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Optimized KPI quality markers

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Summary

Over 1 billion juveniles of European sea bass (BSS) and gilthead sea bream (SBG) are produced every year in hatcheries across the Mediterranean (FEAP, production report). The quality of the juveniles and the feeding regime are the two critical determinants of the outcome of the on-growing phase of production. Deliverable 2.2 was directed at identifying, optimizing and validating robust indicators of larval and juvenile quality (KPIs) for commercial hatcheries by testing multiple batches of eggs and larvae classified as "Good" and "Bad" based on the KPIs of incidence of skeletal malformation and length growth and variability. To maximise the scope and success of the developed tool a suite of methodologies were utilised; histology, tissue and organ histopathology, proteomics and molecular biology. The outcome was two tools, one, a multiparametric semiquantitative scoring system (MSSS) composed of 18 descriptors belonging to 6 organ districts: gills, liver, anterior intestine, posterior intestine, pancreas and adipose tissue and excretory system. The MSSS does not have predictive utility but can be used to monitor production quality and give an early warning of production quality allowing timely corrective measures. The second tool is based on molecular markers and multi-tier statistical analysis. The analytical approach acts as a filter to identify and validate markers linked to quality. The application of the multi-tier statistical approach to the BSS larval samples reveals good correlation with juvenile quality when multiple markers are used. The analytical approach developed and the filtered and selected genes (UTH so far but further are being added, CCMAR and UNIUD) are the basis of a predictive quality monitoring system for BSS that will be extended to SBG. New knowledge generated and of importance for hatchery managers is that several identified skeletal malformations identified in juveniles are corrected during grow-out. This is the first time that this has been clearly demonstrated and provides the basis for rational management of juvenile selection for grow-out bringing positive benefits for health and welfare and hatchery KPIs.

1 Introduction

Over 1 billion juveniles of European sea bass (BSS) and gilthead sea bream (SBG) are produced every year in hatcheries across the Mediterranean (FEAP, production report). The vast majority of these juveniles are tranferred into sea cages for on-growing up to commercial size. The quality of the juveniles and the feeding regime are the two critical determinants of the outcome of the on-growing phase of production. Healthy, robust juveniles with high growth potential are expected to develop into disease-resistant, fast-growing fish when in the sea (**Figure 1**). This is particularly important as it can shorten the production cycle, leading to an economic advantage and a reduced ecological impact. Furthermore, benefits may be gained from the higher disease and stress resistance and the potentially increased attractiveness for the consumers of the final product.

Even though it is largely recognized that the success of marine aquaculture strictly depends on the production of good quality larvae/juveniles, this rearing phase still remains one of the main bottlenecks. Although current managerial practices aim at rearing fish under conditions that guarantee the maximum growth rate, feed conversion efficiency, survival and at the same time the minimum problems related to infections, nutrition and environmental diseases (Plumb and Hanson, 2011 quoted by Saraiva *et al*., 2015), poor growth of individuals, decreased survival, and malformations are often observed (Valdstein *et al*., 2004; Støttrup, 1993 quoted by Valdstein *et al*., 2013). Many of the problems associated with juvenile quality may not be perceptible with current methods of monitoring and observation of the larval stages of production and/or are only visible in later stages (Logue et al. 2000 quoted by Valdstein *et al*., 2013), so that it is important to establish the means for recognition of “good” and “bad” quality larval production cycles and the factors associated with the larval quality so that action can be taken against factors that can damage production.

Previously funded National and European projects (EU projects; ORCIS, FINEFISH, LIFECYCLE, FINDiT, SUSHIFISH, National; BASSGEN, EPIFARM) and scientific literature report detailed studies of the biology of BSS and SBG and have developed resources and an extensive set of markers, including molecular (e.g. biochemical, genes and proteins), morphological (e.g. external morphology, skeleton, swim bladder etc), histopathological (e.g. gastrointestinal tract condition), immunological (e.g. Toll-like receptor and MHC) and functional (using a challenge), all of which are indicative of juvenile quality. PerformFISH is set to optimize and validate existing juvenile quality markers in the context of commercial hatchery conditions according to their degree of correlation with Key Performance Indicators (KPIs) of hatchery production that are described in Deliverable 7.1. The overall objective is to improve predictability, quality and sustainability of the hatchery phase of BSS and SBG by exploiting existing technical and biological knowledge and when necessary carry out further original research to fill gaps.

Task 2.2 in PerformFISH is aimed at the optimization and validation of larval and juvenile quality indicators and it is uniquely designed to use biological samples derived only from commercial hatchery production. This strategic decision is endorsed by five hatcheries across the Mediterranean (Spain, France, Italy, and Greece) and provides the advantage of comparing biological variation at all the levels represented by the markers employed, along with environmental variation and variation in hatchery operating procedures. A multi-tier comparison between developmental stages, environment and practice has been adopted and a

unique sampling strategy was employed as described in Deliverable 2.1. Based on the overarching aim, the specific objectives of Task 2.2 in relation to the present Deliverable are to:

- investigate the correlation between egg and larval quality with juvenile performance by identifying, optimizing and validating robust indicators of larval and juvenile quality (KPIs) for commercial hatcheries,
- estimate the correlation of fish quality between different ontogenetic stages at the hatchery phase, and
- correlate fish quality between different ontogenetic stages in several hatcheries and correlate morphological quality between the juvenile (1.5g) and fish of commercial size.

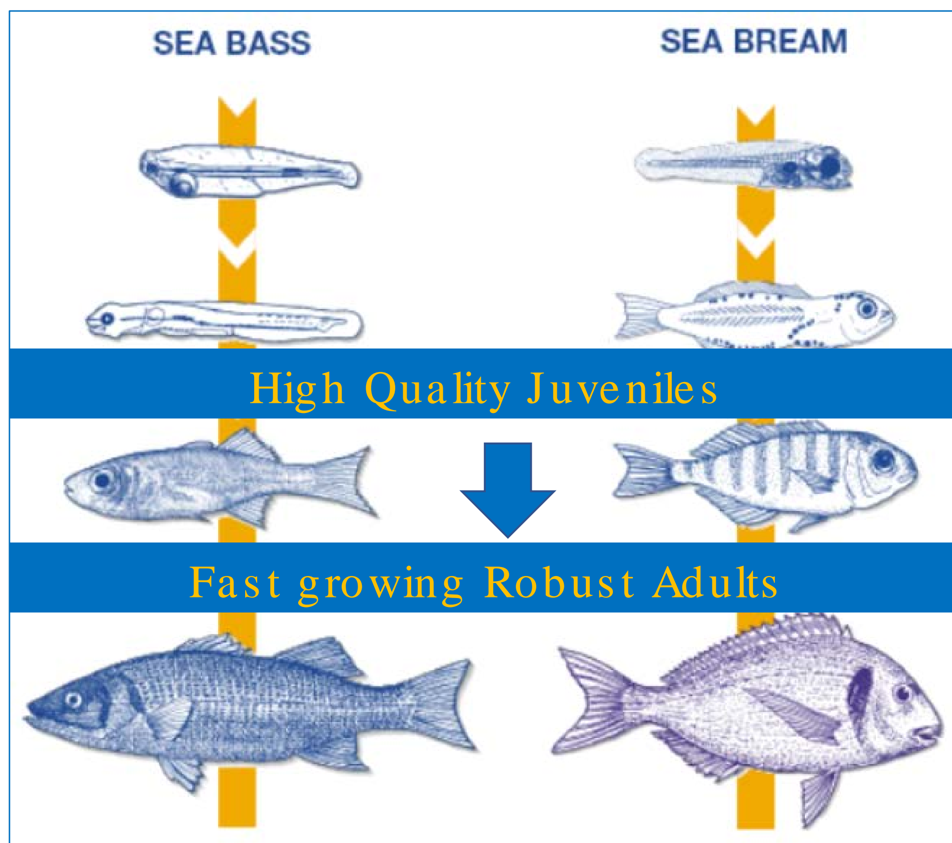


Figure 1. The Mediterranean Marine Fish Farming sector relies on high quality juveniles

The results presented in this Deliverable will feed into Deliverable 2.3 and Deliverable 2.6. The ultimate aim is the identification of a suite of markers that they tightly correlate with KPIs and are predictive of juvenile performance, to constitute the nucleus of the first certification scheme of juvenile quality in Mediterranean Marine Fish Farming (MMFF). The work described in the present deliverable is the analytical work required to identify markers for testing by correlative analysis with production conditions

reported in the zootechnical excel recording sheet each of the hatchery kept associated with the collected egg and larval samples (see D2.1). It should be noted that an important consideration is that the suite of markers is practical and cost-effective and that the material required is easily collected and part of the normal production cycle of hatcheries. The output of the task will only be useful and applicable if it can be deployed with samples collected in a real production setting and takes into account the restraints this places on the material that can be collected, stored and transported to the sites of analysis. The outcome of the analysis so far and the tools generated are being viewed from the perspective of an integrated tool of commercial interest and as such IP or licensing is under consideration. For this reason, since the present deliverable is public the more commercially relevant information has masked.

2 Approach

Optimization is aimed at generating a few markers strongly correlated with larval and subsequent juvenile quality for use as a management tool for quality monitoring and control of hatchery conditions and practices. From the onset, markers do not necessarily have to be linked with specific processes but rather be well correlated with biological trait quality and this considerably widens the scope of potential markers.

Aiming at optimizing and validating quality markers that perform consistently across different production conditions and reflect the outcome of the hatchery production, namely, biological value and potential of the juveniles, it was decided that the markers should:

1. be indicative/correlated with biological traits strongly linked to production quality and KPIs;
2. discriminate between good and bad batches independent of the species and the commercial hatchery.
3. take into consideration the variability in production parameters across Mediterranean hatcheries and be of consistent predictive value.

Building an extensive collection of commercial batches from hatcheries across the Mediterranean was important to cover variability in production based on location, broodstock, and time of year and to identify sufficient numbers of good and bad batches for comparison. The details of the sample collection are presented in Deliverable 2.1.

It should be noted that from the project planning phase prior knowledge about skeletal malformations formed the basis of the total number of population/batches selected for analysis. The high and difficult to control variability of SBG, meant that 20 independent batches/populations were collected by the collaborating LTPs. In the case of BSS production is less variable and the incidence of malformation less frequent and so only 15 independent batches/populations were collected by the collaborating LTPs (furthermore, one less LTP provided BSS).

Tasks 2.2.1 and 2.2.2 dealt with the comparison of good vs bad batches from different angles. Task 2.2.1 was set to determine how egg quality affects the quality of larvae and juveniles. Eggs giving origin to an excellent or poor production cycle were analyzed to identify diagnostic markers. High throughput omics methods were employed to map the egg molecular markers that signify quality of offspring (CCMAR).

Task 2.2.2 followed an integrated approach to correlate the fish quality between different ontogenetic stages, namely first feeding (FF), flexion (FL), end of larval rearing (10-14mm TL; ELR), and middle metamorphosis (15-20mm TL; MM). Meristic and morphological analysis of larvae and juveniles was performed to establish malformation indices and larval quality (UoC). This formed the basis of classification of “good” and “bad” batches/populations of larvae. However, it was decided to advance with other parameters and not just skeletal malformations for the classification of “good” and “bad” batches/populations of BSS and GSB. To this end a multiparametric semiquantitative scoring system (MSSS) based on 18 histological descriptors was developed (UNIUD). To acheive a robust outcome for the MSSS tool all batches/populations of BSS and GSB were analysed (some delay has occurred due to covid-19). Another parameter considered for classification of “good” and “bad” batches/populations was growth (weight and length) at MM. This permitted initiation of molecular analysis, which was planned to be carried out using the 2 best and 2 worst batches/population of larvae coming from each LTP. The reason for selecting this approach was to contain the costs of analysis. This means that 3 different parameters were chosen for classification of “good” and “bad” batches/population of GSB and BSS. Malformation incidence and MSSS was established by analysing all samples of larvae and juveniles collected, in the case of molecular analysis only the 2 “best” and 2 “worst” batches/populations were analysed. Although non-consensual scoring with the different methods for quality analysis means that more batches/population are being analysed using molecular methods.

Marker genes indicative of larval/juvenile robustness and health status were targeted to assess the ontogenetic state of immune system and competence (UNIUD/CCMAR). Molecular markers linked to growth, metabolism and cellular energetics were analysed to establish an index of physiological and developmental quality. The ontogenetic patterns of these markers were established along with the patterns of coordinated expression. Validation of existing and development of further biomarkers was employed to identify those that fulfil the criteria set above (UTH/CCMAR).

A further parameter analyzed was the global microbiome of the tank water at critical stages of production to assess the correlation between microbiological indicators and production quality and decipher the parameters that shape the larvae and juvenile microbiome (CCMAR).

In addition, the needs of hatcheries to establish and validate criteria to evaluate the quality of juvenile morphology and accurately predict the phenotypic quality of table-size fish was addressed. Task 2.2.3 examined whether the initial variability of juvenile phenotype is conserved during the on-growing period in an attempt to demonstrate the spectrum of juvenile shapes and sizes that result in good quality and consumer acceptable fish, contribute to the reduction in discards and in this way increase hatchery KPIs and facilitate marketing of the product. Juvenile seabream and sea bass morphology linked to phenotypic quality of table-size fish will be incorporated into the e-guidelines and photo-atlas (Deliverable 2.3).

3 Batch Rating

All BSS and SBG batches received from the LTPs (**Table 1**) were evaluated according to Key Performance Indicators established for the hatchery phase in Deliverable 7.1. All BSS and SBG batches were inspected and rated according to a) degree of malformations present (KPIs H7-H10), and b) length growth (KPI H11). Approximately 20 batches/ populations were delivered as foreseen in the DoA for SBG and approximately 15 for BSS.

Table 1. The number of batches received from LTP (coded as A, B, C and D) and analysed for morphological malformations. LTP – linked third party

HATCHERY	SBG		BSS	
	Received Batches	Analyzed Batches	Received Batches	Analyzed Batches
LTP A	20	20	15	15
LTP B	20	20	--*	-- *
LTP C	27	20	15	15
LTP D	15	15**	20**	20**
Total	82	75	50	50

**no BSS production in hatchery B; **Hatchery joined WP2 task in Y2 and so sample delivery to RD partners occurred later than other LTP*

3.1 Malformations

In commercial BSS and SBG hatcheries, quality control is performed at various steps of the rearing production, targeting different aspects of morpho-anatomy. **Table 2** summarizes the features most frequently examined, with some information on the applied methodology and the critical rearing/ontogenetic stages.

Estimation of the incidence of skeletal abnormalities in the samples from the participating commercial hatcheries

Quality control focused on the samples of 10-14 mm TL (SBG) and 14-18 mm TL (BSS) and was performed after the double staining of the specimens for bone and cartilage (Walker and Kimmel 2007). Recorded skeletal abnormalities were categorized according to the affected anatomical area and typology (e.g. crossbite, pugheadness, lateral displacement of the upper jaw, etc, for jaw abnormalities), as well as their severity (i.e. severe *vs* light) (Koumoundouros et al. 2010, Fragkoulis et al. 2017, 2018).

Following the request of the participating commercial hatcheries, all data were handled exclusively by UoC (Koumoundouros lab) and treated as strictly confidential and anonymous. Each participating hatchery has already received a confidential detailed report for all the deformity types and severity levels, which were recorded by UoC in their own samples. Mean reported values of overall rates (sum of light and severe) for skeletal abnormalities are given in **Table 3**. In general, SBG samples presented (at varying rates and severity degrees) abnormalities of the gill-cover, jaws and fins. In BSS, abnormalities of the jaws and fins were mostly observed.

Table 2. Morpho-anatomical features included in the quality control assessment during the hatchery and pre-growing phases. CV, coefficient of variation. ds, double staining. fs, counting of sinking to floating fish. st, stereoscopic examination. vs, macroscopic visual examination. x-ray, mammography (Koumoundouros 2010, Koumoundouros et al. 2018)

Feature examined	Rearing period	Method	Notes
Swimbladder inflation rate	During the first ca 2-3 w after yolk-consumption	st	always
Swimbladder inflation rate	During salinity floating test	fs	always
Swimbladder inflation rate	End of hatchery phase	xr	always
Fish behavior	Larval rearing phase	*in situ*	always
Skeletal abnormalities (haemal lordosis excl)	End of larval rearing phase (33-40 dph)	ds	*
Skeletal abnormalities (haemal lordosis incl)	> 0.3-0.5 g W	xr, vs	always
Size heterogeneity	End of hatchery phase	CV	always
Miscellaneous (e.g. notochord, epidermis, stomach, lipid globule)	Embryonic and larval stages	st	*

*, rarely to always, depending on the hatchery, species, production cycle, etc

Table 3. Mean reported values of overall rates (sum of light and severe) for skeletal abnormalities, grouped by gross anatomical areas. Data concerns seabream samples from eight hatcheries (three aquaculture companies) (Kourkouta, PhD thesis)

	Frequency (%, ±SD)
Cranial abnormalities (total)	19±22
Fin abnormalities (total)	3±7
Vertebral abnormalities (total)	1±2

3.2 Length growth

In the PerformFISH document of work (DoW), it was initially foreseen that analysis would be targeted to production batches/populations that had elevated or low incidence of skeletal malformations. However, it became evident during the advance of the projects that this was delaying the analysis of other markers since all larvae collected had to be analysed for skeletal malformation, which is a very labour intensive and slow process. It was also evident that the available budget and time meant that it was not tenable to conduct all the foreseen analysis on all the collected samples. Moreover, the inclusion of one principal classification parameter, skeletal malformations, although an important quality KPI did not cover other

KPI. For this reason it was decided to include a further classification parameter that could be more rapidly and easily recorded and is also an important KPI, growth. For this reason a new batch classification factor linked to "good" and "bad" performing batches was used to identify those for analysis and which could also be cross-referenced with the skeletal quality analysis as it became available. Growth parameters were determined for each larvae contained in the samples of middle metamorphosis stage as received from the LTPs. Weight and total length were measured and condition factor (K) was calculated. Total length was considered the robust metric to use for growth assessment and basic statistics were employed based on total length. Batches with the highest mean values ± standard deviation of total length were classified as "good", as opposed to those with the lowest mean values of total length that were classified as "bad". Large differences and variability was observed between batches in terms of mean and distribution of total length. **Figure 2** shows the distribution of total length values in indicative "good" and "bad" batches.

This classification resulted in a list of 24 and 18 batches of SBG and BSS, respectively, that were used for the validation and optimization of molecular markers.

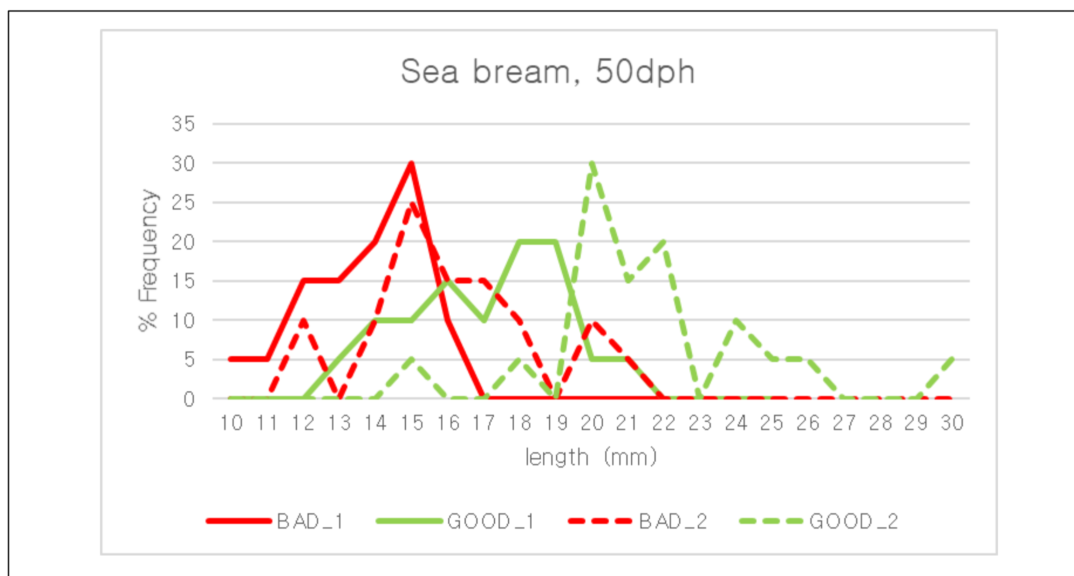


Figure 2. Distribution of total length (mm) in batches of SBG classifed as "good" and "bad" in terms of growth performance. All samples were collected at 50dph.

