all primers available			**Step 2**-Worked in larvae (big pool mix of all stages) and <35cycles			**Step 3**- qPCR Cdna big pool <29cycles			**Step 4**- Best vs Poor of ***Sparus aurata***																	
									SA_ELR									SA_MM								
IN-HOUSE	ALP	Bone related	IN-HOUSE	ALP	Bone related	IN-HOUSE	ALP	Bone related	**Primer sel**	**Patway**	**Gene**	**Company**	**G&B DIF**	**+ expressed**	**>1,5**	**<29 Cts**	**Primer sel**	**Patway**	**Gene**	**Company**	**G&B DIF**	**+ expressed**	**>1,5**	**<29 Cts**
IN-HOUSE	Angptl7	Immune	IN-HOUSE	Angptl7	Immune	IN-HOUSE	Angptl7	Immune																	
IN-HOUSE	APOH	Lipidic metabolism	IN-HOUSE	BMP2	Bone related	IN-HOUSE	CATD1	Immune	IN-HOUSE	bone related	**ALP**	1	**-0,9**	GOOD		30,5	IN-HOUSE	bone related	**ALP**	1	**-1,3**	GOOD		32,0
IN-HOUSE	BMP2	Bone related	IN-HOUSE	CATD1	Bone related	IN-HOUSE	CATK	Immune				2	**1,9**	BAD						2	**2,4**	BAD		
IN-HOUSE	CATD1	Bone related	IN-HOUSE	CATK	Immune	IN-HOUSE	COLaV	Immune				3	**0,2**	BAD						3	**4,3**	BAD		
IN-HOUSE	CATK	Immune	IN-HOUSE	CAV3	Immune	IN-HOUSE	ColIa1F	Bone related	IN-HOUSE	immune system	**Angptl7**	1	**-2,5**	GOOD		28,5	IN-HOUSE	immune system	**Angptl7**	1	**-1,8**	GOOD		31,3
IN-HOUSE	CAV3	Immune	IN-HOUSE	COLaV	Immune	IN-HOUSE	csf1r	Immune				2	**1,2**	BAD						2	**2,8**	BAD		
IN-HOUSE	COLaV	Immune	IN-HOUSE	ColIa1F	Bone related	IN-HOUSE	CYTC	Immune				3	**0,2**	BAD						3	**0,9**	BAD		
IN-HOUSE	ColIa1F	Bone related	IN-HOUSE	csf1r	Immune	IN-HOUSE	GHRI	Growth	IN-HOUSE	immune system	**Cathepsin D1**	1	**-1,0**	GOOD		28,3	IN-HOUSE	immune system	**Cathepsin D1**	1	**-1,4**	GOOD		29,0
IN-HOUSE	csf1r	Immune	IN-HOUSE	CYTC	Immune	IN-HOUSE	GR	Immune				2	**0,4**	BAD						2	**1,7**	BAD		
IN-HOUSE	CYTC	Immune	IN-HOUSE	FGF6	Larvae development	IN-HOUSE	GST	Immune				3	**0,1**	BAD						3	**-0,3**	GOOD		
IN-HOUSE	dct	Immune	IN-HOUSE	GHRI	Growth	IN-HOUSE	IGF-I	Growth	IN-HOUSE	immune system	**catheps inK**	1	**-1,6**	GOOD		26,0	IN-HOUSE	immune system	**catheps inK**	1	**-1,4**	GOOD		28,7
IN-HOUSE	Era	Endocrine	IN-HOUSE	GHRII	Growth	IN-HOUSE	MAMDC2	Growth				2	**1,7**	BAD						2	**0,1**	BAD		
IN-HOUSE	Erba	Endocrine	IN-HOUSE	GR	Immune	IN-HOUSE	m-cadherin	cell structure				3	**-0,2**	GOOD						3	**-1,0**	GOOD		
IN-HOUSE	FGF6	Larvae development	IN-HOUSE	GST	Immune	IN-HOUSE	Murf1	Growth	IN-HOUSE	immune system	**COLaV**	1	**-2,2**	GOOD		26,2	IN-HOUSE	immune system	**COLaV**	1	**-2,2**	GOOD		27,5
IN-HOUSE	Follistatin	Endocrine	IN-HOUSE	IGF-I	Growth	IN-HOUSE	OGN2	Bone related				2	**1,0**	BAD						2	**1,7**	BAD		