4 Egg quality indicators

4.1 Introduction

Early development of oviparous organisms, such as fish, reptiles, birds and monotremes, that lay eggs that mature and hatch, is dependent on maternally derived structural, nutritional and regulatory molecules. The maternal factors that accumulate in eggs influence their quality and this determines the future developmental potential of the embryo, a characteristic that is of high interest for fish aquaculture (Bizuayehu et al., 2019). Embryogenesis in teleosts has been extensively studied and has contributed to

the success of the teleosts of which over 28,000 extant species exist (Cucchi et al., 2012, Honji et al., 2012). Since most fish are oviparous, egg and embryo development is directly influenced by the environment and despite the almost impermeable outer chorion, temperature, oxygen availability, salinity, pH etc are all reported to affect embryogenesis. For this reason, although a stage frequently overlooked, fertilization through to hatch is of high importance as this is when the embro and larval fish is formed and epigenetic imprinting can occur.

Maternally inherited mRNAs and proteins accumulate and support early embryonic development until activation of zygotic transcription and thus play a pivotal role during oogenesis and early embryogenesis (Howley and Ho, 2000, Bobe and Labbe, 2010). Proteins are an important molecular cargo of fish eggs since they account for > 50% of the egg and are important as a source of cellular energy and for the formation of fish embryos and larvae (Lubzens et al., 2017). A number of studies have assessed the role of specifically derived maternal proteins contributing to immune protection of the egg, such as complement 3 (C3) protein in Atlantic salmon (*Salmo salar*) (Lovoll et al., 2007) and phosvitn (Pv) in zebrafish embryo (Wang et al., 2011). Since the protein content of the egg may determine the success of the subsequent embryo we decided to conduct an in-depth study of the egg proteome, with the view to defining a "normal" egg cargo of protein and a modified egg cargo of protein. The approach was exploratory as few or no protein markers exist for fish eggs and the link between egg protein and larval quality egg has yet to me made. The aim was to come up with a core set of markers for screening of "good" and "bad" quality egg batches.

A number of practical difficulties exist with studies of protein, namely their instability, the ease with which they degrade and the absence of cheap stabilising solutions, such as exist for nucleic acids (eg. RNALater). Taking into consideration that the egg protein content might contain critical markers of function it was decided to develop comparative proteome analysis using several different species to identify core marker proteins common across eggs from different species. It was reasoned that the common proteins should have crucial functions and so changes in them could lead to reduced quality and so they would be correlated with egg quality. Challenges for this task were technical, linked to sample collection, storage and transport. The criteria for collection was that proteins should be stabilised without requiring expensive reagents or dry ice freezing, which is not readily available in many LTP. Moreover, transport with dry ice adds to the logistical difficulties and cost and so transport at 4°C was preferred.

4.2 Methods

Experimental fish and sample collection

Comparative analysis was carried out using frozen eggs (24h before hatch or at hatch) obtained from broodstock maintained in the Portuguese Institute for Sea and Atmosphere (IPMA, Olhão, Portugal) or eggs from LTP collected into RNALater. Eggs for freezing were snap frozen in liquid nitrogen after they were netted, washed in clean, sterile seawater and put into a 1.5 ml microcentrifuge tube. Eggs collected for RNA later were netted, rinsed in sterile, filtered seawater, the excess water drained off and then eggs placed into a 15 ml sample tube full of RNA Later (Sigma-Aldrich). Eggs were allowed to equilibrate with the RNA later overnight at 5°C and were then frozen at -20°C.

Total protein extraction

Triplicate samples for each of the developmental stages (1 day before hatching and hatching) were collected simultaneously from the same broodstock tank to ensure all samples of a given species were at the same stage. The frozen samples were from white sea bream (DS, *Diplodus sargus,* n=3 before hatch, BH and at hatch, H), meagre (AR, *Argyrosomus regius,* n = 3 BH and n = 3 H) and SBG (SA, n = 3 BH and n = 3 H) and RNAlater samples were from SBG (n = 3 BH and H) and BSS (n = 3 BH and H).

Protein was extracted from the samples using denaturing buffer (protein extraction buffer: consist of 1.7% SDS solution, 50mM Tris HCl pH 6.8, 100mM DTT). All steps of protein extraction were carried out on ice. Samples of eggs (100 mg) were homogenized in 500ul of protein extraction buffer. After homogenization 5 µl of protease inhibitor (ten times concentrated, Sigma-Aldrich) was added to each sample. Protein extraction from the egg homogenates was promoted by vortexing the samples at 10-minute intervals over 30 minutes and then heating the samples at 95 °C for 10 min, mixing every 5 min and then freezing at -80oC. Before polyacrylamide gel electrophoresis samples were thawed on ice and then centrifuged at 14000rpm for 15min at 4 °C. The pellet and the supernatant were collected and the pellet was resuspended in 250 µl of 8M UREA, 50mM Tris HCl pH 8.0, 100mM NaCl to give a concentration of 7 µg/µl. All extracted protein samples were store at -80°C until analysis.

Proteomic analysis

SWATH-MS (Sequential Window data independent Acquisition of the Total High-resolution-Mass Spectra) was used in the present study to determine the egg total proteome and the identity and relative quantification of the constituent proteins. Briefly, 50 µg of protein from three independent samples per species representing the stages BH (n = 3) and H (n = 3) and a single sample composed of a pool of the individual samples (50 µg) per group were subject to a partial SDS-PAGE run. Proteins in the selected gel regions were excised and subject to in-gel digestion using trypsin as described in Santa et al., 2016. Peptides were recovered from gels by solid phase extraction with C18 sorbent (OMIX tip, Agilent Technologies) and analysed on a Triple TOF™ 5600 System (Sciex®, Framingham, MA). The LC-MS information was acquired in two different acquisition modes: information-dependent acquisition (IDA) of the pooled samples and SWATH (Sequential Windowed data independent Acquisition of the Total High-resolution Mass Spectra) acquisition of individual samples.

Protein analysis

Protein identification and library construction was performed using ProteinPilot™ (v5.0.1, Sciex) software and the following search parameters: 1) comparison of mass spectra against the predicted proteins from the sea bass genome database (June 2012 draft assembly dicLab v1.0c with annotation from July 2013; file diclab1_pep.faa.gz downloaded from http://seabass.mpipz.mpg.de/DOWNLOADS/ (Tine et al., 2014); 2) acrylamide alkylation; 3) trypsin digestion (Paragon™ Algorithm). Relative quantification was performed using the SWATH™ processing plug-in for PeakView™ (v2.2, Sciex). For SWATH™ quantification up to 15 peptides were chosen per protein and peptide retention time was adjusted using malE-GFP peptides. Protein levels were estimated from all the peptides and normalized to the total proein intensity per sample.

Statistical Analysis

To perform statistical comparisons between groups of samples the software SPSS v23 (IBM) was used. A non-parametric Mann Whitney U-test was used for the comparisons of experimental groups two-by-two and a Kruskal-Wallis followed by a post-hoc Dunn's test corrected by FDR using the Benjamini-Hochberg adjustment, due to the reduced number of samples per comparison group.

4.3 Results

Quality of Proteome Sequencing library

The SWATH-MS technology yielded a total 2407 proteins for frozen eggs from DS, AR and SBG and 1845 proteins from eggs from ESB and SBG that had been stored in RNA later (**Table 4**). The number of proteins quantified for the frozen eggs were 960, 1149 and 922 for DS, AR and SBG, respectively. For eggs maintained in RNA later 961 and 1495 proteins were quantified for SBG amd BSS, respectively. The differentialy expressed proteins when BH and H were compared were 331, 575 and 400 for DS, AR and SBG, respectively.

Table 4. Results of SWATH analysis and proteome generated from fish eggs before hatching (BH) and after hatching (H). Note that the frozen eggs had 24% more identified proteins than the samples fixed in RNA later. Nonetheless, the overall analysis was similar in relation to the proteins quantified

	FROZEN			RNA LATER	
	SBG	**DS**	**AR**	**SBG**	**BSS**
Identified proteins	**2407**	**2407**	**2407**	**1845**	**1845**
Quantified proteins	**960**	**1149**	**922**	**961**	**1495**
Total modified proteins	**331**	**575**	**400**		
Proteins whose levels increase (BH versus H)	**284**	**414**	**363**		
Proteins whose levels decrease (BH versus H)	**47**	**161**	**37**		

* SBG – Gilthead seabream; DS – white seabream; AR – meagre; BSS – European seabass.

Protein expression pattern of DS, AR and SBG frozen eggs

The BSS (*Dicentrarchus labrax*) genome was used as a reference genome for identification of the proteins in all the species. Preliminary analysis of the SWATH data revealed that the close phylogeny of these fishes and the good conservation of genes and protein sequence meant that the confidence level was high for protein identification irrespective of species. A global proteome was generated for the quantified proteins identified in each of the species without discriminating between the egg stage. Comparison of the quantified and identified proteins between the three species revealed that 694 proteins were common indicating that there is a high conservation of the proteins in the eggs during early development of these species (**Figure 3**).

The proteins that were differentially expressed (DE) between the different frozen egg stages (BH and H) were determined on a species by species basis. The list of proteins that were differentially expressed was then compared between species and a Venn diagram generated (**Figure 4**). This revealed greater specificity for each species since fewer common proteins were identified. For example only 107 common proteins were found between all three species. This more variable DE dataset may in part reflect differences in ontogeny linked to differences in the timing of sample collection. This means that when all proteins in eggs irrespective of stage are compared, then greater similarity exists as the time frame is longer and the coverage of ontogeny is similar. However, small difference in timing of egg collection for BH and H and then DE analysis may generate larger difference if the relative ontogeny between species is different.

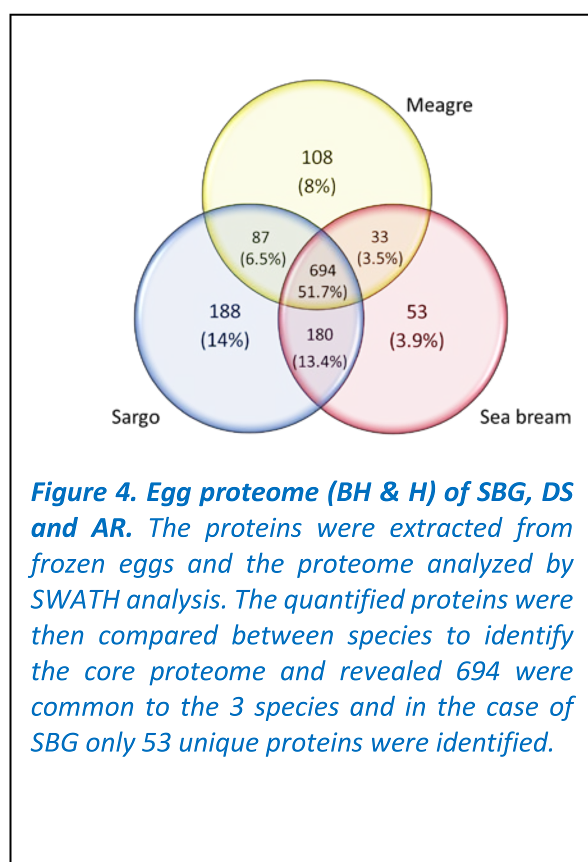


Figure 4. Egg proteome (BH & H) of SBG, DS and AR. *The proteins were extracted from frozen eggs and the proteome analyzed by SWATH analysis. The quantified proteins were then compared between species to identify the core proteome and revealed 694 were common to the 3 species and in the case of SBG only 53 unique proteins were identified.*

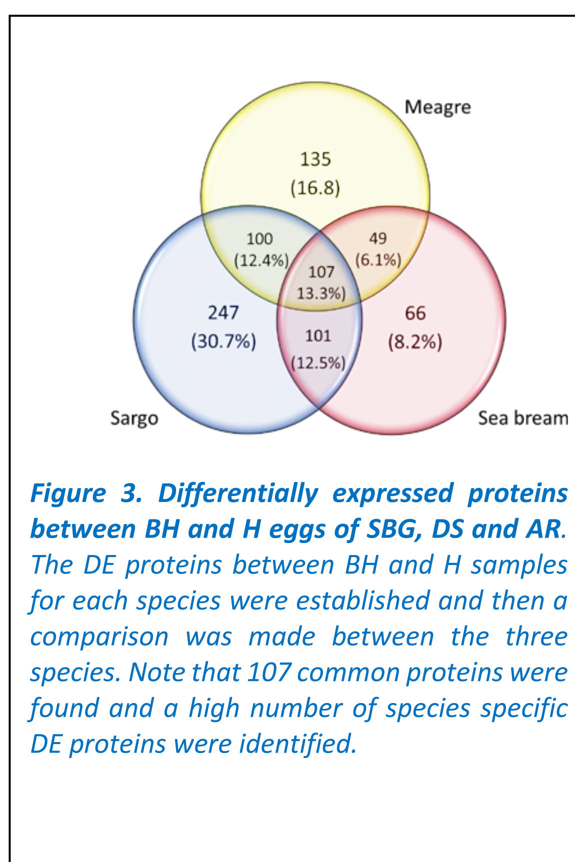


Figure 3. Differentially expressed proteins between BH and H eggs of SBG, DS and AR. *The DE proteins between BH and H samples for each species were established and then a comparison was made between the three species. Note that 107 common proteins were found and a high number of species specific DE proteins were identified.*

Establishment of markers of egg quality

The species-specific comparison has highlighted a core of marker proteins that are highly conserved irrespective of species and presumably are linked to essential functions and in this way egg quality. However, the DE proteins identified between the egg stages (BH and H) resulted in far fewer common proteins being identified and revealed far greater variability between species. Nonetheless, when the top 26 proteins (13 up-regulated and 13 down-regulated) that were modified between BH and H stages were

compared between each of the species SBG, DS and AR they shared 80% of the proteins. This suggests that these *proteins are markers of the shift between BH and H and are important for the quality of the eggs*.

Comparisons of the proteome of frozen and RNA Later preserved eggs

Another of the objectives of the comparative analysis was to establish if RNA Later is a suitable preservation method and ensures protein stability and minimises degradation or modifications of the proteome. For this reason the proteome was determined for frozen SBG and RNA Later preserved SBG eggs at H (**Figure 5**). RNA Later preserved BSS eggs were also generated to confirm comparability with the proteome of the frozen eggs.

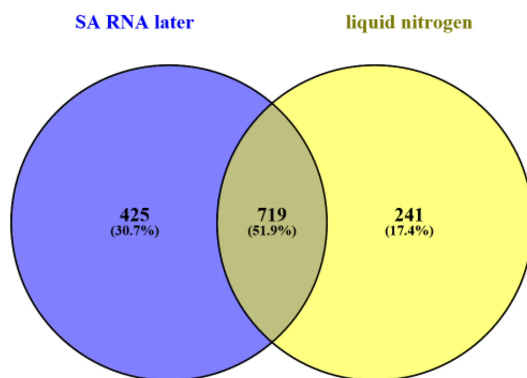


Figure 5. Comparative analysis of the proteome of frozen or RNA later fixed SBG egg. Note that a high proportion of common proteins are identified between frozen and fixed SBG eggs. However, 30% of the proteins in RNA later fixed eggs are different.

Comparison of the proteins identified as potential markers of quality, since they are common and linked to the developmental transition between BH and H in SBG, AR and DS revealed they were highly represented in the frozen versus RNA later preserved eggs. Thus, although the analysis of DE proteins between BH and H was not the objective of the comparison in frozen versus RNA later SBG eggs, it was possible to see a similar pattern of expression in relation to highly abudant or low abundance proteins.

4.4 Conclusion

RNA Later was found to be a good preservative of egg proteins and is a viable method for studies of proteins in eggs. This was an important observation as many of the LTPs do not have access to dry ice and do not have appropriate facilities (-80°C) for storage of samples until shipping. Furthermore the use of RNA later removes logistical limitations and reduces the cost of shipping.

A suite of marker proteins were identified that can be used for egg quality screening but are still under validation using material collected in WP2 and also samples from field trials (WP6). It is not practical to run proteomics by LTPs for batches/populations of eggs during routine production due to the cost and time it takes for analysis. However, the application of a selection of protein bioquality indicators identified by proteomics may be feasible and can be an additional marker contributing to hatchery quality monitoring.

*validation work is pending as the PhD student has been retained in China since January 2020.

5 Histological quality indicators

5.1 Introduction

Organ microanatomy reflects the overall effect of biotic and abiotic factors (Saraiva *et al.,* 2015), and for this reason in many studies histology has been used to assess fish heath status and toxicants, diet, and temperature effects (Sirri *et al*., 2018; Manera *et al*., 2016a; Saraiva *et al.*, 2016; Puvanendran *et al*., 2015). Histological grading systems lead to a standardized quantification and allows the possibility of legitimate comparison between different samples and studies (Bernet *et al.*, 1999). To the best of our knowledge no histological tools have been specifically built with the purpose of evaluating fish larval quality. The aim of this study was to develop a specific prognostic histological system to allow estimates of larval and juvenile quality in gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*). This approach could be used to highlight problems in larval batches and provide farmers with information about the impact of their managerial practices.

5.2 Material and Methods

5.2.1 Fish and sampling procedures

Larvae and Juvenile European sea bass (*BSS*) and Gilthead sea bream (*SBG*) were sampled and fixed in Bouin's solution (Bio-Optica, Milano) by the participating LTP. After fixation, samples were preserved in EtOH 70% until processing. All these procedures were carried out by the LTP in accordance with the PerformFISH Sampling Manual "Juvenile Quality and Growth Potential Standard Operating Procedures for Sampling and Data Recording". Once in EtOH, samples were delivered by courier to the University of Udine facilities. It should be noted that from a "health and safety" perspective not all the participating LTP had the necessary facilities to handle Bouin fixative and so they did not provide samples as it is indicated in Deliverable 2.1.

The numbers of samples received and analysed by UNIUD for histological quality indicators is indicated in **Table 5** and **Table 6**. Samples were either shipped from CCMAR or directly by the LTP to UNIUD where they were checked and then preserved at 4ºC. Among the received batches, those defined as the "best" or "worst" from a given company were selected for histological analysis, more batches/populations were analysed from what is foreseen in the DoA, to define robust MSSS score attribution criteria and to compensate for samples with failure in fixation. The number of fish samples used for the score scale definition was affected by problems in sampling and histological preservation quality, so it was not always possible to analyze 20 fish per stage; moreover, not all the stages were sampled in some batches, due to farms internal matters.

Table 5. Number of batches received and analyzed divided for species and LTP hatchery.

HATCHERY	SBG		BSS	
	Received Batches	Analyzed Batches	Received Batches	Analyzed Batches
A (Greece)	8	7	8	5
B (France)	20	8	--*	-- *
C (Italy)	27	12	6	2
D (Greece)	15	0**	20	0**
Total	60	27	34	7

**no BSS production in hatchery B*
*** Hatchery D joined WP2 in Y2 and substituted a Spanish LTP that was unable to participate as the site was closed for production and were not analyzed for the purpose of defining the score*

Before running full analysis, the preservation quality of specimens was considered and in some cases was only partially acceptable and compatible for the purposes of the study. This was the case when non appropriate fixation or preservation affected one or more tissues in a fish, or, when particularly marked, the whole specimen. Fish or organs were excluded from the evaluation in case of poor cellular details (or difficult interpretation of the cellular type). In 6 first feeding groups received, 90% of larvae showed poor preservation so these groups were totally excluded from the evaluation. In the middle of metamorphosis and juvenile groups the poor histological preservation quality was limited to some organs (e.g. in the intestine the epithelium was missing, or evident cytoplasmic and nuclear alterations were detectable), but this event did not compromise the whole specimen evaluation. The most affected organ was the gut (from 0,5% to 88% within the groups), more rarely pancreas, liver and kidney. Generally, gills were well preserved, only in one group were the gills poorly fixed in 88% of the individuals. The total numbers of fish analyzed is shown in **Table 6**.

Table 6. Summary of samples (individuals) assessed for MSSS analysis.

STAGE	SBG	BSS
FIRST FEEDING	400	127
FLEXION	540	105
END OF LARVAL REARING	533	140
MIDDLE OF THE METAMORPHOSIS	540	140
JUVENILE	413	80
Total	**2426**	**592**

5.2.2 Histology

At the UNIUD laboratories, 20 larvae/juveniles per stage and batch were placed in an automatic tissue processor (Tisbe DiaPath) and were dehydrated through an ethanol series, treated with xylene and *in toto* embedded in paraffin. Paraffin blocks were serially sectioned at 4 µm on a microtome (Leica Biosystems 2135), and sections were stained with hematoxylin and eosin (H&E) and coverslipped using a synthetic resin (Eukitt, O, KindlerGmbH). The specimens were blindly evaluated, by a single trained operator, under a light microscope (Leica DMLB) and relevant images were captured using a digital camera (Nikon Fi3) with imaging software NIS-Elements BR (Nikon instruments Italia). In some case Periodic Acid Shiff (PAS), Alcian blue, PAS-Alcian blue, Schmorl’s stain were performed to further confirm the findings with H&E staining. In order to assess the adequacy of the samples for the histological evaluation, parameters related to tissue alterations (structural conservation, and cytoplasmic and nuclear detail) by autolysis as a results of slow or inadequate fixation were overall considered by assigning a score from 1 to 5. When this score was greater than 3, the organ or the whole fish was excluded from the evaluation.

5.2.3 Literature review on histological grading systems

During the first months of the PerformFISH project, UNIUD carried out a meta-analytic literature review on the use of histological markers in fish studies, in order to evaluate the investigation methods, in particular the choice of descriptors, the organs/tissues to be evaluated, and the grading systems. For the accomplishment of this goal, different search engines were used for the search, including PRIMO (Copyright © 2015), CAB Direct (©Copyright 2020 CAB International), Google (Copyright ©2020 Google), PubMed (Copyright © 2020 NCBI), in Scopus (Copyright © 2020 Elsevier B.V.), Web of Science (Copyright © 2020 Clarivate) and ScienceDirect (Copyright © 2020 Elsevier B.V.) and using specific keywords (see **Table 7**). Data on fixatives, sampling weights, studied organs, grading systems and descriptors obtained from literature were defragmented and re-aggregated into an Excel sheet.

Table 7. A list of the key words used to elaborate a bibliographic search and to identify publications about histological and other markers that exist for GSB and BSS in order to identify preexisting research or activities used as a quality marker

KEY WORDS				
Larvae Fish European seabass Gilthead seabream *Dicentrarchus labrax* *Sparus aurata*	AND/OR	Markers Biomarkers Histology Histological markers	AND/OR	Quality Development Growth Ontogenesis

5.2.4 Design of the Multiparametric Semiquantitative Scoring System (MSSS)

The information obtained from the literature analysis and the preexisting UNIUD experience in histology applied to fish constituted the basis for the design of an original Multiparametric Semiquantitative Scoring System (MSSS), for evaluation of BSS and SBG larval and juvenile stages. A score from 1 to 5 is semi-quantitatively attributed to each descriptor identified for each organ/tissues analyzed based on the presence and severity of different pathological events or quantitative phenomena.

Figure 6. Extract of the Excel sheet used to record the information obtained from the search with the keywords in Table 3.

			MATHERIAL and METHODS			TARGET ORGAN										
PDF	AUTORI	ANNO	FIXATIVE	NUMBER	STAINING	OC	BR	SV	INT	FE	PA	RE	MI	CU	CUO	GON
	Samanta et a	2018	NBF	3/gruppo	EE		1			1		1				
	Chatigny et al	2018	NBF	3/gruppo	EE											
	Sirri et al.	2018	NBF-CRIO-GLU	9-6-1/gruppo	EE-ORO-BT				1	1						
	Liu et al.	2018	NBF	7/gruppo	EE	1										
	Bonvini et al.	2017	NBF	15/gruppo	EE				1							
	Murali et al.	2017	NBF	30/gruppo	EE					1						
	Annamalai et a	2017	NBF	10/gruppo	EE		1			1						
	Carraschi et al.	2017	NBF	9/gruppo	EE-PAS		1			1		1				
	Fu et al.	2017	NBF	13-18/gruppo	EE					1						
	Hamed et al.	2017	NBF	12/gruppo	EE					1		1				
	Kostic et al.	2017	NBF	5-12/gruppo	EE		1			1						
	Piccinetti et al	2017	PFA	30/gruppo	EE			1	1	1						
	Robson et al.	2017	BO	4/gruppo	EE		1			1		1			1	1
	Manera et al.	2016c	GLU		BT		1									
	Manera et al.	2016b	GLU		BT		1									
	Manera et al.	2016a	GLU	4/gruppo	BT		1									
	Marcon et al.	2016	PFA	10/gruppo	EE		1									
	Murussi et al.	2016		12/gruppo	EE		1									
	Tabassum et al	2016	BO	10/gruppo	EE		1			1		1				
	Liu et al.	2016								1						
	Popovic et al.	2016	NBF	19-45/gruppo	EE-PAS		1			1		1	1			
	Saraiva et al.	2016	NBF	30	EE		1		1	1		1		1		
	Evans et al.	2016	NBF		EE							1	1			
	Raskovic et al.	2016	NBF	12/gruppo	EE-PAS-AB/PAS		1		1	1		1				
	Martins et al.	2016	BO		EE-AB-WHI-VG		1									

5.2.5 Immunohistochemistry

Immunohistochemistry (IHC) assays were set up for a panel of mono- or polyclonal antibodies (CD68 - ab 199000, Abcam; GM-CSFR – 690 Santa Cruz Biotechnologies; G7 anti-gilthead sea bream acidophilic granulocytes, courtesy of prof. Mulero, Univ. of Murcia; Histamine - H7403 Sigma Aldrich; ESB IgM – University of Trieste; GSB IgM – University of Trieste) in order to characterize the immune system cells. Four µm histological sections were collected on adhesive glass slides (TOMO® Matsunami, Germany), left to dry overnight (ON) and used for immunohistochemical (IHC) procedures. IHC was conducted following a conventional protocol or using the EnVision™ FLEX system (K8009, K8021 and K8023, Dako, Agilent Technologies) to amplify the signal. Detection was performed using 3,3′-diaminobenzidine tetrahydrochloride (DAB) (D5905, Sigma-Aldrich) as the chromogen and slides were counterstained with hematoxylin. Slides were examined under a light microscope (Leica DMLB). Appropriate controls were included in all assays, and primary antibody was replaced by dilution buffer in negative control sections.

1.2.5.1. Conventional IHC

Sections were de-waxed in xylene baths (2x20 min) and gradually rehydrated through graded alcohols (100% ethanol 2 baths for 3 min each, 95% ethanol for 3 min, 80% ethanol for 3 min, 50% ethanol for 3 min) and a final bath in MilliQ water (ddH2O) for 5 min. Endogenous peroxidase activity was blocked by the addition of 3% (v/v) hydrogen peroxide (H2O2) in ddH2O for 30 min at room temperature (RT), then slides were washed in ddH2O (3 min). In some case antigen retrieval was performed using a 0.5% trypsin solution for 15 min at 37°C. All incubations were performed in a closed-lid humidified box. Nonspecific antibody binding was blocked by 1:20 normal goat serum (S26-100 EMD Millipore) in 1× PBS for 30 min at RT. After incubation with the primary antibodies (**Table 10**) appropriately diluted in 1% (w/v) bovine albumin serum (BSA) (A9647, Sigma Aldrich), 10% (v/v) normal goat serum in 1× PBS for 2h at RT, sections were washed in TRIS buffer (3×5 min). Thereafter they were incubated for 30 min at RT with a biotinylated secondary antibody: goat-anti-mouse Ig (SAB3701051, Sigma Aldrich) diluted 1:400 in 1× PBS; or goat-anti-rabbit Ig (A9169, Sigma Aldrich) diluted 1:1,000 in 1× PBS; or rabbit-anti-goat Ig (B7014, Sigma Aldrich) diluted 1:800 in 1× PBS. Washes in TRIS buffer (3 × 5 min) followed the incubations with the primary and secondary antibodies. The reactions were developed using an ABComplex horseradish

peroxidase (HRP) kit (PK-4000, Vectastain, Vector Laboratories) for 30 min at RT, followed by staining with DAB for 7 min and counterstaining with haematoxylin (1 min). Sections were washed with tap water and gradually dehydrated [50% ethanol for 3 min, 80% ethanol for 3 min, 95% ethanol for 3 min, 100% ethanol (2×3 min) and 2 successive xylene baths, 5 min each], coverslipped using a synthetic resin (Eukitt, O, KindlerGmbH).

1.2.5.2 EnVision™ FLEX

For some antibodies it was necessary to apply the EnVision™ FLEX protocol as conventional IHC was not effective for labelling of the antigens. The protocol was the same as that described above for conventional IHC until the step of endogenous peroxidase inhibition. Then slides were washed with TRIS buffer (2×3 min). Antigen retrieval was performed using High pH or Low pH solutions (K8004 and K8005, Dako, Agilent) for 10 min at 90°C. Thereafter, slides were left for 15 min to cool to RT and washed with TRIS buffer (2×3 min). Targeted sections were encircled with paraffin to keep reagents localized over the sections. All incubations were performed in a closed-lid humidified box. Nonspecific antibody binding was blocked using a 1:20 dilution of normal goat serum (S26-100 EMD Millipore) in 1× PBS for 30 min at RT. After incubation with the primary antibodies (**Table 10**) appropriately diluted in 1% (w/v) bovine albumin serum (BSA), 10% (v/v) normal goat serum in 1× PBS for 2h at RT, sections were washed in TRIS buffer (3×5 min). The labelling was carried out using a horseradish peroxidase (HRP)-based anti-rabbit or anti-mouse linker (EnVision™ FLEX; Dako, Agilent) for 20 min at RT. Followed by two washes with TRIS buffer (5 min each), then the sections were treated as described for conventional IHC, starting from the DAB step.

5.3 Results

5.3.1 Histology

The histological quality of the preservation of the samples was only in part acceptable and compatible for the purposes of the study. Non appropriate fixation or preservation affected one or more tissues in a fish, or, when particularly marked, the whole specimen. In 6 first feeding groups, 90% of larvae showed poor cellular detail (or difficult interpretation of the cellular type), so these groups were excluded from the evaluation. In the middle of metamorphosis and juvenile groups the poor histological quality preservation was limited to some organs (e.g. in the intestine the epithelium was missing or cytoplasmic and nuclear alterations were evident), but this event did not compromise the evaluation of the whole specimen so it was included in the analysis. The most affected organ was the gut (from 0.5% to 88% within the group), more rarely pancreas, liver and kidney. Generally, gills were well preserved and only in one group of larvae were 88% of the gills non-evaluable using histological analysis.

5.3.2 Literature review on histological grading systems

Literature analysis (including 91 scientific articles) allowed the extrapolation of significant data concerning the use of histology and histopathology in fish. The major part of these studies used histology in toxicological investigations (46%) or to evaluate effects of diets (35%), but also to assess the effects of temperature, to study health status and the response to pathogens (24%). Methods that are used are qualitative description (43%), semiquantitative scores (44%), quantitative morphometry (36%) and fractal analysis (5%). Ninety-three descriptors or parameters, belonging to 16 organ districts and quantitatively

or semi-quantitatively assessable, were extrapolated from the papers. Fifty-two of them were discarded as not useful for the purpose of the present project, the remaining 43 were critically discussed on the basis of previous studies conducted by UNIUD group and then tested on preliminary SBG and BSS samples in order to select the best descriptors for use in a larval/juvenile quality assessment tools.

5.3.3 Multiparametric Semiquantitative Scoring System

The first multiparametric semiquantitative scoring system (MSSS) version developed included 43 descriptors belonging to 7 organ districts. As the PerformFISH study progressed, the less informative descriptors and organs were eliminated, so as to make the system simpler but at the same time more robust in determining histopathology. The definitive tool obtained is composed of 18 descriptors belonging to 6 organ districts: gills, liver, anterior intestine, posterior intestine, pancreas and adipose tissue and excretory system. All the descriptors are organ/tissue-specific, except for inflammatory infiltrates which is taken into consideration for each organ district. A score from 1 to 5 is semi-quantitatively attributed to each descriptor on the basis of the extent of the phenomenon. For some of them, the scoring criteria were first defined quantitatively by means of image analysis, then the measurements were divided into ranges corresponding to the scores from 1 to 5 (**Table 8**). By using software, the operator was also trained to assign the correct score to the 18 descriptors selected. For purely descriptive purposes, all the samples are also evaluated for the presence or absence of yolk sac and swim bladder. The cell infiltration parameters evaluated in the considered organs were cell type (supplemented by an estimate of the relative percentages), tissue distribution (focal, multifocal and diffuse) and structural type (perivasal/periductual or not perivasal/periductal). For each organ district, one descriptor included in the MSSS is briefly described in the following section (5.3.3.1 – 5.3.3.6) to provide an example of the overall construction and application of MSSS.

5.3.3.1 Gills

Chloride cells (hypertrophy and hyperplasia)

These are plump and slightly hypereosinophilic cells concentrated in the afferent region of the filament epithelium of the gill filament, within the lamellar sulci (Wolf et al., 2015; Wilson and Laurent., 2002). The term ‘‘chloride cell’’ relates to their function in Cl^- elimination (Wilson and Laurent., 2002) but they are also known as “mitochondrion-rich cell” or “ionocytes” because they are involved not only in chloride secretion in seawater, but also in acid-base regulation and ammonia excretion (Hiroi and McCormick, 2012). Changes of these cells occur in response to exposure to irritating factors, especially when chronic. The effects of heavy metals, ammonia intoxication, excessively high or low water pH, and parasitic infestations are thought to be the cause of excessive proliferation (Strzyzewska *et al*., 2016). For the purpose of this study, first feeding larvae were not evaluated for chloride cell hypertrophy/hyperplasia since they are insufficiently developed in this stage. Concerning the other stages, chloride cells were considered hyperplastic when proliferating along the lengths of lamellae in a basal to apical direction as indicated in Wolf *et al*., 2015, and hypertrophic when they altered the normal architecture of gill filaments.

Table 8. Score attribution system. The scoring method of one descriptor for each organ district is shown

ORGAN DISTRICTS and DESCRIPTORS	SCORING				
	1	2	3	4	5
GILLS					
Chloride cells (Hypertrophy/Hyperplasia)	Absent	Scarce	Moderate	Abundant	Higly abundant
LIVER					
Hepatocyte fat accumulation	Absent	Scarce	Moderate	Abundant	Higly abundant
ANTERIOR INTESTINE					
Mucous cells (density)	Absent	Scarce	Moderate	Abundant	Higly abundant
POSTERIOR INTESTINE					
Supranuclear vacuoles (density and dimension)	Absent	Scarce	Moderate	Abundant	Higly abundant
PANCREAS and VISCERAL ADIPOSE TISSUE					
Inflammatory infiltrate	Absent	Scarce	Moderate	Abundant	Higly abundant
EXCRETORY SYSTEM					
Calculi (dimension)	Absent	Small	Medium	Large	Very large

5.3.3.2 Liver

Hepatocyte fat accumulation

The liver is the most important regulator of lipid metabolism, including both the synthesis and degradation of fatty acids, thus imbalances in the dietary fatty acids could modify the functioning and morphology of this organ. In certain fish species, including BSS and SBG, the liver functions as a main energy reservoir, frequently in the form of triacylglycerols (TGs) (Kaushik, 1997 quoted by Caballero et al., 2004). Also in fish larvae, as in adult fish, the liver plays a role as an energy reservoir. Mature hepatocytes have been observed in the liver of several species from first feeding (Ronnenstad *et al*., 2013; Micale *et al*., 2008; Guyot *et al*., 1995; Tanaka, 1969). When dietary lipid or energy exceed the capacity of the hepatic cells to oxidize fatty acids, the result is the large synthesis and deposition of TG in vacuoles, leading to a morphological pattern known as steatosis or lipidosis (Caballero et al., 2004). In SBG, this condition has been observed when the dietary lipid content increases (Caballero *et al*., 1999 quoted by Caballero *et al*., 2004), as result of an essential fatty acid deficiency (Montero *et al*., 2001 quoted by Caballero *et al*., 2004), the use of artificial diets (Spisni *et al*., 1998 quoted by Caballero *et al*., 2004) and the inclusion of vegetable oils (Alexis, 1997 quoted by Caballero *et al*., 2004). Anyway, since the point at which hepatic lipid accumulation becomes deleterious to fish is currently undetermined and probably quite variable within fish species, the term steatosis/lipidosis should be used only when it is possible to demonstrate cellular alterations induced by an excessive accumulation of lipids, such as cell membrane rupture or saponification (Wolf et al., 2015). For the purpose of the study PAS staining was performed in doubtful cases to avoid diagnosing glycogen storage as fat storage and evaluating the lipid accumulation in quantitative terms.

5.3.3.3.Anterior intestine

Mucous cells

Mucins play an important role in protecting the mucosa against pathogens attack and physical and chemical damage, moreover in the digestive tract, mucus also acts as enzymatic support, and not only as a lubricant. The time of appearance of mucous or goblet cells in the teleost digestive tract varies among species. In SBG, digestive regions are histologically differentiated from 4 days post hatching (Sarasquete *et al*., 1995) and goblet cells appear in the anterior intestine from the exotrophic phase; some of them contain neutral mucins and others acid (sialomucin-type and sulphated) or mixed mucin types (Ebal *et al.*, 2004; Sarasquete *et al*., 1995). Also, in BSS intestinal goblet cells are first found in the intestine during the exotrophic phase and are rich in neutral (García Hernández *et al*., 2001) or acid muco-substances (Tan Tue, 1980 quoted by García Hernández *et al*., 2001). According to Sarasquete *et al*. (1995), inter- and intraspecific differences in the content of muco-substances in the digestive goblet cells could be related to different feeding habits. The number of mucous cells in the digestive tract normally increases with larval development, moreover the number of these cells varies due to several factors including, diet (Torrecillas et al., 2017; Baeza-Ariño *et al*., 2016), microplastic and toxicant exposure (Pedà *et al*., 2016) and the presence of pathogens (Xu *et al*., 2019; Redondo and Álvarez-Pellitero, 2010).

5.3.3.4 Posterior intestine

Supranuclear vacuoles

During the transition from endogenous to exogenous feeding, the posterior intestine has a basic nutritional role in absorbing protein macromolecules by pinocytosis as an alternative pathway until the development of the stomach and acid digestion takes place. Thus, the presence of supranuclear vacuoles with acidophilic inclusions is a typical feature of the posterior intestine of fish larvae in early life stages (Gisbert *et al*., 2008; Sarasquete *et al*., 1995; Yúfera et *al*., 1993). These inclusions are strongly positive with PAS stain and slightly with Alcian blue (pH 2.5) indicating the presence of neutral mucopolysaccharides (and/or glycoproteins) and carboxylated acid mucopolysaccharides. They are also positive to bromophenol blue, confirming the presence of proteins (Sarasquete *et al*., 1995; Yúfera et *al*., 1993). Preliminary histochemical studies on supranuclear vacuoles carried out by UNIUD group, showed that, in some cases, the content of these vacuoles appears as a brownish lipofuscin-like pigment when observed in white sections; moreover, they gave a positive reaction with Lillie's Nile blue, Sudan black B and Schmorl's stain, confirming the presence of oxidized lipids or lipoproteins probably due to a diet-enzyme imbalance during larval development. Although in most of the studied species the number and size of supranuclear vacuoles decreased as the stomach differentiated and extracellular digestion take place (Gisbert *et al*., 2008), they have been described in epithelial cells of the BSS rectum during phase IV (55-61 days post hatching) (García-Hernández *et al*., 2001) as well as in the rectum of larvae and adults of other teleosts (Boulhic and Gabaudan, 1992; Govoni *et al*., 1986 quoted by Sarasquete *et al*., 1995) including *S. aurata* (Cataldi *et al*., 1987; Elbal and Agulleiro, 1986 quoted by Sarasquete *et al*., 1995).