IN-HOUSE	FOXO3	Immune		IN-HOUSE	IGFIBP1	Growth		IN-HOUSE	OSN	Bone related				3	**-0,1**	GOOD								3	**0,2**	BAD		
IN-HOUSE	GHRI	Growth		IN-HOUSE	MAMDC2	Growth		IN-HOUSE	OSTC	Bone related	IN-HOUSE	Bone related	**Colla1**	1	**-0,8**	GOOD		23,4		IN-HOUSE	Bone related	**Colla1**	1	**-2,3**	GOOD		22,6
IN-HOUSE	GHRII	Growth		IN-HOUSE	m-cadherin	cell structure		IN-HOUSE	RBP	Immune				2	**1,0**	BAD				IN-HOUSE			2	**1,4**	BAD		
IN-HOUSE	GR	Immune		IN-HOUSE	Murf1	Growth		IN-HOUSE	trα	Endocrine				3	**0,5**	BAD							3	**-0,0**	GOOD		
IN-HOUSE	GST	Immune		IN-HOUSE	OGN1F1	Bone related		Literature	AhR/bmal1	growth	IN-HOUSE	immune system	**csf1r**	1	**-1,4**	GOOD		30,6		IN-HOUSE	immune system	**csf1r**	1	**-2,1**	GOOD		31,2
IN-HOUSE	IFI56	Immune		IN-HOUSE	OGN2F1	Bone related		Literature	BMP4	bone related				2	**0,6**	BAD							2	**1,5**	BAD		
IN-HOUSE	IGF-I	Growth		IN-HOUSE	OSN	Bone related		Literature	CAT	bone related				3	**0,7**	BAD							3	**-0,5**	GOOD		
IN-HOUSE	IGFIBP1	Growth		IN-HOUSE	OSTC	Bone related		Literature	CILP2	bone related	IN-HOUSE	immune system	**CYTC**	1	**-2,0**	GOOD		27,3		IN-HOUSE	immune system	**CYTC**	1	**-1,6**	GOOD		27,4
IN-HOUSE	IGFIBP4	Growth		IN-HOUSE	p22Phox	Immune		Literature	clock	larval development				2	**0,1**	BAD				IN-HOUSE			2	**1,7**	BAD		
IN-HOUSE	IGF-II	Growth		IN-HOUSE	RBP	Immune		Literature	COX2	bone related				3	**-0,8**	GOOD							3	**0,0**			
IN-HOUSE	IGF-II	Growth		IN-HOUSE	TRAP	Bone related		Literature	CRY1	larval development	IN-HOUSE	growth/endocrine system	**GHRI**	1	**-1,9**	GOOD		30,5		IN-HOUSE	growth/endocrine system	**GHRI**	1	**-1,4**	GOOD		30,9
IN-HOUSE	Ihh	Immune		IN-HOUSE	trα	Endocrine		Literature	FN1A	bone related				2	**0,3**	BAD							2	**2,0**	BAD		
IN-HOUSE	MAMDC2	Growth		IN-HOUSE	trβ	Endocrine		Literature	GH	growth				3	**0,4**	BAD							3	**0,0**			
IN-HOUSE	m-cadherin	cell structure		IN-HOUSE	tyr	skin		Literature	GK	metabolism	IN-HOUSE	immune system	**grf**	1	**-1,8**	GOOD		28,7		IN-HOUSE	immune system	**grf**	1	**-1,9**	GOOD		29,8
IN-HOUSE	MSTN	Growth		Literature	AhR/bmal1	Growth		Literature	GPX	bone related				2	**0,4**	BAD							2	**1,7**	BAD		
IN-HOUSE	Murf1	Growth		Literature	BMP4	bone related		Literature	MGP	bone related				3	**0,3**	BAD							3	**-0,5**	GOOD		
IN-HOUSE	newSox8	Immune		Literature	CAT	bone related		Literature	PER3	larval development	IN-HOUSE	immune system	**GST**	1	**-2,2**	GOOD		28,5		IN-HOUSE	immune system	**GST**	1	**-2,0**	GOOD		29,6
IN-HOUSE	OGN1F1	Bone related		Literature	CILP2	bone related		Literature	PFK-1	metabolism				2	**1,0**	BAD							2	**1,4**	BAD		
IN-HOUSE	OGN2F1	Bone related		Literature	clock	Larvae development		Literature	PK	metabolism				3	**-0,1**	GOOD							3	**-0,9**	GOOD		
IN-HOUSE	OSN	Bone related		Literature	COX2	bone related		Literature	PRL	growth	IN-HOUSE	growth/endocrine system	**IGF-I**	1	**-2,5**	GOOD		33,0		IN-HOUSE	growth/endocrine system	**IGF-I**	1	**-1,6**	GOOD		34,4