5.3.3.5 Pancreas and visceral adipose tissue

Inflammatory infiltrate

A previous study carried out in UNIUD laboratory has shown that, apparently healthy SBG post-larvae reared in Mediterranean countries in recent years have a significantly higher number of pancreatic inflammatory cells than recorded 20 years ago. This could be the result of a changed nutritional scenario, both in terms of diet composition due to the replacement of animal origin ingredients with others of vegetable origin, and in the management of feeding, characterized by zootechnical forcing in order to promote rapid growth (Beraldo *et al*., 2018). The score attribution to this feature was based on the count of inflammatory cells (**Table 9**).

Table 9. Criteria for score attribution to pancreatic inflammatory infiltrates. Cells are counted in 4 fields at 40X magnification

DESCRIPTOR	SCORING				
	1	2	3	4	5
PANCREAS and VISCERAL ADIPOSE TISSUE					
Inflammatory infiltrate	Absent (from 1 to 6 cells)	Scarce (from 6 to 20 cells)	Moderate (from 21 to 50 cells)	Abundant (from 51 to 150 cells)	Higly abundant (more than 150 cells)

5.3.3.6 Excretory system

Renal calculi

Nephrocalcinosis is a chronic condition affecting the kidney and is characterized by the presence of mineral deposits within the renal tissue. Farmed fish are more sensitive to environmental factors as they live in a restricted environment and they are also often subject to other contingent stressors. Nephrocalcinosis is usually associated with increased levels of free CO_2 in the water and coexisting conditions, such as decreased levels of dissolved oxygen and reduced pH (Vatsos and Angelidis, 2017). It has been suggested that a mechanism that leads to these lesions in the kidney could be the precipitation of the calcium hydrogen phosphate present in the urine when the urinary pH increases (Roberts and Rodgers, 2001). This change could occur in fish exposed to elevated CO_2 concentration. However, several studies showed that other mechanisms are involved in the onset of the nephrocalcinosis, such as high levels of calcium coupled with phosphorous in the diet (Richardson *et al.*, 1985 quoted by Vatsos and Angelidis, 2017)**.** Within the nephrocalcinosis condition, urinary calculi, mainly calcium phosphate crystals, Ca5(PO4), can be observed in the urethra or urinary bladder. According to FAO (Food and Agriculture Organization) in the "Manual on Hatchery Production of Seabass and Gilthead Seabream" (1999), calculi are the earliest signal of poor quality in a larval batch and they are correlated with environment-induced stress. When a large calculosis, (more than 40% of the fish), is observed, it can be interpreted as a sign of poor rearing conditions which typically will result in a low survival rate and environmental and feeding parameters (excessive water currents due to wrong aeration or water inflow, disproportionate prey size, insufficient light intensity, dangerous levels of some water quality parameters), or even, when the total observation are higher than 30% during the first feeding days (5 to 15) it has been recommended cultures be restarted with a new population of larvae. In the MSSS calculi are considered as a descriptor related to the excretory system. The score attribution is based on their dimension as indicated in **Table 10**.

Table 10. Score attribution criteria for calculi in the excretory system

DESCRIPTOR	SCORING				
	1	2	3	4	5
EXCRETORY SYSTEM					
Calculi (dimension)	Absent	Small (From traces to 50µm)	Medium (From 51µm to 200µm)	Large (From 201µm to 400µm)	Very large (More than 400µm)

5.3.4 Histological Index calculation

Through a weighted summation based on the general architecture of each organ district, the descriptors that are part of the MSSS are summarized in a single cumulative histological index (CHI) that returns immediate information on the histological quality of the sample. To the general architecture, a score from 1 to 5 is also attributed as shown in **Table 11**. Within the general architecture are eventually included regressive and progressive alterations.

Table 11. Criteria for score attribution to the organ general architecture

	SCORING				
	1	2	3	4	5
ARCHITECTURE	Normal	Mild alterations	Moderate alterations	Severe alterations	Unrecognasible

5.3.4.1 Good quality and bad quality batches examples

Table 12 shows an example of the evaluations of two SBG batches. All the MSSS descriptors were evaluated and summarized to obtain a single value per organ district. These values were finally summed to obtain a CHI for each stage. What can be observed in the last column is that in all developmental stages, except for the first feeding stage, the batch of good quality SBG has a lower CHI value compared with the batch of SBG of bad quality. According to the assumptions of our evaluation system, this means that the good batch had a better histological quality than the bad. It can be noted that the high CHI values found in the poor quality batch are, in this case, influenced above all by the gills, the intestine (both anterior and posterior) and the excretory system. The value recoded for the gills probably indicates inadequate water parameters, this hypothesis appears to also be confirmed by the results of the excretory system data. Moreover, the values related to the anterior and posterior intestine are particularly high in the juvenile phase of the second batch probably due to metabolic difficulty; based on this data, the indication that could be given to the farmer would be to check water quality, to review the feeding strategy and try to

adapt it. Further details on the batches histological quality can be extrapolated from the analysis of the individual descriptors (data not shown).

Table 12. Example of the evaluation of a good quality and a bad quality GSB batch

BATCH	STAGE	G	L	AI	PI	PAT	E	CHI
GOOD BATCH	FIRST FEEDING	/	3	5	6	2	2	18
	FLEXION	3	2	5	4	2	2	18
	END LARVAL REARING	3	2	5	4	3	2	19
	MIDDLE METAMORPHOSIS	8	4	14	6	3	2	37
	JUVENILE	13,75	3,5	10	6,5	5	6	47,5
BAD BATCH	FIRST FEEDING	/	2	5,5	6	2	2	17,5
	FLEXION	6	4	6	5	2	2	25
	END LARVAL REARING	8	3	9	5	2	3	30
	MIDDLE METAMORPHOSIS	15	4	18	7	2	4	50
	JUVENILE	12	4	24	12	7	16	75

Abbreviations: G – gills; L – liver; AI – Anterior intestine; PI – posterior intestine; PAT – pancreas and adipose tissue; E – excretory system; CHI – cumulative histological index.

5.3.5 Immunohistochemistry

Only 2 out of the 4 antibodies tested effectively reacted with the BSS tissues and 2 out of 5 with the SBG tissues. Protocols and optimal dilutions are shown in **Table 13**. The cell types identified with these antibodies are: SBG acidophilic granulocytes (G7); BSS mast cells (Histamine); BSS and SBG B lymphocytes and plasma cells (IgM).

Table 13. Primary antibodies used in conventional and EnVision™ FLEX (Dako, Agilent) IHC tests, listed in alphabetical order

MARKER	COMPANY	DEVELOPED IN	TESTED SPECIES	PROTOCOL	ANTIGEN RETRIVIAL	OPTIMAL DILUITION
CD68	abcam ab199000	Mouse	ESB	EnVision™ FLEX	Low pH	nd
					High pH	nd
			GSB	EnVision™ FLEX	Low pH	nd
					High pH	nd
GM-CSFR*	Santa Cruz Biotechnologies 690	Rabbit	ESB	Conventional	/	nd
			GSB	Conventional	/	nd
G7	Prof. Mulero, Univ. of Murcia	Mouse	GSB	EnVision™ FLEX	Low pH	1:200
Histamine	Sigma Aldrich H7403	Rabbit	ESB	EnVision™ FLEX	Low pH	1:200
			GSB	EnVision™ FLEX	Low pH	nd
					High pH	nd
				Conventional	/	nd
					0,5% Trypsin	nd
					Low pH	nd
IgM (ESB)	Univ. of Trieste	Rabbit	ESB	Conventional	/	1:4000
IgM(GSB)	Univ. of Trieste	Rabbit	GSB	Conventional	/	1:2000

*no other protocols were tested with this antibody since it is out of production. *nd – not detected.*

5.4 Discussion and Conclusion

The hatchery phase still represents one of the main bottlenecks in marine larval rearing and one of the aims of the H2020 PerformFISH project is to find new quality markers applicable to ESB and SBG larvae and juveniles. In this light, and according to the literature revision performed, histology could be a useful tool for early recognition of poor quality larval and indicate actions that can be taken on specific factors that can limit production. Indeed, each biotic and abiotic factor and each managerial decision has a potential impact and can positively or negatively affect the fish health condition.

In the present study an original histological scoring system, specifically designed for the evaluation of marine larvae and juveniles, has been created. However, UNIUD underlines that histology provides indications of the fish morphological condition in a precise instant of their life history and, therefore, it is not possible to obtain predictive information of the fish quality at the end of the production cycle. Furthermore, fish larval and juvenile stages have an extraordinary growth potential which is also expressed as high competence in repairing or regenerating (process by which damaged or lost structures are perfectly or near-perfectly replaced) tissue due to pathological events (infectious and non-infectious) (Poss et al., 2003; Beraldo and Canavese, 2011; Zupanc and Sîrbulescu, 2013; Choi *et al*., 2015; Wolf *et al*., 2015; Bates *et al*., 2018). If these characteristics are not taken into account it will lead potentially to the overestimation of the long-term impact of certain lesions on the health of individual fish and fish populations (Wolf *et al*., 2015). This is why the developed **MSSS should not be used for a predictive long-term quality evaluation but rather as an effective tool in the early highlighting of health problems in a production batch/population and therefore helps farmers to solve critical problems in the hatchery phase**.

An algorithm that allows to summarize all the descriptors in a single number (Cumulative Histologic Hindex – CHI) has been developed. Having a single value that indicates the histological status of a batch is useful to obtain a first glance on the status of the batch itself. The CHI does not give information on which organ district is most affected by a problem, but if high, it suggests that something may be wrong and it can act as an “early warning system” for the farmer. More specific information is given by individual descriptors, which also provide a way to understand what is specifically causing the problem. Immunohistochemical techniques have been applied in this work to characterize and describe the inflammatory infiltrate found in the organ districts, believing that this may be a further interesting information. Only 2 antibodies per species reacted with tissue antigens, allowing the identification of mast cells in ESB, acidophilic granulocytes in SBG and B lymphocytes and plasma cells in both species. The cause of the failure of some antibodies used could have been the lack of cross-reactivity with the tested species antigens (as in the case of CD68, which is considered a validated marker for mammalian macrophages), or antigen “masking” promoted by the fixative used in the study or by long preservation in ethanol of the samples.

6 Molecular quality indicators

6.1 Introduction

The contribution of juvenile quality to production sustainability is widely recognized and a big volume of research and alternative husbandry practices have been dedicated in mastering the production at the early stages of development. The ultimate goal is the production of robust, fast-growing juveniles to increase MMFF competitiveness. All these factors that establish a high growth potential and promote the development of a mature, highly responsive immune system are of high importance in the hatchery phase of production. Quality indicators that are linked to all these processes underlying growth and immunity were validated.

UTH focused on marker genes underpinning energy balance and muscle and adipose development and growth, important components contributing to the muscle quantity and quality when fish are harvested. UNIUD complemented by some targeted work by CCMAR aimed to identify marker genes indicative of the immunocompetence status of BSS and SBG larvae and juveniles. All candidate genes were studied from a comparative perspective in batches classified as good and bad using the growth parameter as the indicator.

6.2 Growth Indicators

White muscle is approximately 50% of the fish body, the biggest growing tissue and the final product in fish farming. To a great extent, growth is the synthesis and deposition of proteins in white muscle. This process is finely regulated according to the energy supply and reserves. Myogenesis is common to all vertebrates and consists of serial complex events involving the specification, proliferation, differentiation, migration and fusion of precursor cells to form multinucleated muscle fibres. Hyperplastic growth dominates the first stages of white muscle development, whereas hypertrophic growth becomes the major growth mechanism at the later stages (Valente et al., 2013).

In SBG, a clear stage of stratified hyperplasia was identified early in the development but ceased by 35 dph when hypertrophy took over. Mosaic recruitment of new white fibers began as soon as 60 dph. The expression of genes encoding for structural and regulatory molecules appeared well orchestrated in the early SBG development (see **Table 14**) (Georgiou et al., 2016).

The genes myosin light chain, phosphorylatable, fast skeletal muscle b (*mylpfb*) and myosin light chain, phosphorylatable, fast skeletal muscle a (*mylpfa*) encoding for myosin light chain 2 (MLC2) isoforms were expressed at various levels during the main phases of hyperplasia and hypertrophy. The genes myogenin (*myog*) and *mylpfb* were significantly up-regulated during the intensive stratified formation of new fibers and their expression was significantly correlated. Expression of myostatin 1 (*mstn1*) and insulin-like growth factor 1 (*igf1*) increased at 35 dph, appeared to regulate the hyperplasia-to-hypertrophy transition, and might stimulate the expression of *mylpfa, mylpfb* and collagen alpha-1(I) chain-like (*col1a1*) at the onset of mosaic hyperplasia. The up-regulation of *mstn1* at transitional phases in muscle development indicates a dual regulatory role of myostatin in fish larval muscle growth (Georgiou et al., 2016). Moreover, the two isoforms of MLC2, *mylpfb* and *mylpfa,* marked hyperplasia and hypertrophy, respectively, while the ratio of *mylpfb/mylpfa* expression exhibited a tight, significantly negative

correlation to total length of juveniles, independent of age, origin or rearing conditions providing evidence for the robustness of MLC2 expression as a growth marker (Georgiou et al., 2014).

Table 14. Genes targeted in SBG larvae and juveniles as growth indicators

Gene name	Complete gene name	accession number	database
MYLPFB	myosin light chain, phosphorylatable, fast skeletal muscle b	XM_030399973.1	NCBI
MYLPFA	myosin light chain, phosphorylatable, fast skeletal muscle a	XM_030408088.1	NCBI
LPL	lipoprotein lipase	XM_030434118.1	NCBI
mstn1	Myostatin; growth/differentiation factor 8	ENSSAUG00010004791	ENSEMBL
MYL1	myosin, light chain 1, alkali; skeletal, fast	XM_030428754.1	NCBI
MYLZ3	myosin, light polypeptide 3, skeletal muscle	AF149756.1	NCBI
MYOG	myogenin	XM_030419701.1	NCBI
PPARG	peroxisome proliferator-activated receptor gamma	XM_030421187.1	NCBI
col1a1a	collagen alpha-1(I) chain-like	ENSSAUG00010019190	ENSEMBL
RPS18	40S ribosomal protein S18	XM_030410417	NCBI
RPL13	ribosomal protein L13	XM_030415490.1	NCBI

Similarly, in BSS *col1a1* expression in development was positively correlated with bone development (Darias et al., 2008) suggesting that *col1a1* is of huge biological importance by providing mechanical support in both the growing skeleton and the increasing muscle fiber organization.

Myostatin is considered to be an inhibitor of proliferating myoblasts and a major negative regulator of fiber number (Ostbye et al,. 2001). Two *mstn* paralogs have been identified in gilthead sea bream, namely *mstn1* and *mstn2; mstn1* is predominantly expressed in muscle and *mstn1* gene polymorphism has been associated with growth traits (Sánchez-Ramos et al., 2012).

White muscle and adipose tissue represent the physiological compartments of energy storage and protein accretion, respectively, and a series of molecules have been implicated in the cross-talk between the two tissues and the regulation of fatty acid metabolism and protein turnover (**Figure 7**). Moreover, dietary polyunsaturated fatty acids (FA) have been recognized of crucial importance in early development of fish as building blocks and to energy suppliers. Several genes encoding for key-players have been identified (**Figure 7**). Genes that are involved in the uptake and mobilization of FA stores and the cross-talk between white muscle and adipose tissue, and their correlation with the mechanisms of muscle development in SBG were targeted. Myostatin is a member of the TGF-β family that besides an inhibitory role in muscle growth it is known to promote adipogenesis in mesenchymal multipotent cells (Artaza et al., 2005).

Lipoprotein lipase (LPL) catalyses the hydrolysis of the triacylglycerol component of circulating chylomicrons and very low density lipoproteins, thereby providing non-esterified fatty acids and 2-monoacylglycerol for tissue utilisation (see **Table 15** for gene candidates). Comparative genomics analysis has revealed the presence of a paralogue *lpl* gene, named lipoprotein lipase-like (*lpl-like*), expressed in fish muscle at high levels (Simó-Mirabet et al., 2018). Our preliminary results showed that *lpl-lik*e is expressed in the early stages of larval development with the maximum peak occuring at 25 dph, an age which coincides with the beginning of exogenous feeding and active swimming. By metamorphosis

(58dph) the levels of *lpl-like* expression were significantly higher in larger individuals compared with the smaller.

Peroxisome proliferator activated receptor gamma (PPARγ) is a major transcription factor regulating genes implicated in lipid metabolism (Cruz-Garcia et al., 2009). Our work in SBG has revealed that *PPaRγ* expression remained significantly high during the stage of hyperplasia between 5 and 25 dph and declined towards the transition stage from hyperplasia to hypertrophy, exhibiting a lower expression level during the hypertrophic stage compared with that observed during the hyperplastic stage.

AMPK senses the ATP availability and acts as a central metabolic switch in the cell to govern major metabolic decisions. It is a complex molecule built by three subunits, α, β and γ (Mihaylova et al., 2011). Multiple paralogues encode for each subunit in SBG and BSS. The sufficiency of ATP produced by oxidative phosphorylation in the mitochondria drives AMPK responses. Thus, cellular energetics are very closely monitored before protein accretion and growth is allowed.

UTH welcomed a PhD trainee (Andreas Tsipourlianos) to their research team to work on marker discovery associated with nuclear genes encoding for proteins of the OxPhos complexes. OxPhos is central to cellular energetics and its function and efficiency is considered crucial to energy-consuming processes such as development and growth. OxPhos genes that retain multiple copies (paralogues) in gilthead sea bream and European sea bass have been identified and characterized using comparative genomics in all of the complexes. The pattern of expression of the paralogue genes at different developmental stage and in larvae with different growth rates was studied to identify those paralogues most closely linked to increased growth rate.

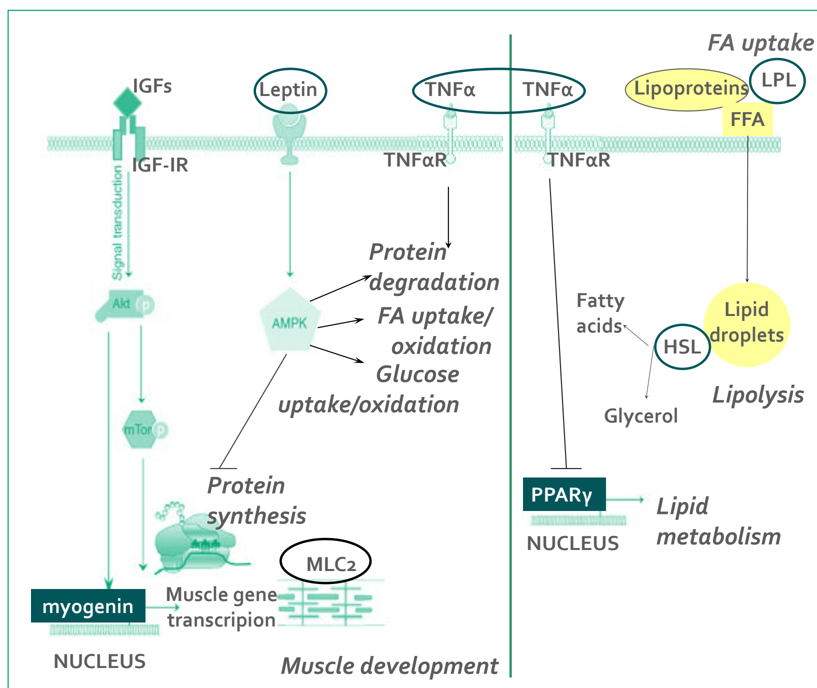


Figure 7. Molecular links between muscle development and growth and lipid metabolism. Adapted from Johnston 2006 and Cruz-Garcia et al. 2009.

6.2.1 Materials and Methods

Samples

cDNA collections derived from larvae from SBG and BSS at FF, Fl, ELR and MM were delivered to UTH by CCMAR. To date, 22 and 7 batches of SBG and BSS (6 individuals / stage/batch/ LTP), respectively have been analyzed.

Candidate gene number

Basic comparative genomics analysis was followed to verify gene copy number and putative isoforms of candidate genes and to ensure that multiple forms are considered during the analysis, as previous work has shown differential expression of isoforms may be important (Georgiou et al., 2014). The genomes of fifteen (15) species were investigated for paralogs of the genes encoding for the candidate genes. Specifically, the genomes of *Callorhinchus milii* (elephant shark) as a representative of Chondrichthyes, *Latimeria chalumnae* (coelacanth), *Homo sapiens* (human), *Mus musculus* (mouse) and *Gallus gallus* (chicken) as representatives of Sarcopterygii and finally *Lepisosteus oculatus* (spotted gar), *Oreochromis niloticus* (tilapia), *Oryzias latipes (*medaka), *Poecilia Formosa* (amazon molly), *Xiphophorus maculatus* (platyfish), *Gasterosteus aculeatus (*stickleback*), Sparus aurata (*gilthead sea bream), *Dicentrarchus labrax* (European sea bass), *Takifugu rubripes* (fugu) and *Tetraodon nigroviridis* (tetraodon) as representatives of Actinopterygii. Homolog genes were identified and retrieved from their corresponding databases using BLAST. Orthology and paralogy relationships were estimated by a reciprocal BLAST strategy. In several cases pairs of paralogues in the BSS and SBG genomes were selected for expression analysis.

Gene expression

The expression of target genes was determined by real time-qPCR. Primers were originally designed for nine (9) genes in SBG (**Table 14**) and their expression was normalized against the expression of RPL13a and RPS18 that were used as reference genes. Similarly, primers were originally designed for eighteen (18) genes were targeted for optimization and validation in BSS (**Table 15**). Their expression was normalized against the expression of RPL13a and FAU that were used as reference genes. Five (5) genes were common between SBG and BSS.

Real-time polymerase chain reaction (Q-PCR) was conducted in a Step-One Real-Time PCR system (Applied Biosystems) in duplicate by using the Applied Biosystems SYBR PCR Master mix. All reactions comprised 150-300 nmol/l of each primer and 0.15 μg/μl cDNA (1:30 diluted) in a reaction volume of 10 μl. The following PCR conditions were used: an initial denaturation step at 95 °C for 3 min, 40 cycles of amplification (each cycle was 30 s at 95 °C, 1 min at 61 °C, 1 min at 72 °C), followed by the dissociation curve step (1 min at 95 °C, 30 s at 55 °C, 30 s at 95 °C) to verify the amplification of a single product. Efficiency curves were obtained for each cDNA template by plotting CT values against the $\log_{10}$ of six serial dilutions of a cDNA pool created from all samples analyzed. Q-PCR efficiency (E) was calculated according to $E = 10^{[-1/slope]}$ (Pfaffl 2001) and varied between 96 % and 104 %. The normalization factor was calculated as the geometric mean of two housekeeping genes.

Statistical Analysis

A multi-tier statistical analysis was followed 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 can differentiate good from bad batches.

Table 15. Genes targeted in BSS larvae and juveniles as growth indicators

Gene name	Complete gene name	accession number	database
MYL1	myosin light chain 1	DLAgn_00049580	UCSC
MYLPFA	myosin light chain 2	DLAgn_00193180	UCSC
MYLPFB	myosin light chain 2	DLAgn_00101680	UCSC
MYLZ3	myosin light chain 3	DLAgn_00137220	UCSC
PPARG	peroxisome proliferator-activated receptor gamma	DLAgn_00094900	UCSC
PRKAA2	5 -amp-activated protein kinase catalytic subunit alpha-2	DLAgn_00152080	UCSC
AAKB1	5-amp-activated protein kinase subunit beta-1	DLAgn_00114660	UCSC
PRKAB1	5 -amp-activated protein kinase subunit beta-1	DLAgn_00086660	UCSC
PRKAG3	5 -amp-activated protein kinase subunit gamma-3-like	DLAgn_00139480	UCSC
PRKAG2	5 -amp-activated protein kinase subunit gamma-2-like	DLAgn_00005960	UCSC
NDUFS1A	nadh-ubiquinone oxidoreductase 75 kda mitochondrial isoform 1	DLAgn_00051250	UCSC
NDUFS1B	nadh-ubiquinone oxidoreductase 75 kda mitochondrial-like	DLAgn_00139760	UCSC
UQCR11A	cytochrome b-c1 complex subunit 10	DLAgn_00004580	UCSC
UQCR11B	cytochrome b-c1 complex subunit 10	DLAgn_00150950	UCSC
UQCRC2A	cytochrome b-c1 complex subunit mitochondrial-like	DLAgn_00189790	UCSC
UQCRFS1A	cytochrome b-c1 complex subunit mitochondrial precursor	DLAgn_00163360	UCSC
UQCRFS1B	cytochrome b-c1 complex subunit mitochondrial-like	DLAgn_00165490	UCSC
UQCRHB	cytochrome b-c1 complex subunit mitochondrial precursor	DLAgn_00003700	UCSC
RPL13	60s ribosomal protein l13	DLAgn_00158470	UCSC
FAU	ubiquitin-like protein fubi	DLAgn_00067980	UCSC

6.2.2 Results

Candidate gene number

Comparative genome analysis allowed the determination of the actual number of genes encoding for markers in SBG and BSS genomes. The analysis undertaken for genes encoding for Complex III of OxPhos is provided here as an example. A total of fifteen (15) species were investigated for paralogs of the nine (9) nuclear encoded subunits of OXPHOS complex III (**Figure 8**). Some species display gene losses. In four genes, cytochrome b-c1 complex subunit mitochondrial-like (*uqcrc2*)*,* cytochrome b-c1 complex subunit mitochondrial precursor (*uqcrh*)*,* cytochrome b-c1 complex subunit 10 (*uqcr11*), and cytochrome b-c1 complex subunit mitochondrial precursor (*uqcrfs1*), paralogs were identified at numerous taxa of the lineage of teleosts, while in the rest of the species, only taxa specific duplications were observed. Gene *uqcrc2* is a paralog of *uqcrc1* and of 5 more genes belonging to the peptidase M16 family. Neither *uqcrc1* nor the other members of M16 peptidase were investigated further due to the absence of paralogs in teleosts and lack of functional contribution to the OXPHOS complex III in focus. The four (4) complex III nuclear genes with extra copies in teleosts appear to play distinct roles in the function of complex III, based on human genes function annotation and they are characterized by a variety of gain/loss patterns amongst investigated species.

A single copy of *uqcrh* gene was identified at the genomes of Elephant shark, Sarcopterygii and Spotted gar, with the exception of human, where two copies exist. Two genes copies were identified in both BSS and SBG. *uqcr11* gene is not predicted at the genomes Spotted gar and Stickleback. A single copy of the gene was identified at the genomes of Elephant shark, Platyfish, Medaka and Sarcopterygii with the exception of human that has two (2) copies of the gene, with one of them (ENSG00000267059) being a pseudogene according to the annotation. Additionally, in the 6 out of the 9 teleost species that were investigated, two copies of *uqcr11* were found including BSS and SBG. A single copy of *uqcrc2* gene was identified in the genomes of Sarcopterigii, Elephant shark, Spotted gar and Gilthead sea bream. Two (2) copies of the gene were identified in the genomes of all teleost species that were examined, including BSS. A single copy of *uqcrfs1* gene was identified in the genomes of, Elephant shark, Sarcopterigii, Spotted gar and Gilthead sea bream. Two copies were identified in the genomes of most of the teleost species, BSS including. Finally, three (3) copies were identified in the genome of Fugu.

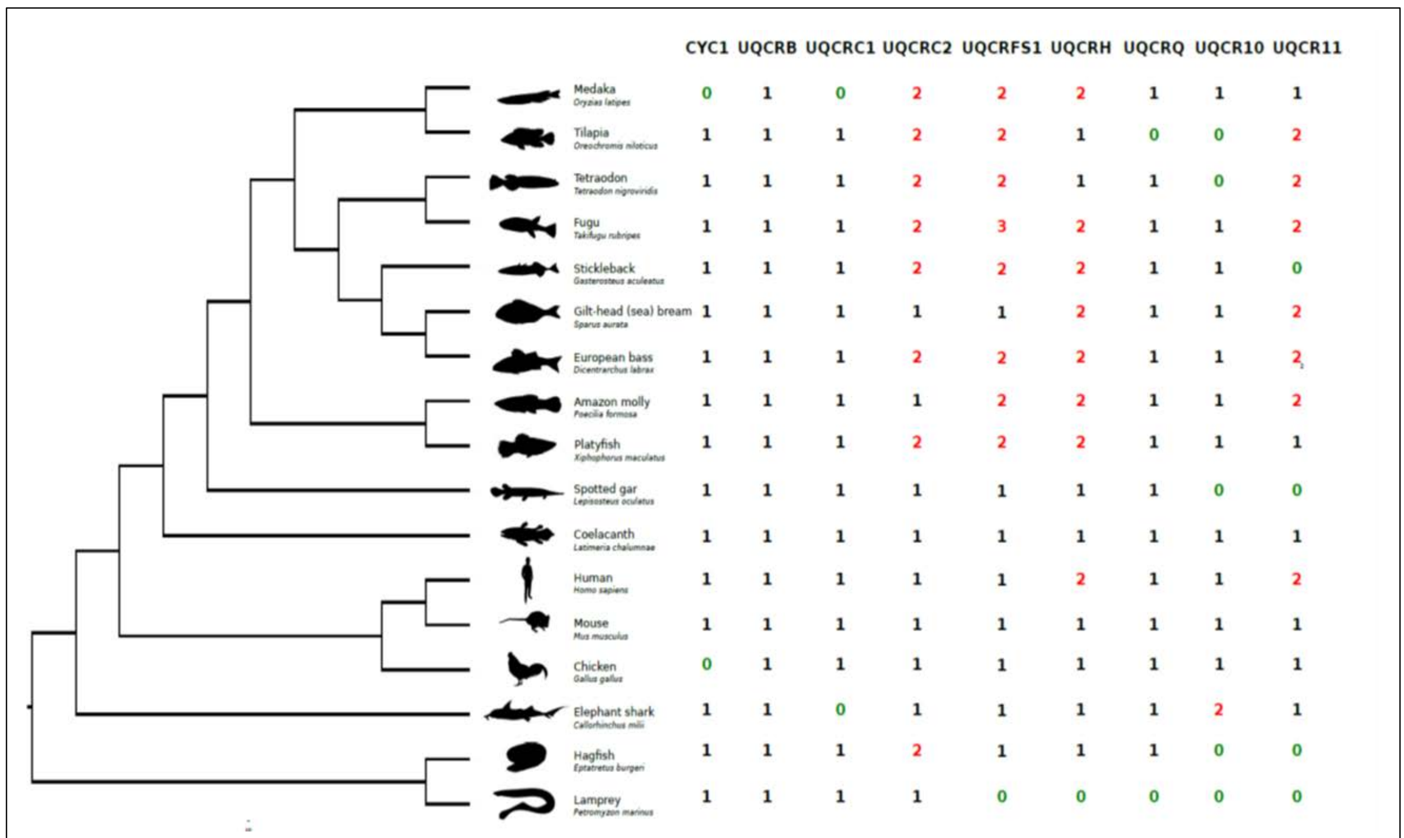


Figure 8. Species tree constructed with complete MT-DNA sequences. Complex III genes and their copy number for each species. Genes with paralogs are marked red while gene absence is marked green

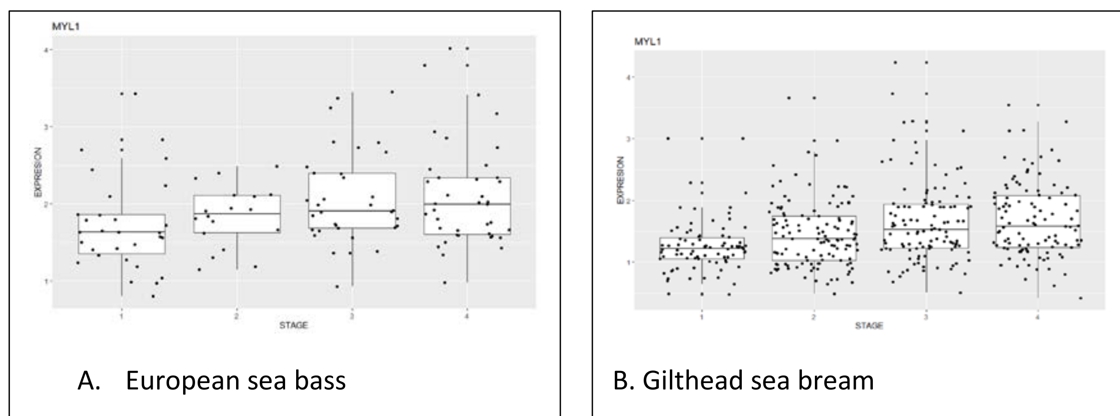
Gene expression

The genes examined both in SBG and BSS exhibited similar ontogenetic patterns when all samples from all batches were considered together (**Figure 9**). However, significant differences were apparent in the ontogenetic pattern between batches and when samples from each stage were compared (**Figure 10**), indicative of a great degree of plasticity at each stage. However, this plasticity did not reflect the quality of the batch.

Significant coordination in the expression of the different genes was observed (**Figure 11**, **Figure 12**) in both species. Correlation matrices revealed a tight coordination of gene expression at the early developmental stages that weakens as development progresses. In SBG, 14 significant correlations at FF were observed, whereas only 5 were detectable at MM. A similar trend was also observed in BSS (**Figure 12**). In SBG, significant and stable correlations across developmental stages were observed between *col1a1* and *myl1*, *lpl* and *myog*, *myl1* and *mylZ3*, *myl1* and *mylfpb*, *mylZ3* and *mylfpa*. Interestingly, the expression of *myl1* and *mylfpb*, *myl1* and *mylZ3* were also significantly correlated across developmental stages in BSS.

Combining markers increased the resolution of comparison between batches. Each two markers with significant correlation at MM stage were plotted in the same graph (**Figure 13**) including all the individuals from all batches in comparison. The first and third quartile of each marker are indicated on the plot in vertical and horizontal lines. A fairly consistent trend was observed; individuals falling outside the box outlined by the quartiles and in the lowest left part of the plot belonged to batch with high average total length, ie a good batch. On the contrary, individuals positioned in the highest right part of the plot belonged to a bad batch.

The trend was particularly consistent for marker genes encoding for OxPhos complexes in BSS. No such trend was explored in SBG. The multi-tier statistical analysis developed has generated a robust multi-gene analysis approach that has been used to 1) identify informative markers genes that 2) define “good” and “bad” hatchery batches/ populations.



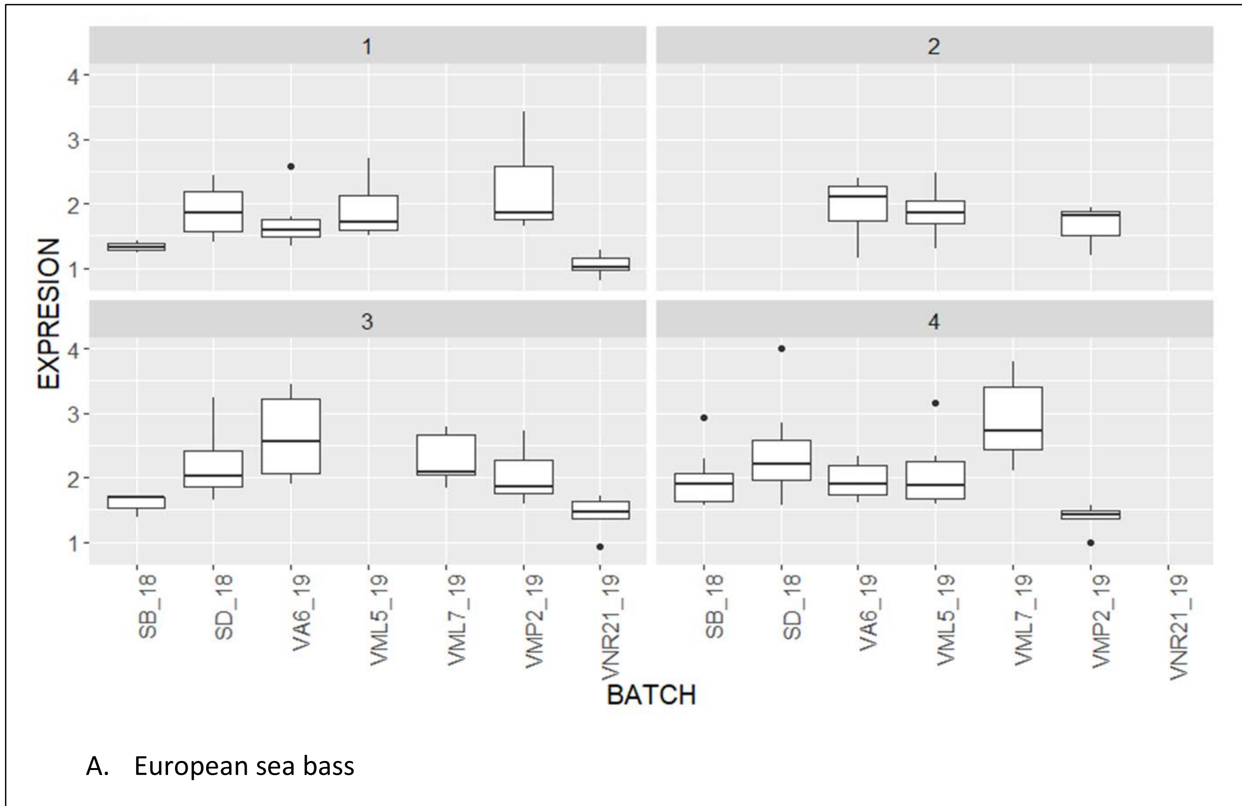
A. European sea bass

B. Gilthead sea bream

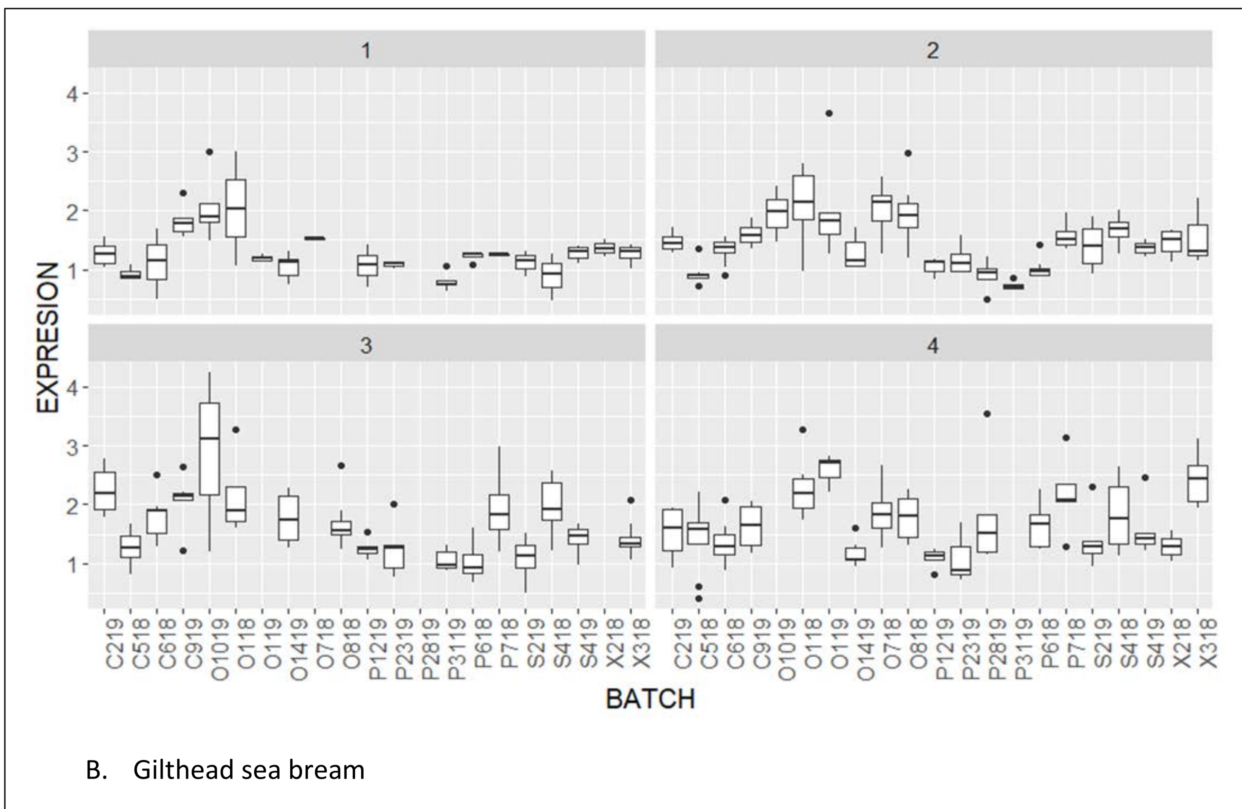
Figure 9. Expression of myl1 in BSS (A) and SBG (B) larvae at FF, Fl, ELR and MM stages.