IN-HOUSE	OSN	Bone related		Literature	CRY1	Larvae development		Literature	RUNX2	bone related																									
IN-HOUSE	OSTC	Bone related		Literature	FN1A	bone related		Literature	TRY	digestive																									
IN-HOUSE	p22Phox	Immune		Literature	GH	Growth																													
IN-HOUSE	pomc2	Growth		Literature	GK	metabolism																													
IN-HOUSE	pomcα1	Growth		Literature	GPX	bone related																													
IN-HOUSE	RBP	Immune		Literature	MGP	bone related																													
IN-HOUSE	RPL27	Housekeeping		Literature	PER3	Larvae development																													
IN-HOUSE	Shh	larvae deveopment		Literature	PFK-1	metabolism																													
IN-HOUSE	star	Endocrine		Literature	PK	metabolism																													
IN-HOUSE	TRAP	Bone related		Literature	PRL	Growth																													
IN-HOUSE	trα	Endocrine		Literature	RUNX2	bone related																													
IN-HOUSE	trβ	Endocrine		Literature	TRY	digestive																													
IN-HOUSE	tyr	skin																																	
Literature	AhR/bmal1	growth																																	
Literature	AMY2A	digestive																																	
Literature	BHMT3	bone related																																	
Literature	BMP4	bone related																																	
Literature	CAT	bone related																																	
Literature	CILP2	bone related																																	
Literature	clock	larval development																																	
Literature	COX2	bone related																																	

			2	0,9	BAD														2	1,6	BAD														
			3	-1,2	GOOD														3	-0,4	GOOD														
IN-HOUSE	growth/development	MAMDC2	1	-1,8	GOOD		30,7												1	-0,6	GOOD		30,6														
			2	0,9	BAD														2	2,5	BAD																
			3	0,1	BAD														3	-0,0	GOOD																
IN-HOUSE	cells strcuture	m-cadherin	1	-4,9	GOOD		32,2												1	-0,2	GOOD		33,6														
			2	0,9	BAD														2	2,2	BAD																
			3	0,9	BAD														3	1,0	BAD																
IN-HOUSE	growth/development	Murf1	1	-2,4	GOOD		30,1												1	-2,7	GOOD		31,3														
			2	0,6	BAD														2	1,4	BAD																
			3	-1,8	GOOD														3	-1,6	GOOD																
IN-HOUSE	bone related	OGN2	1	-0,4	GOOD		32,0												1	-2,5	GOOD		31,9														
			2	0,3	BAD														2	1,0	BAD																
			3	1,2	BAD														3	-0,7	GOOD																
IN-HOUSE	bone related	OSN	1	-1,3	GOOD		25,5												1	-2,1	GOOD		26,6														
			2	0,8	BAD														2	1,8	BAD																
			3	0,3	BAD														3	0,5	BAD																
IN-HOUSE	bone related	OSTC	1	1,5	BAD		28,2												1	-4,1	GOOD		28,5														
			2	0,3	BAD														2	0,5	BAD																
			3	0,8	BAD														3	0,7	BAD																
	immune system	RBP	1	-2,0	GOOD		28,7												1	1,9	BAD		30,2														

Literature	CRY1	larval development
Literature	FN1A	bone related
Literature	GH	growth
Literature	GK	metabolism
Literature	GPX	bone related
Literature	MGP	bone related
Literature	PER3	larval development
Literature	PFK-1	metabolism
Literature	PK	metabolism
Literature	PRL	growth
Literature	RUNX2	bone related
Literature	spp1	bone related
Literature	TRY	digestive
Literature	Δ6D	growth

IN-HOUSE			2	**1,3**	**BAD**		
			3	**0,2**	**BAD**		
IN-HOUSE	growth/endocrine system	**trα**	1	**-1,0**	GOOD		29,7
			2	**0,3**	**BAD**		
			3	**0,3**	**BAD**		
Literature	Growth	PRL	1	**-0,3**	GOOD		31,7
			2	**-0,4**	GOOD		
			3	**0,1**	BAD		
Literature	Growth	GH	1	**-2,0**	GOOD		30,1
			2	**0,5**	BAD		
			3	**-0,3**	GOOD		
Literature	Metabolism	GK	1	**-4,7**	GOOD		34,8
			2	**-2,4**	GOOD		
			3	**-1,7**	GOOD		
Literature	Metabolism	PFK-1	1	**-2,0**	GOOD		30,4
			2	**1,1**	BAD		
			3	**-0,1**	GOOD		
Literature	Metabolism	PK	1	**-3,4**	GOOD		30,2
			2	**-0,1**	GOOD		
			3	**-0,9**	GOOD		
Literature	Growth	AhR	1	**-1,6**	GOOD		27,5
			2	**1,9**	BAD		
			3	**0,3**	BAD		
Literature	Larval development	clock	1	**-1,6**	GOOD		30,7
			2	**0,8**	BAD		
			3	**0,3**	BAD		