6.2.3 Conclusion

Significant deviations in the ontogenetic pattern of the candidate genes were recorded between SBG and BSS commercial batches at all four developmental stages analyzed. The expression of many of the candidate genes appeared well coordinated, particularly at the early developmental stages. No single marker differentiated between good and bad batches. Combined analysis of markers in BSS using the developed multi-gene analysis approach revealed a differentiating trend. Analysis in SBG will be enriched with markers that displayed discriminatory capacity in BSS. COVID-19 outbreak has brought a halt in the work on SBG, which is delayed relative to BSS.



A. European sea bass



B. Gilthead sea bream

Figure 10. Expression of myl1 in batches of BSS (A) and SBG (B) larvae at FF, Fl, ELR and MM stages.

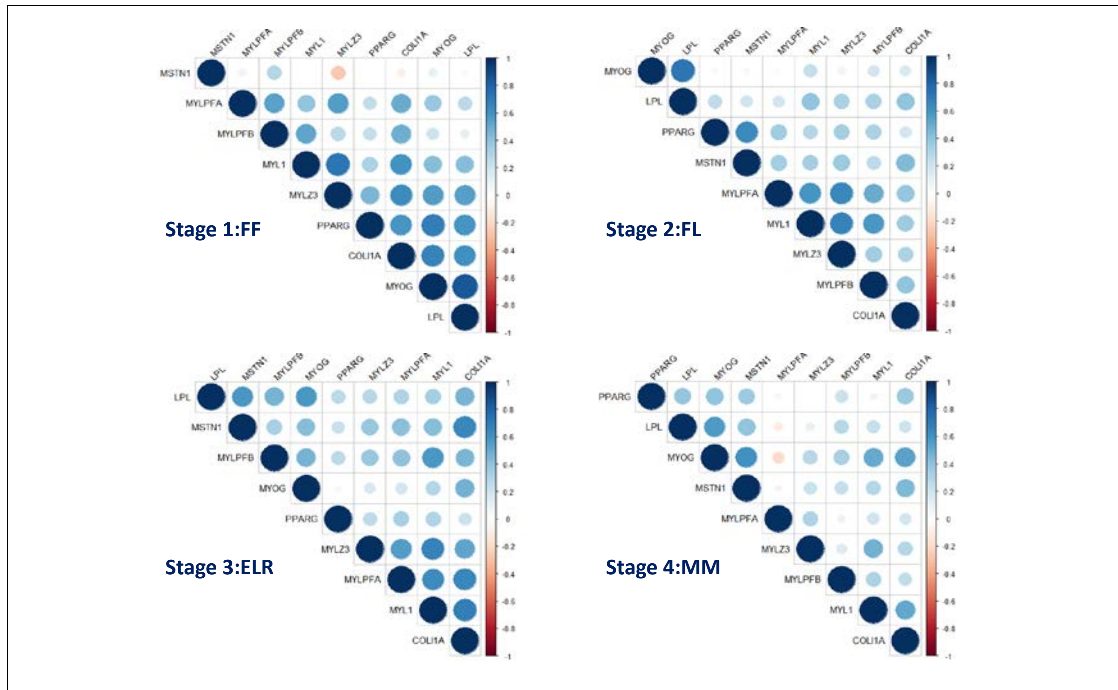


Figure 11. Correlation matrices of marker gene expression SBG larvae at FF, Fl, ELR and MM stages. Positive and negative correlations are indicated as blue and pink dots, respectively. The size of the dot is indicative of the significance of correlation.

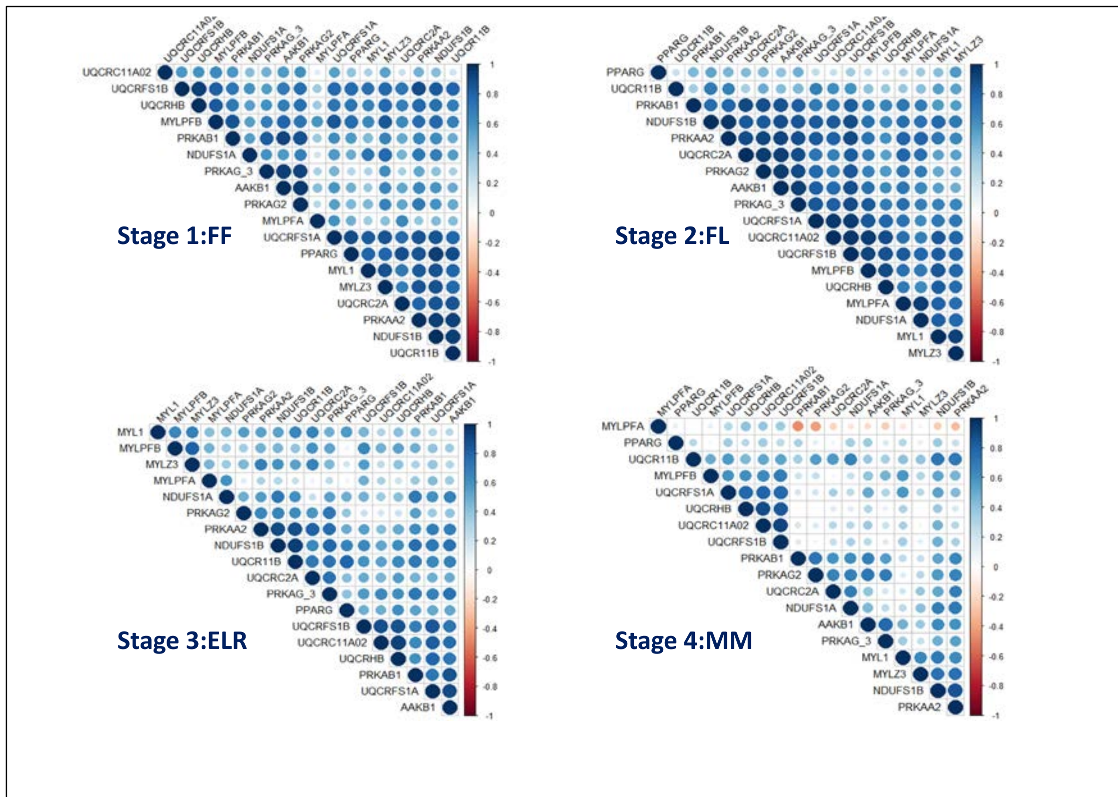


Figure 12. Correlation matrices of marker gene expression BSS larvae at FF, Fl, ELR and MM stages. Positive and negative correlations are indicated as blue and pink dots, respectively. The size of the dot is indicative of the significance of correlation.

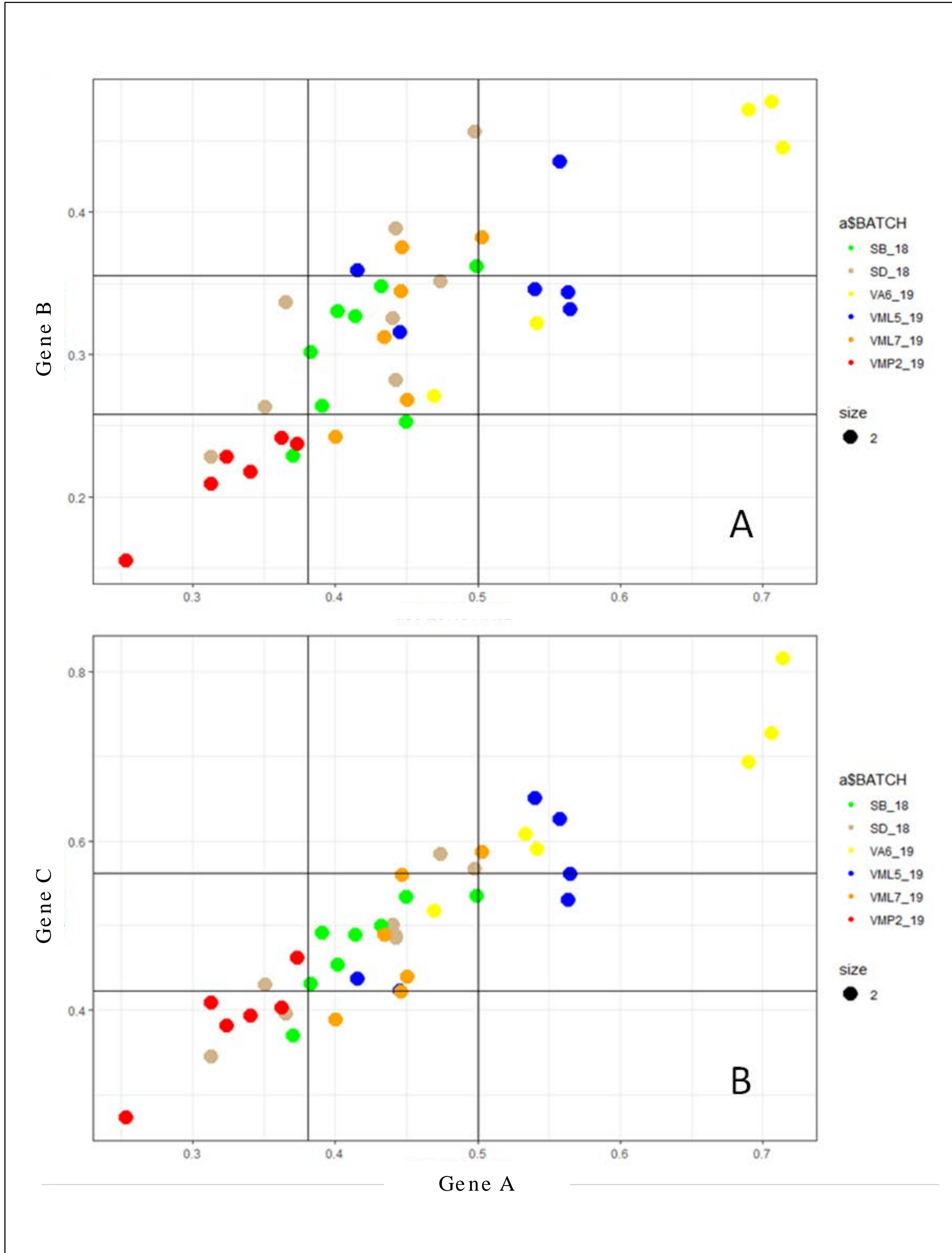


Figure 13. Scatter plot of combined individual expression of A) Gene A and Gene B, and B) Gene A and Gene C in BSS batches at MM stage. VMP2_19 is a good batch, whereas VML5_19 is a bad batch.

6.3 Immunity markers

6.3.1 Introduction

The study aims at investigating the potential of immune gene indicators as markers that could predict larval quality, based on their specific expression in the different stages of larval development. In vertebrates, immune protection is provided by both the innate and adaptive branches of the immune system (Medzhitov and Janeway, 2000). The adaptive immune response mediated by B and T lymphocytes, first emerged in vertebrates and is characterised by its specific recognition of pathogens and antigens, immunological memory and "self and non-self" recognition (Flajnik and Kasahara, 2010). In fish, innate immunity appears to have the dominant role in combatting pathogens (Bergljot, 2006) and this has led to a great diversification of genes and proteins of the innate immune system, which can make analysis more complex. Following the initial philosophy of the project to identify and utilise existing knowledge, a literature review (**Table 16**) was performed to identify known immune gene sequences to be used as markers in BSS and SBG.

In the context of immunity markers two main objectives were pursued, a) to better characterise immune gene families (to validate and improve precision of analysis), and b) to evaluate the immune-related gene markers (innate immunity, adaptive immunity, and stress) in good and bad batches of BSS and SBG using quantitative real-time polymerase chain reaction. ***To better characterise immune gene families*** (to improve precision of analysis), two main systems were evaluated, *the complement system*, a large family of plasma proteins that play a central role in innate immunity. Activation of complement occurs through three different pathways that converge on complement 3 (C3) and end in pathogen lysis. The second system analysed was the *lysozymes* (LYZs, a.k.a muramidase or N-acetylmuramide glycanhydrolase) family an ancient group of antimicrobial enzymes that break-down the peptidoglycan layer of bacteria and have a central role in innate immune defence (reviewed by Magnadottir, 2010). The teleost specific whole genome duplication together with the increased importance of the innate immune system in teleosts led to increased complexity. For this reason and to facilitate identification of the gene transcripts studied by other scientists and those that appear to be most abundant during the hatchery stage in depth analysis of these gene families were carried out.

To to evaluate the immune-related gene markers (innate immunity, adaptive immunity, and stress) in good and bad batches of BSS and SBG a literature review of reports (see **Table 16**) about immune gene expression in different fish species, including BSS and SBG was performed and candidate genes selected (**Table 17** and **Table 18**). The salient observations were that there is expression of trypsin (*tryp*) in eggs of both BSS and SBG, with an increase in abundance from 6 dpf onward (Cordero *et al*., 2016). Genes related to organogenesis, energy pathways, biosynthesis, and digestion were over-represented in BSS larvae (Darias *et al*., 2008). Interleukine 8 (*il8*), cyclooxygenase-2 (*cox 2*) and caspase 1 (*casp1*) were expressed in eggs and early stages of BSS larvae but interleukine 1β (*il1β*), tumour necrosis factor α (*tnfα*) and interleukine 6 (*il6*) were not expressed in BSS eggs. The expression of immunoglobulin M (*ighm*) and teleost immunoglobulin H (*ight*) genes were detected in eggs and from 3 dpf onwards in SBG and BSS (Darias *et al*., 2008). Some immune-relevant molecules, such as *Il1β* and macrophage colony stimulating factor receptor (*mcsfr*), are produced by germ cells and ovarian and testicular somatic cells in SBG (Chaves-Pozo *et al*., 2008). Analysis of immune gene

transcripts was established in the larval samples fixed in RNA later® from the LTP, hatcheries A, B and C. Only larval batches providing good quality total RNA were processed/analysed. The protocol for evaluating immune gene marker from larval samples was optimised. The primers use (to study the immune markers from BSS and SBG by qPCR) was standardized and validated for quantitative analysis. Overall, the expected outcome from this work on immune gene markers will be to accurately predict the specific gene expression during selected stages of the larval rearing, as well as to identify the best or poor quality larval batches in different hatcheries. This will help to understand and differentiate the larval quality status and to screen batches to be addressed to the subsequent farming phases.

The workshop to be organized (delayed due to covid-19 restrictions) for the harmonization of the markers for the juvenile certificate of quality will give the opportunity to cross-check and compare the markers tested in the collaborating partner institutions.

Table 16. Highlights of research carried out on immune gene indicators during the ontogenic development of different fish species

NO	Species	Results	Reference
1	Asian seabass (*Lates calcarifer*)	Activity of all digestive enzymes increased from around 18 dph and onwards	Srichanun *et al*., 2013 https://www.ncbi.nlm.nih.gov/pubmed/23458902
2	Meagre (*Argyrosomus regius*)	Demonstrated *defb*, *hep2*, *pisc* expression in all stages of larval development and in juvenile tissues (kidney, spleen, gut and gill).	Campoverde *et al*., 2017 https://www.ncbi.nlm.nih.gov/pubmed/28842373
3	SBG (*Sparus aurata* and BSS sea bass (*Dicentrarchus labrax*)	Expression of *tryp* demonstrated in eggs of both species, with higher values found from 6 dpf onward.	Cordero *et al*., 2016 https://www.ncbi.nlm.nih.gov/pubmed/27317010
4	SBG (*Sparus aurata* L.) and BSS (*Dicentrarchus labrax*)	Genes related to organogenesis, energy pathways, biosynthesis, and digestion were over-represented. *il8*, *cox 2* and *casp1* are expressed in eggs and early stages of larva, but *il1β*, *tnfα* and *il6* are not expressed in eggs. Expression of *ighm* and *ight* genes from eggs and from 3 dpf onwards in SBG and BSS.	Darias *et al*., 2008 https://www.ncbi.nlm.nih.gov/pubmed/18246396
5	SBG (*Sparus aurata* L.)	Some immune-relevant molecules, such as *Il1β* and Mcsfr, are produced by germ cells (*Il1β*) and ovarian and testicular somatic cells (Mcsfr).	Chaves-Pozo *et al*., 2008 https://www.ncbi.nlm.nih.gov/pubmed/18325594
7	Cod *(Gadus morhua)*	Out of 24 genes analyzed five genes were highly expressed in the unfertilized eggs, namely, *mcl1* and *NR-13*; *sod*, fortilin (*tpt*), and *glut1* Out of 24, 8 genes in larval *bcl2*, *mcl1* and *NR-13*, *mt*; an antiviral gene, *tpt* and three antioxidant genes, glutathione peroxidase (*gpx*), *hsp70* and *sod*.	Caipang and Fagutao 2015 https://onlinelibrary.wiley.com/doi/epdf/10.1111/are.12350
8	Brown trout (*Salmo trutta*)	*il1β* and *tnf-α* transcripts were detected from 4 weeks post-fertilization onwards, whereas *tgf-β* transcript was detected only from 7 weeks post-fertilization. Lysozyme c-type transcript was detected early from unfertilized egg stage onwards.	Cecchini *et al*., 2013 https://www.ncbi.nlm.nih.gov/pubmed/23765117
9	Rohu (*Labeo rohita*)	Transferrin and *tlr22-like* mRNA transcripts were detected by RT-PCR from 6 h post-fertilization to 31 days post-fertilization, whereas β-2 macroglobulin transcripts were detected only from 7 days post-fertilization onwards.	Nayak *et al*., 2011 https://www.ncbi.nlm.nih.gov/pubmed/21362484

		Lysozyme C mRNA transcripts were detected from 24 h post-fertilization to 31 days post-fertilization. Lysozyme G mRNA transcripts were detected early from unfertilized egg stage onwards.	
10	Rainbow trout (*Oncorhynchus mykiss*)	Early onset of expression was seen for all immune genes at very low levels. The amount of mRNA slowly increased and peaked around and after hatching. The highest increases were seen for *mhcII*, *c3*, *c5* and *saa*.	Heinecke *et al.*, 2014 https://www.ncbi.nlm.nih.gov/pubmed/24561127
11	Common sole (*Solea solea*)	Transcripts encoding relevant members of innate immune repertoire, such as lysozyme, AMPs (hepcidin, β-defensin), PPRs and complement components are generally characterized by high expression levels at first stages (i.e. hatch and first feeding). Transcription of adaptive immune genes (i.e. Class I and class II *mhc*, *tcr*) differs from that of the innate immune system.	Ferraresso *et al.*, 2016 https://www.ncbi.nlm.nih.gov/pubmed/27554393

Table 17. Candidate immune gene analyzed in different ontogenetic stages of "good and bad" batches/populations of GBS collected from LTP. The system in which genes are principally involved are indicated along with the gene symbol, name and principal function.