IN-HOUSE			2	**1,4**	**BAD**		
			3	**-0,1**	GOOD		
IN-HOUSE	growth/endocrine system	**trα**	1	**-2,0**	GOOD		31,0
			2	**2,6**	**BAD**		
			3	**-0,7**	GOOD		
Literature	Growth	PRL	1	**-0,5**	GOOD		30,9
			2	**0,8**	BAD		
			3	**0,0**			
Literature	Growth	GH	1	**-0,9**	GOOD		29,8
			2	**1,4**	BAD		
			3	**-0,4**	GOOD		
Literature	Metabolism	GK	1	**-1,6**	GOOD		32,7
			2	**3,5**	BAD		
			3	**1,2**	BAD		
Literature	Metabolism	PFK-1	1	**-3,1**	GOOD		31,1
			2	**2,0**	BAD		
			3	**-0,6**	GOOD		
Literature	Metabolism	PK	1	**-2,2**	GOOD		30,8
			2	**2,1**	BAD		
			3	**-0,3**	GOOD		
Literature	Growth	AhR	1	**-2,1**	GOOD		28,2
			2	**1,9**	BAD		
			3	**-0,5**	GOOD		
Literature	Larval development	clock	1	**-2,6**	GOOD		30,2
			2	**1,9**	BAD		
			3	**-1,1**	GOOD		

Literature	Larval development	CRY1	1	**-1,9**	GOOD		30,7	Literature	Larval development	CRY1	1	**-1,6**	GOOD		30,5
			2	**1,2**	BAD						2	**2,0**	BAD		
			3	**-0,2**	GOOD						3	**-0,9**	GOOD		
Literature	Larval development	PER3	1	**-1,5**	GOOD		28,6	Literature	Larval development	PER3	1	**0,7**	BAD		30,0
			2	**0,7**	BAD						2	**1,9**	BAD		
			3	**-0,1**	GOOD						3	**-0,1**	GOOD		
Literature	Metabolism	TRY	1	**-2,0**	GOOD		23,3	Literature	Metabolism	TRY	1	**-0,9**	GOOD		22,7
			2	**-0,0**							2	**0,3**	BAD		
			3	**1,4**	BAD						3	**-3,4**	GOOD		
Literature	Bone related	RUNX2	1	**-2,2**	GOOD		30,6	Literature	Bone related	RUNX2	1	**-2,1**	GOOD		30,2
			2	**0,8**	BAD						2	**1,6**	BAD		
			3	**1,1**	BAD						3	**0,4**	BAD		
Literature	Bone related	COX2	1	**0,3**	BAD		30,9	Literature	Bone related	COX2	1	**-1,2**	GOOD		31,4
			2	**1,8**	BAD						2	**2,0**	BAD		
			3	**-1,8**	GOOD						3	**-0,2**	GOOD		
Literature	Bone related	CAT	1	**-2,3**	GOOD		29,8	Literature	Bone related	CAT	1	**-1,6**	GOOD		31,1
			2	**0,2**	BAD						2	**0,4**	BAD		
			3	**-0,2**	GOOD						3	**-1,1**	GOOD		
Literature	Bone related	BMP4	1	**-2,1**	GOOD		32,8	Literature	Bone related	BMP4	1	**-2,1**	GOOD		33,8
			2	**0,8**	BAD						2	**1,6**	BAD		
			3	**0,5**	BAD						3	**0,4**	BAD		
Literature	Bone related	FN1A	1	**-1,8**	GOOD		28,2	Literature	Bone related	FN1A	1	**-2,3**	GOOD		29,1
			2	**0,8**	BAD						2	**1,8**	BAD		
			3	**0,7**	BAD						3	**-0,6**	GOOD		
Literature	Bone related	CLIP2	1	**-2,1**	GOOD		30,8	Literature	Bone related	CLIP2	1	**-2,2**	GOOD		32,0
			2	**1,0**	BAD						2	**1,6**	BAD		
			3	**0,7**	BAD						3	**-0,7**	GOOD		

Literature	Bone related	MGP	1	**-0,3**	GOOD		27,4	Literature	Bone related	MGP	1	**-2,3**	GOOD		27,2
			2	**0,4**	BAD						2	**0,9**	BAD		
			3	**0,1**	BAD						3	**-0,1**	GOOD		
Literature	Bone related	GPX	1	**-0,9**	GOOD		27,5	Literature	Bone related	GPX	1	**-2,0**	GOOD		27,5
			2	**0,8**	BAD						2	**0,6**	BAD		
			3	**-0,2**	GOOD						3	**-0,2**	GOOD		