System	Gene symbol	Gene Name	Function
Innate immunity	*il1b*	Interleukin 1-beta	Pro inflammatory cytokines, which are predominantly activated by macrophages and involved in the inflammatory reactions during invading pathogens as an innate immune defense in the host.
	il6	Interleukin 6	
	il8	Interleukin 8	
	tnfa	Tumor necrosis factor alpha	
	csfr	*colony-stimulating factor-1 receptor*	
	il10	*Interleukin-10*	IL-10 is an anti-inflammatory cytokine that maintains the balance of the immune response, allowing the clearance of infection while minimizing damage to the host.
	hep	*Hepcidin*	Hepcidin is one of the antimicrobial peptides involved in the innate immune system of fish against a variety of infectious agents like bacteria, virus, fungus, and is also involved in an iron metabolism
	cox2	Cyclooxigenase 2	Cox-2 is an inflammatory related enzyme, which is responsible for alteration of Arachidonic acid into Prostaglandins often associated with closely connected to the fish innate immune response.
	tlr9	Toll-like receptor 9	TLR9 is an intracellular pathogen recognition receptor (PRR) which is responsible for recognizing unmethylated DNA from genome of bacteria.
	tlr5	Toll-like receptor 5	TLR5 as PRR recognizes flagellin of bacteria and activates innate immune response in the host.
Adaptive immunity	*ighm*	Immunoglobulin mu heavy chain	Role in adaptive immune response, which are involved in neutralizing the invading pathogen.
	ight	Immunoglobulin tau heavy chain	
	tgf-β	Transforming growth factor beta 1	Its function is to regulate the lymphocyte proliferation in fish.
Complement	*c3*	Complement 3	Important central molecule of the complement system, leading to inflammatory reactions, such

			as opsonisation, chemotaxis, and cell lysis of pathogens.
Stress	*casp1*	Caspase 1	Although they are inflammatory Caspase but the functional activates is still not clear.
Reference	*ef1a*	Elongation factor 1-alpha	Reference genes used to normalize the target molecule.
	actb	Beta-actin	

Table 18. Candidate immune gene analyzed in different ontogenetic stages of "good and bad" batches/populations of BSS collected from LTP. The system in which genes are principally involved are indicated along with the gene symbol, name and principal function.

System	Gene symbol	Gene Name	Function
Innate immunity	*cc1*	CC chemokine 1	Pro inflammatory cytokines, which are predominantly activated by macrophages and involved in the inflammatory reactions during invading pathogens as an innate immune defense in the host.
	Ifn-γ	Interferon gamma	
	il1b	Interleukin 1 beta	
	il8	Interleukin 8	
	tnfα	Tumor necrosis factor *α*	
	fer	Ferritin	Ferritin and Hepcidin are antimicrobial peptides involved in the innate immune system of fish against a variety of infectious agents like bacteria, virus, fungus, and is involved in an iron metabolism.
	hep	Hepcidin	
	tlr1	Toll-like receptor 1	PRR known to recognize bacterial lipoproteins and initiate innate response.
	tlr9	Toll-like receptor 9	TLR9 is an intracellular pathogen recognition receptor (PRR) which is responsible for recognizing methylated DNA from genome of bacteria.
Adaptive immunity	*mhc class ia*	Major Histocompatibility Complex I α	MHC I and II proteins share the task of presenting peptides on the cell surface for recognition by T cells and activates adaptive response.
	mhc class iia	Major Histocompatibility Complex II α	
	mhc class iib	Major Histocompatibility Complex II ß	
	ighm	Immunoglobulin mu heavy chain	Role in adaptive immune response, which are involved in neutralizing the invading pathogen.
	ight	Immunoglobulin tau heavy chain	
Complement system	*cla*	C-Lectin-A	Involved in lectin pathway, recognizes carbohydrates and establish homeostasis during host-microbe interaction.
	gal	Galectin	
	casp3	Caspase 3	Caspase-3 and 9 are one of the major caspases operating in apoptosis.
	casp9	Caspase 9	
Stress	*cat*	Catalase	Stress proteins and predominantly induced when fish are subjected to stress.
	hsp70	Heat shock protein 70	
Reference	*actb*	Beta-actin	Reference genes used to normalize the target molecule.
	l13a	Ribosomal protein L13 a	
	hsp90	Heat shock protein 90	

6.3.2 Materials and Methods

B) Characterization of immune gene families (to validate and improve precision of analysis)

The methodological approach followed for the characterisation of the complement system and lysozyme system was the same. There two systems were chosen as they are important in the

hatchery stages of larvae before the acquired innate immune response is fully functional. They have also been identified in eggs and so are of potential interest in relation to egg quality. The improved characterisation of gene families is essential as if there are multiple copies of a gene then it is important to verify that the most appropriate gene is being analysed in a given species and stage and also corroborate the existing gene identification in the literature and generally improve annotation of fish genes.

Evolutionary screening of the complement system and lysozyme family

Orthologues of human genes (lysozyme and complement) were procured in ray-finned fish genomes using tBLASTn (Altschul et al., 1990). We also interrogated genomes of fish species that diverged earlier from vertebrate radiation and thus are considered to possess less rearranged genomes: the lobe-finned coelacanth (*Latimeria chalumnae*), that diverged basal to the tetrapods; two cartilaginous fishes, the elephant shark (*Callorhinchus milii)* and whale shark (*Rhincodon typus*), that are basal to the bony vertebrates and the cyclostome (jawless fish), the Sea lamprey (*Petromyzon marinus*) which diverged prior to the gnathostomes. For comparative analysis to other vertebrates the mouse (*Mus musculus*), the chicken (*Gallus gallus*), the reptile (*Anolis carolinensis*) and the amphibian clawed African toadfish (*Xenopus tropicalis*) were also included in the analysis as well as two basal deuterostome genomes, the urochordate Ciona (*Ciona intestinalis*) and the cephalochordate Amphioxus (*Branchiostoma floridae*) to infer the likely evolutionary origin of the vertebrate complement and lysozyme proteins.

Sequence comparisons and phylogenetic analysis

Multiple sequence alignments were performed using the MUSCLE algorithm (Edgar, 2004) in the Aliview platform (Larsson 2014) and conserved regions were identified. GeneDoc (http://www.nrbsc.org/gfx/genedoc) was used to calculate identity/similarity between the sequences. Phylogenetic trees were constructed using Bayesian Inference (BI) methods.

Gene structure and protein motif annotation

To predict the effect of evolution on protein function, the aa sequence of selected fish complement and lysozyme deduced proteins from ENSEMBL were annotated and compared. Sequence alignments were performed using the MUSCLE algorithm in the Aliview platform and identification of protein domains was carried out using Uniprot (https://www.uniprot.org) and Interpro (https://www.ebi.ac.uk/interpro/) and the signal peptide was deduced using the SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/).

Expression of Lysozyme in eggs and early larval stages

Total RNA extraction and cDNA synthesis

Larval Sample collection and shipment is described below and was common for all partners with shipment coming to Faro and then being routed on to the different partners. So far only validation of the lysozyme system in SBG was established due to the failure to fully validate all the forms of lysozyme in BSS. These genes were also optimised for adult immune response in the context of thermal regulation of development (Task 2.2).

All samples were collected in RNAlater (Sigma) and stored at -20°C for RNA extraction. Total RNA (tRNA) was extracted from SBG using the E.Z.N.A kit (VWR, USA) according to the manufacturer's instructions. Tissues were defrosted in lysis buffer and homogenized by mechanical disruption with two iron beads (5 mm) using a Tissue lyser II Qiagen and 4 cycles of 30 seconds at room

temperature. DNase I digestion was performed directly on the columns for RNA isolation following the manufacturer's instructions. For cDNA synthesis, 500 ng of DNAse treated total RNA was used and reactions were performed with RevertAid-RT (Thermo Fisher, USA) for a 20 µL final volume with 100 pmol random hexamers, 1 mM dNTPs, 200 U of enzyme and 20 U RNase Inhibitor. Reaction conditions were 25°C, 10 min; 42 °C, 60 min; 70 °C, 10 min. The quality of cDNA was initially assessed by amplification of ribosomal subunit *18s* (**Table 19**) using the following cycle: 95°C, 3 min; 25 cycles x (95°C, 20 sec; 62 °C, 20 sec; 72 °C, 20 sec); 72 °C, 5 min. Samples for RNA extraction included eggs (n= 6 with 10 eggs per sample) and a series of consecutive post-hatching (dph) stages: 4-8 dph (n= 3 with 20 individuals per sample) when 100% of the yolk sac was resorbed; 19-23 dph (n = 6, 3 individuals per sample) when flexion occurred; 36-43 dph (n = 6, 1 individual per sample) the end of larval rearing and at 50-52 dph (n = 6, 1 individual per sample), mid-metamorphosis.

Table 19. Primers used in the amplification reactions for lysozyme in SBG. * no amplification was obtained

Primer	Sequence (5'-3')	T (°C)	Efficiency (%)	R^2
lalba-F	GCAGGAGGAAGTGAAGCAGA	62	100.2	0.99
lalba-R	GCTATGCTGAACAAGCCCGC			
lyzg1-F	TGGAGGTGGACACACTAAACGG	64	97.9	0.99
lyzg1-R	AACATCAACACCTGCAACGGTC			
lyzg2-F	TGGAGGTGGACACACTAAACGG	62	100.5	0.99
lyzg2-R	GCCGTCGACATTTTCATATGAA			
lyzg3-F*	CTGGCTGGAGCAAGGAGCAACA	-	-	-
lyzg3-R	TAGTCTCCACCATAGGTTTTGG			
lyzg4-F*	CTGGCTGGAGCAAGGAGCAACA	-	-	-
lyzg4-R	CCCTCCTGGAAAGACAAATACA			
Housekeeping genes				
18s-F	TGACGGAAGGGCACCACCAG	60	99.4	0.99
18s-R	AATCGCTCCACCAACTAAGAACGG			
ef1a-F	TCAAGGGATGGAAGGTTGAG	62	92.8	0.99
ef1a-R	AGTTCCAATACCGCCGAT			

Quantitative real-time PCR

Real-time quantitative PCR (RT-qPCR) was used to determine changes in *lyz* expression in SBG eggs and subsequent developmental stages. Specific primers were designed for the SBG *lyz* transcripts identified in this study, lysozyme C (*lalba*) and several forms of lysozyme G numbered 1 – 4 (*lyzg1*, lyzg2, lyzg3, lyzg4), but amplification products were only obtained for *lyzg1*, *lyzg2* and *lalba* (**Table 19**). Reactions were performed in duplicate (< 5% variation between replicates) using a BioRad CFX Connect Real Time System and SsoFast EvaGreen supermix (Bio-Rad, Portugal). The final reaction volume was 10 µl with 200 nM of both primers and 2 µl of template cDNA (diluted 1:5) in low volume 96-well microplates (Axygen). Optimized cycling conditions were 95 ºC, 30 sec followed by 45 cycles of 95 ºC, 5 sec; 58 ºC, 10 sec. Melting curves were performed to detect non-specific products and primer dimers. Control reactions were included to confirm the absence of contaminating genomic DNA. For this study the gilthead sea bream elongation factor 1-alpha (*ef1α*) and the 18 ribosomal subunit (*18s*) were selected as reference genes as they showed stable expression levels in the larval stages and egg analysed. Expression

data was normalized against the geometric mean of the two reference genes. PCR efficiencies and R^2 (coefficient of determination) were established with standard curves prepared in duplicate from a 10-fold serial dilution series of the purified PCR product of each of the target genes.

To associate *lyz* gene expression with gilthead sea bream egg/larval quality, samples from two egg and larval production batches from two brood stocks that had a divergent growth performance by mid-metamorphosis were compared (see 3.2).

Statistical Analysis

For q-PCR analysis significant differences between *lyz* transcript expression levels in the egg samples were detected using an unpaired t-test (two-tailed). All data is presented as the mean ± SEM and statistical analysis performed using SPSS 25.0 and $p < 0.05$ was considered significant different.

B) immune-related gene markers (innate immunity, adaptive immunity, and stress) in good and bad batches of BSS and SBG using quantitative real-time polymerase chain reaction

Larval Samples collection and shipment

Larvae and Juvenile European sea bass (*Dicentrarchus labrax, BSS*) and Gilthead sea bream (*Sparus aurata, SBG*) coming from different batches of 4 European hatcheries (identified as A, B, C, D), were sampled at 5 different developmental stages: first feeding, flexion, end of the larval rearing (10-14 mm TL), middle of the metamorphosis (15-20 mm TL) and early juvenile (0,8-1,5 g). In the hatchery, fish were caught with appropriate nets, sacrificed with an overdose of MS-222 (50-100mg/L) and immediately transferred in RNALater® (ThermoFisher Scientific) in 1:10 ratio. All these procedures were carried out in accordance with the PerformFISH Sampling Manual "Juvenile Quality and Growth Potential Standard Operating Procedures for Sampling and Data Recording". Then, samples were delivered to UNIUD facilities.

Total RNA extraction and cDNA synthesis

Total RNA was extracted using TRIzol® reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA purity and degradation was checked on a 1% agarose gel. The quality of total RNA was analyzed by a spectrophotometer using 260/280nm UV. From individual larvae sample total RNA was dissolved equally in RNase-free water. For q-PCR, 2 µg of total RNA was reverse-transcribed in a 20 µL reaction system according to the manufacturer's protocol (iScript™ cDNA synthesis kit, Bio-Rad).

Quantitative real time PCR

One microliter of each cDNA synthesis reaction was employed as the template in the qPCR reactions to analyze each gene transcription. Primers to detect expression of genes from BSS and SBG are presented in **Table 20** and **Table 21**, respectively. The primers efficiency was determined and multiple reference was used for normalizing the target gene. Amplification was performed in a final volume of 10 µL. Reaction mixture contained 5 µL of SsoAdvanced SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 0.5 µL of each primer set (10 mM), 1 µL of template cDNA and 3 µL of DEPC-water. Real time PCR determinations have been performed in triplicate in 96-well PCR plates and carried out in an CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with an initial denaturation cycle of

95 oC for 30 s, followed by 40 cycles of 95 oC for 5 s and 68 oC for 10 s. Amplification was followed by a standard melting curve from 65 oC to 95 oC, in increments of 0.5 oC for 5 s at each step, to confirm that only one product was amplified and detected. Samples were run in parallel with two reference genes, beta-actin (ACTB2) and hsp 90, for cDNA normalization. Relative mRNA expression was calculated using 2(-DDCt) method, normalizing with geometric average of two reference genes (ACTB2 and hsp90) and relative to total RNA from egg stage.

Table 20. Primers used for evaluating immune gene markers in BSS (see **Table 18** for the full name and function).

Functional group	Name	Forward Sequence	Name	Reverse Sequence
Innate immunity	*cc1-F*	TGGGTTCGCCGCAAGGTTGTT	*cc1-R*	AGACAGTAGACGAGGGGACCACAGA
	ifn-F	GTACAGACAGGCGTCCAAAGCATCA	*ifn-R*	CAAACAGGGCAGCCGTCTCATCAA
	il1b-F	CAGGACTCCGGTTTGAACAT	*il1b-R*	TTGTCCCCTTTTGAATGGAC
	il8-F	GTCTGAGAAGCCTGGGAGTG	*il8-R*	GCAATGGGAGTTAGCAGGAA
	il6-F	ACTTCCAAAACATGCCCTGA	il6-R	CTCCTTAGACTGACCAGCGG
	IL-10-F	CAGTGCTGTCGTTTTGTGGAGGGTTTC	*IL-10-R*	TCTCTGTGAAGTCTGCTCTGAGTTGCCTTA
	fer-F	ATGCACAAGCTCTGCTCTGA	*fer-R*	TTTGCCCAGGGTGTGTTTAT
	hep-F	AAGAGCTGGAGGAGCCAATGAGCA	*hep-R*	GACTGCTGTGACGCTTGTGTCTGT
	tlr1-F	GCCTCTGCCTCAATACCTGATCCCA	*tlr1-R*	AACAACCTGTGCTTGGCCCTGTC
	tlr9-F	TCTTGGTTTGCCGACTTCTTGCGT	*tlr9-R*	TACTGTTGCCCTGTTGGGACTCTGG
	tnfa-F	AGCCACAGGATCTGGAGCTA	*tnfa-R*	GGACAGCTACAGAAGCGGAC
	cox2-F	AGCACTTCACCCACCAGTTC	cox2-R	AAGCTTGCCATCCTTGAAGA
Adaptive immunity	*mhc class ia-F*	TGTACGGCTGTGAGTGGGATGATGAG	*mhc class ia-R*	AGCCTGTGGTCTTGGAGCGATGAA
	mhc class iia-F	AGTCCGATGATCTACCCCAGAGACAAC	*mhc class iia-R*	ACAGGAGCAGGATAGAAACCAGTCACA
	mhc class iib-F	GCTGGCAGACGCTGATTGGTTCT	*mhc class iib-R*	TAACCAGAGGTTCTCTCAGGCTGGC
	Ighm-F	AGGACAGGACTGCTGCTGTT	*ighm-R*	ACAACAGCAGACAGCAGGTG
	Ight-F	TCACTTGGCAAATTGATGGA	*ight-R*	AGAACAGCGCACTTTGTTGA
Complement system	*cla-F*	GATGGCAGCAAGCTCCGGTATTCA	*cla-R*	TCTGACCTATGACCCCAGCCAACA
	gal-F	TGCAACTCTTACCAGGGAGGCAACT	*gal-R*	GTCACGAGGAACTCTGTAGGGGTGA
	casp3-F	CTGATTTGGATCCAGGCATT	*casp3-R*	CGGTCGTAGTGTTCCTCCAT
	casp9-F	GGCAGGACTCGACGAGATAG	*casp9-R*	CTCGCTCTGAGGAGCAAACT
Stress	*cat-F*	TGATGGCTACCGCCACATGAACG	*cat-R*	TTGCAGTAGAAACGCTCACCATCGG
	hsp70-F	ACAAAGCAGACCCAGACCTTCACCA	*hsp70-R*	TGGTCATAGCACGTTCGCCCTCA
Reference	*actb-F*	TGAACCCCAAAGCCAACAGGGAGA	*actb-R*	GTACGACCAGAGGCATACAGGGACA
	l13a-F	TCTGGAGGACTGTCAGGGGCATGC	*l13a-R*	AGACGCACAATCTTGAGAGCAG
	hsp90-F	GCTGACAAGAACGACAAGGCTGTGA	*hsp90-R*	AGATGCGGTTGGAGTGGGTCTGT

***Table 21. Primers* used for evaluation immune gene markers in SBG** (see **Table 17** for the full name and function).

Functional group	*Name*	Forward Sequence	*Name*	Reverse Sequence
Innate immunity	*GSBil1b-F*	GGGCTGAACAACAGCACTCTC	*GSBil1b-R*	TTAACACTCTCCACCCTCCA
	GSBil6-F	AGGCAGGAGTTTGAAGCTGA	*GSBil6-R*	ATGCTGAAGTTGGTGGAAGG
	GSBil8-F	GCCACTCTGAAGAGGACAGG	*GSBil8-R*	TTTGGTTGTCTTTGGTCGAA
	GSBil10-F	AACATCCTGGGCTTCTATCTG	*GSBil10-R*	GTGTCCTCCGTCTCATCTG
	GSBhep-F	GCCATCGTGCTCACCTTTAT	*GSBhep-R*	CCTGCTGCCATACCCCATCTT
	GSBtnfa-F	CTGTGGAGGGAAGAATCGAG	*GSBtnfa-R*	TCCACTCCACCTGGTCTTTC
	GSBcox2-F	GAGTACTGGAAGCCGAGCAC	*GSBcox2-R*	GATATCACTGCCGCCTGAGT
	GSBcsfr-F	ACGTCTGGTCCTATGGCATC	*GSBcsfr-R*	AGTCTGGTTGGGACATCTGG
	GSBtlr9-F	GGAGGAGAGGGACTGGAT	*GSBtlr9-R*	GATCACACCGTCACTGTCTC
	GSBtlr5-F	CCTGTCTGCAACTGTCAGGA	*GSBtlr5-R*	TGTGGATCTGGTTCAAGCTG
Adaptive immunity	*GSBighm-F*	CAGCCTCGAGAAGTGGAAAC	*GSBighm-R*	GAGGTTGACCAGGTTGGTGT
	GSBight-F	TGGCAAATTGATGGACAAAA	*GSBight-R*	CCATCTCCCTTGTGGACAGT
	GSBTGF-β-F	AGAGACGGGCAGTAAAGAA	*GSBTGF-β-R*	GCCTGAGGAGACTCTGTTGG
Stress	*GSBcasp1-F*	ACGAGGTGGTGAAACACACA	*GSBcasp1-R*	GTCCGTCTCTTCGAGTTTGC
Reference	*GSBef1a-F*	CTGTCAAGGAAATCCGTCGT	*GSBef1a-R*	TGACCTGAGCGTTGAAGTTG
	GSBactb-F	GGCACCACACCTTCTACAATG	*GSBactb-R*	GTGGTGGTGAAGCTGTAGCC

Statistical analysis

Data were analysed using the SPSS16 (SPSS Inc., Chicago, IL, USA) statistical software and tested for homogeneity of variance before ANOVA. Data distribution was determined using descriptive statistics. Differences in the means among the treatments was investigated using ANOVA.

6.3.3 Results

A) characterization of immune gene families (to validate and improve precision of analysis)

Evolutionary screening of the lysozyme family

In fishes, species-specific evolution of *lyzc* (chicken or conventional) and *lyzg* (goose type) genes occurred (**Figure 14**). A homologue of the mammalian lactalbumin (*LALBA*) gene was identified for the first time in teleosts and was expressed during egg and larval development.

The transcript abundance of *lyzg* and *lalba* in SBG eggs and larvae from different brood stock was divergent indicating differences in maternal innate immune protection (**Figure 15**). This is now being further validated in the context of a broader screen of the larval quality of all the SBG batches and a similar exercise will be established for BSS.

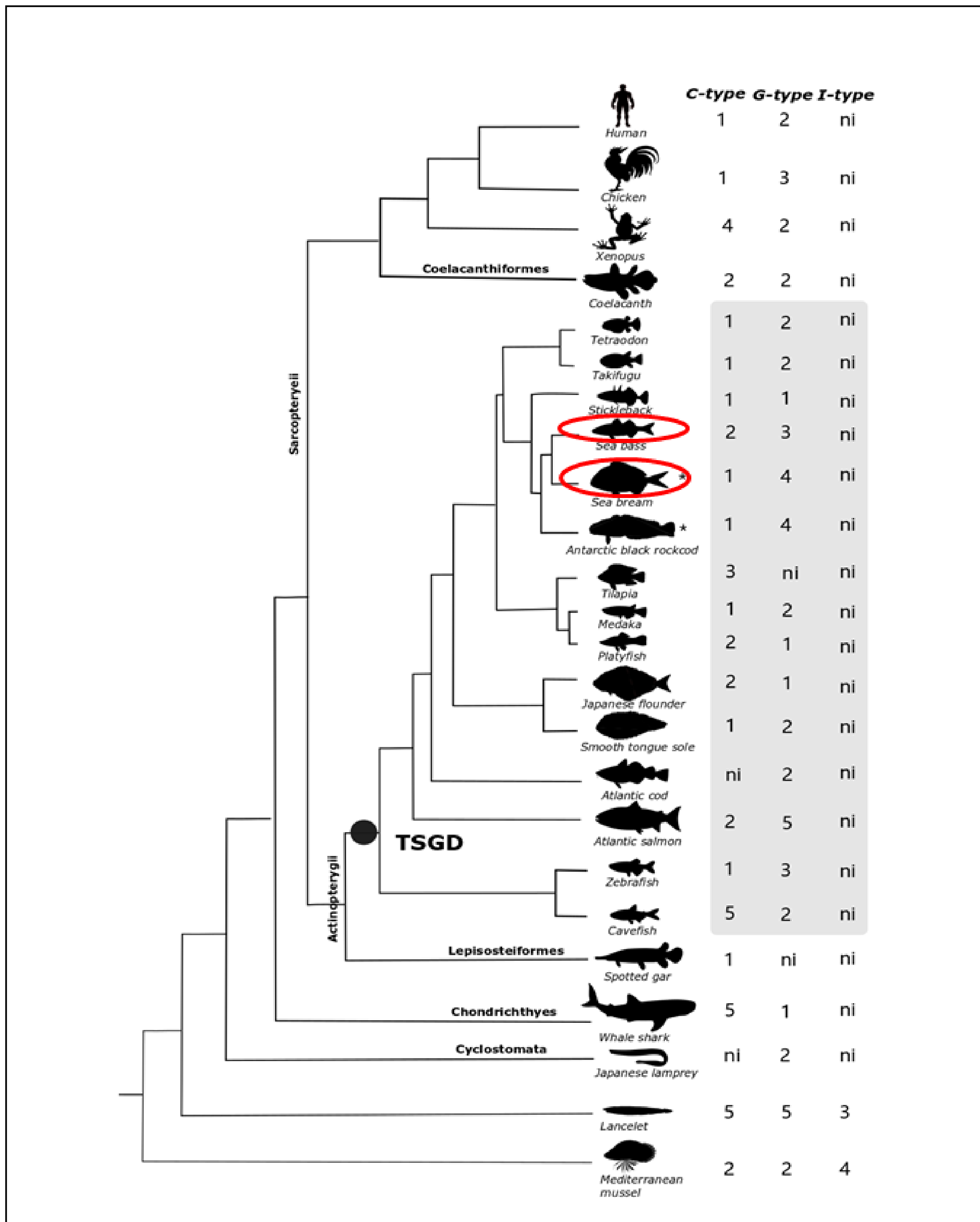


Figure 14. Number of lysozymes (Lyz) in fish. The dendogram describes the number of conventional lysozyme genes and transcripts for putative Lyz c-type and g-type retrieved from fish. Note that SBG has one form of lyzc and 4 forms of lyzg, while ESB has 2 forms of lyzc and 3

forms of lyzg. The invertebrate form of lysozyme (I-type) was only identified in the amphioxus and the Mediterranean mussel. *ni, means not identified.

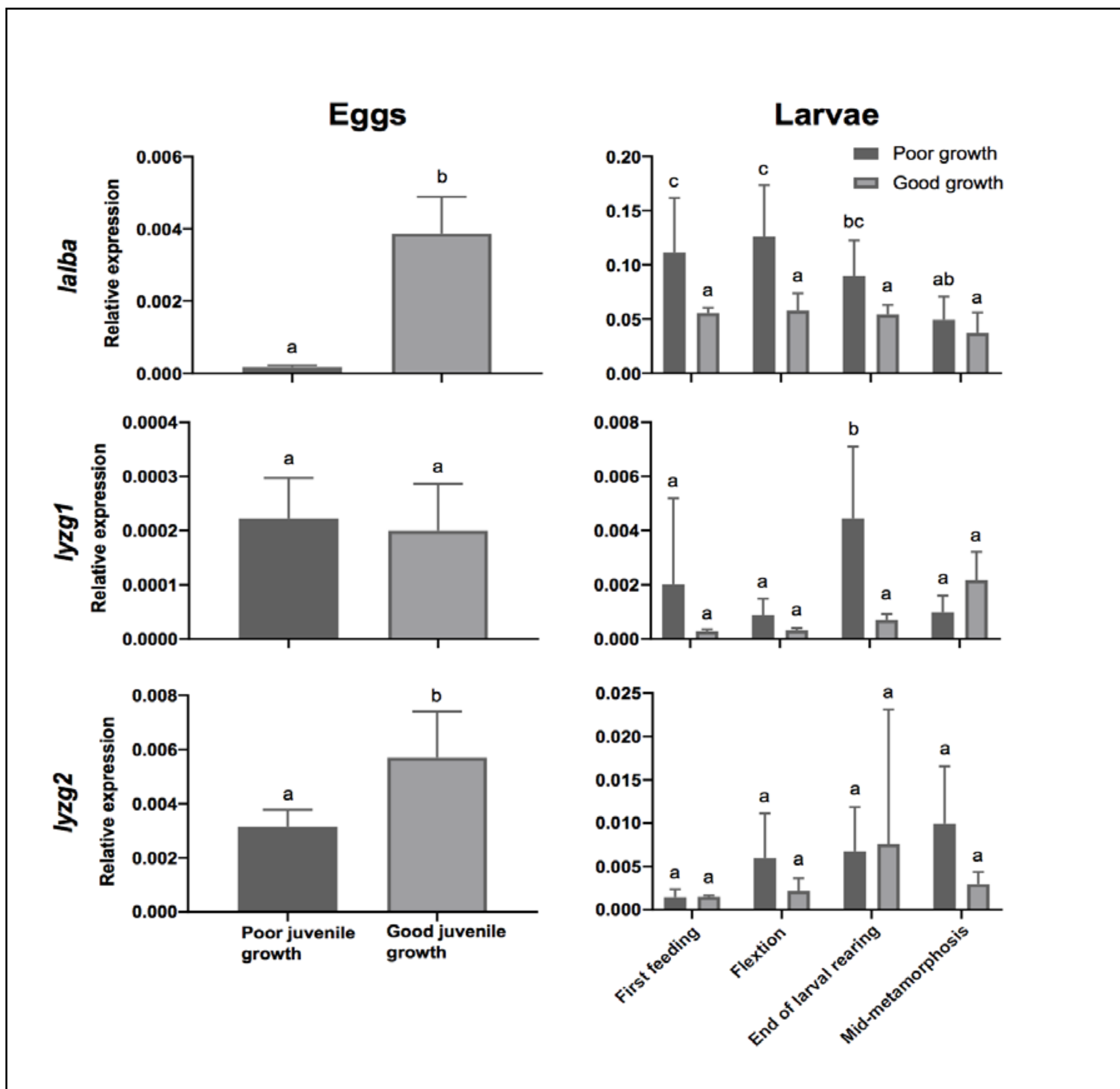


Figure 15. Relative expression of lalba and two lyzg (lyzg1, lyzg2) during gilthead sea bream development. Eggs and larvae had a divergent growth performance by mid-metamorphosis (good juvenile growth - light grey bars and poor juvenile growth - dark grey bars). Data correspond to the mean ± SEM of three (eggs) to six different samples (larvae) and gene expression levels were normalized using the geometric mean of two reference genes (18s and ef1-?). SPSS 25.0 software was used to test for significant differences between the experimental groups using an unpaired student t-test (two-tailed) for the eggs and two-way ANOVA for the different larval stages. Bars with different letters are significantly different ($p < 0.05$).

Evolutionary screening of the complement family

Activation of complement occurs through three different pathways (Classical, MBP-lecitin and alternative) that converge on complement 3 (C3) and end in pathogen lysis. From fish to mammals, the three complement activation pathways are conserved, although the multiple

copies of the *c3* gene found in teleost fish suggests greater complexity. In fish genomes gene number was not conserved and multiple *c3* and the regulatory factor of C3b, factor H (Cfh), genes were encountered. The *c3* genes grouped into two gene clusters, indicating gene duplication ocurred in teleosts. In SBG nine *c3* genes were identified, which is much higher thatn other teleost fish, and four *c3* genes were found in BSS. The sequence related complement factors *c4* and *c5* were single copy in SBG and 2 and 1 copy respectively were present in BSS. The expression of c3 was only assessed in SBG and only 4 of the 9 genes were detected. The validation of the innate immune markers are ongoing by screening eggs and larvae of SBG and BSS classified as "good" and "bad" quality batches. These genes will be required for Task 2.3 - Larval quality indicators.

B) immune-related gene markers (innate immunity, adaptive immunity, and stress) in good and bad batches of BSS and SBG using quantitative real-time polymerase chain reaction

To assess the expression of immunological biomarkers, quantitative polymerase chain reaction (qPCR) was used. Initially to verify the overall protocol, after receiving the larval samples fixed in RNA later from the hatcheries, target genes were amplified and confirmed. The amplified product was sent to Eurofins genomics for DNA sequencing and the sequenced product was tested for the accurate amplification of the target sequence using NCBI Blast. We recorded all the primers from BSS and SBG, amplified the targeted gene and this was used further. In this immunological markers analysis, the expression level of the genes related to innate and adaptive immunity was assessed throughout the early developmental stages of BSS and SBG and used as marker for larval batches evaluation. The majority of the larval batches were processed for immunological marker evaluation, if the quality of the total RNA obtained was adequate. Since some larval batches showed poor quality of the total RNA, they were excluded from the analysis. In synthesis the immunological markers evaluation revealed the following findings. In SBG, among the 14 gene markers tested from innate immunity, adaptive immunity and stress, four markers namely *tnf-alpha*, *cox-2*, *tlr5* and *Igm* were expressed (fold change) in mostly designated good batches (previous communication) Therefore, these could be preferably considered as good markers or bioindicators in larval stages of SBG. Proinflammatory molecules *tnf-alpha* and *cox-2* were upregulated in Flexion, Mid Metamorphosis and Juvenile. Therefore, in these larval stages there is an apparent active promotion of inflammation and they seem to be immunologically prepared for the direct interaction with harmful external stimuli. Although it is still not clear whether the components of the immune gene marker protect against pathogens but the maternal transfer of IgM and early expression of inflammatory response indicate important function as bioindicators in SBG.

Concerning BSS the good batches showed a high fold change of the following genes: Chemokine *cc1*, Interferon gamma, Hepcidin, Toll like receptor 1 and 9, IgM, IgT. Therefore, they can be considered as potential immune markers for further evaluations. A clear balance between innate (cytokines, toll like receptor and antimicrobial peptide) and adaptive (IgM and IgT) response indicates a potential preparation of larval stages towards the external environment. Further evaluation is ongoing, based on principle component analysis (PCA). In this way UNIUD will try to co-relate the immune gene expression patterns of BSS and SBG collected in hatcheries A, B and C, in order to classify the batches as immunologically poor and best. The collected data will be further evaluated in the workshop for the selection of markers that can underpin the

juvenile certificate of analysis. Global data analysis will be required but has not been possible this year due to the restrictions on travel caused by covid-19.

7 Correlating juvenile phenotype with fish phenotype at the end of on-growing

A significant challenge for the most of morpho-anatomical abnormalities is the determination of the critical threshold, over which an abnormality will have significant effects on fish final phenotype, as it will be perceived by consumers. Currently, literature and farm experience suggest that juvenile phenotype at the end of the hatchery phase (i.e. 0.8-8 g W) is a precise predictor of fish phenotype at the end of on-growing. Accordingly, severely abnormal juveniles are sorted-out from the reared stocks at this phase to ensure the high product quality in the following period.

During PerformFish project (Task 2.2.3) it was shown that haemal lordosis, a potentially severe vertebral abnormality, recovers during the on-growing period (Fragkoulis et al. 2019, *Sci. Rep. 9, 9832*, https://doi.org/10.1038/s41598-019-46334-1). Reported recovery rate was ca 50%. Interestingly, body-shape analysis showed that the identification of lordotic juveniles which will recover during on-growing is possible through geometric morphometrics. In future, the automatic sort-out of lordotic juveniles with low recovery potential could thus be possible through the incorporation of computer-assisted systems in the quality control of reared fish.

Given the importance of external phenotype for the quality of the fish which are marketed as a whole, we examined whether the phenotype of normal seabream juveniles can predict the phenotype of the table-size-fish (Task 2.2.3). A total of 959 fish with a normal external morphology that survived during on-growing were selected for geometric morphometric analysis. Procrustes Distances (PDs) were used to estimate the overall body-shape changes of each specimen during growth. Then, Pearson's correlation coefficient between the individual PD values of the first datapoint (1 dpt) and each one of the next four sampling points (77, 282, 371 and 434 dpt) was estimated. This analysis was also repeated for the relative warp (RW) scores, each of which explained a specific part of body shape variation.

Juvenile body shape was significantly correlated with that of the following sampling ages, with the correlation coefficient decreasing with fish growth, from 0.34 between the body shape at 1 and 77 dpt (PD1-PD77), to 0.16 between body shape at 1 and 434 dpt (PD1-PD434, **Table 22**). The low correlation estimates observed might be attributed to the strong strong allometric growth of fish shape with size. As it was demonstrated by the graph of Procrustes distances on SL, there was a significant size-effect on seabream body shape during the on-growing period (**Figure 16**). In the SL range between ca 70 and 192.7±1.9 (±SE) mm SL the PDs increased with the growth of the fish, whereas in the following ontogenetic period it was independent of SL (**Figure 16**).

Table 22. Correlations of the estimated body shape parameters (PD, RW1-RW6) between the first (1 dpt) and the rest four (77-434 dpt) sampling points. All correlations were proven significant ($p<0.05$). (Fragkoulis, PhD thesis)

Datapoint	RW1	RW2	RW3	RW4	RW5	RW6	PD
77	0.40	0.58	0.31	0.25	0.37	0.45	0.34
282	0.33	0.50	0.37	0.22	0.38	0.37	0.19
371	0.32	0.47	0.26	0.23	0.33	0.38	0.19
434	0.35	0.46	0.36	0.26	0.31	0.41	0.16

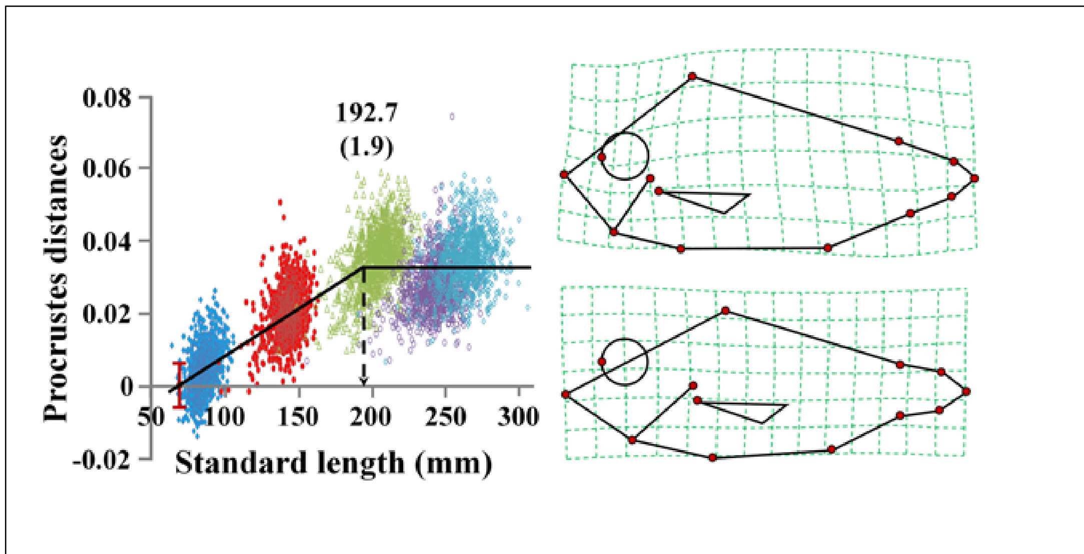


Figure 16. Relationship of the Procrustes Distances (PD) with the fish standard length (SL). Spline diagrams demonstrate body-shape variation across the PD axis (X2). Symbols of different colors represent individual fish at the different sampling ages (Fragkoulis, PhD thesis).

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