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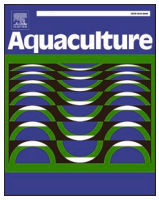

Insights into core molecular changes associated with metamorphosis in gilthead seabream larvae across diverse hatcheries

Babak Najafpour [a], Soraia Santos [a], Manuel Manchado [b], Aurora Vidal [a], Andreas Tsipourlianos [d], Adelino V.M. Canário [a,c], Katerina A. Moutou [d], Deborah M. Power [a,c,*]

[a] Comparative Endocrinology and Integrative Biology, Centre of Marine Sciences, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal
[b] IFAPA Centro El Toruño, Junta de Andalucía, Camino Tiro Pichón s/n, 11500 El Puerto de Santa María, Cádiz, Spain
[c] International Research Center for Marine Biosciences (Ministry of Science and Technology), Shanghai Ocean University, Shanghai, China
[d] Department of Biochemistry and Biotechnology, University of Thessaly, Biopolis, 45100 Larissa, Greece

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ABSTRACT

Early development is a critical period in fish aquaculture and is influenced by biotic and abiotic factors (e.g., temperature, feed) that can vary significantly between hatcheries, making it difficult to identify core factors determining quality. Many of the existing larval transcriptome studies are small-scale and occur under specific rearing conditions that do not mirror the diversity of larviculture practices at an industrial level. In the present transcriptome study, gilthead seabream at the larval to juvenile transition (metamorphosis) from several hatcheries in Europe (Greece, Italy, and France) were analysed in a large-scale RNA-seq study. The aim was to uncover the most significant molecular modifications occurring during metamorphosis, irrespective of differences in biotic or abiotic factors, to address knowledge gaps associated with critical early developmental stages under industrial hatchery conditions. Commonly modified gene transcripts between larval stages were identified based on the clustering of gene expression profiles of 25 gilthead seabream libraries from different hatcheries in a PCA analysis. When larvae at flexion were compared to larvae at mid-metamorphosis, 2243 differentially expressed genes (DEGs) were identified, and when larvae at early to mid-metamorphosis were compared to mid to late-metamorphosis, 2299 DEGs were identified. Comparative analysis across the developmental stages of gilthead seabream revealed genes of importance for the metamorphic transition and adaptation to rearing conditions, including genes related to the nervous system at flexion (24 days post hatch), enteroendocrine cell differentiation, and lipid homeostasis at early to mid-metamorphosis (46 dph), and enrichment of genes indicative of immune competence at mid to late-metamorphosis (51–54 dph). The differential expression of some endocrine-associated genes, *dio1*, *dio2*, *cldn1*, *ing4*, *Pou3f4*, and *fgf22*, highlights their importance in metamorphosis. Meta-analysis of the transcriptomes from two species, the gilthead seabream and Senegalese sole, that have differing symmetry and ecology uncovered common molecular expression patterns that underlie larvae maturation during metamorphosis, and we propose that these represent core gene markers of metamorphosis in these two fish species.

1. Introduction

Early developmental stages are critical in fish aquaculture, as larvae experience significant morpho-physiological changes and stressful conditions like nutritional deficiencies, suboptimal temperatures, high population density and hypoxia can all provoke increased mortality or decreased larvae performance (Pollock et al., 2007; El Kertaoui et al., 2019; Mugwanya et al., 2022). The effect of factors such as temperature and feed on inducing stress or on production traits such as growth performance trigger epigenetic imprinting that can be transmitted between generations (Ho and Burggren, 2010; Pittman et al., 2013; Burggren et al., 2016; Gavery and Roberts, 2017; Robinson et al., 2019; Burggren, 2020; Hou and Fuiman, 2020; Carballo et al., 2020). Despite numerous studies using dietary additives (Chakraborty et al., 2014; Sutili et al., 2018), pre-probiotics (Brugman et al., 2018), and vaccines (Brudeseth et al., 2013) to improve fish health and survival, consistent

* Corresponding author at: Comparative Endocrinology and Integrative Biology, Centre of Marine Sciences, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal.
E-mail address: dpower@ualg.pt (D.M. Power).

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and predictable production of high-quality larvae still remains a challenge for the aquaculture industry. Thus, understanding the regulatory factors that influence development and phenotypic plasticity may help establish rearing practices that enhance larval performance and favour desired adult phenotypes.

The molecular mechanisms explaining body shape during fish development are still enigmatic, although much work has been invested in characterizing this process in flatfish (Power et al., 2008). Niche occupancy, along the benthic-pelagic axis is one of the factors that has imposed body shape evolution in fish, and this is more evident in benthic compared to demersal or pelagic fish (Friedman et al., 2020). Comparative analysis of marine fish eggs and larvae, indicates that the development of flat and round fish has many similarities, such as simple V-shaped myotomes in muscle, a straight undeveloped gastrointestinal tract (GI) at hatch, increasing goblet cell number and the development of a stomach as the GI tract matures, the appearance of neuromast cells with protruding kinocilia and stereocilia on the body surface, and the development of fully pigmented and functional eyes within the first week of hatch (Falk-Petersen, 2005; Rønnestad et al., 2013). Numerous complex developmental changes underpin the larval – juvenile transition (metamorphosis), and the importance of a well-regulated transition for fitness and improved production traits in the grow-out stage makes this developmental transition critical for aquaculture. A better understanding of teleost metamorphosis in general can contribute to a better understanding about how the environment influences endocrine regulation of development, and explain how morphologies and lifestyles have evolved under environmental pressure (McMenamin and Parichy, 2013).

With the advent of high throughput sequencing techniques, it has been possible to map the dynamics of gene expression during larval development (e.g., differentiation, cellular proliferation, growth) in different species and obtain detailed insight into these changes (Mazurais et al., 2011; Qian et al., 2014). Gene clusters that have similar dynamics in different species have been identified, such as those implicated in digestion (e.g., trypsin) and muscle development (e.g., myosin heavy and light chain; Darias et al., 2008; Douglas et al., 2008). Groups of genes that undergo significant modifications at specific developmental stages and under different experimental conditions during larval ontogeny include genes involved in ATP synthesis in the European sea bass (Darias et al., 2008) and in fat digestion and absorption in zebrafish (Xu et al., 2017). Furthermore, in the flatfish Atlantic halibut during metamorphosis contrary to expectation most transcripts (approx. 98%) were not thyroid hormone (TH)-responsive indicating that a relatively small number of genes orchestrate the remarkable transition from a symmetric pelagic larvae to an asymmetric demersal juvenile (Alves et al., 2016).

Previous transcriptome studies of gilthead seabream have mainly focused on juvenile and adult fish due to the commercial importance of this species in the South of Europe. Production challenges are largely linked to disease, diet, growth and physicochemical conditions (Ibeas et al., 1994; Cara et al., 2005; Martos-Sitcha et al., 2014; Mininni et al., 2014; Hampel et al., 2017; Muniesa et al., 2020) and transcriptome studies have focused on adult tissues relevant for these traits like, skeletal muscle, intestine, blood, head-kidney, and the gill (Garcia de la serrana et al., 2012; Calduch-Giner et al., 2013; Vieira et al., 2013; Louro et al., 2016; Pérez-Sánchez et al., 2019). Studies of gilthead seabream hatchery stages are less common and those that exist are focused on very early development (embryo to 168 hph), or have targeted specific tissues (e.g., kidney) or experimental conditions (Yúfera et al., 2012; Sarropoulou et al., 2005, 2016). Such studies have generated important species-specific molecular resources, but there are relatively few comparative studies of different developmental stages.

Common morpho-physiological changes imply sets of core genes drive larval development among different species. However, the diversity of teleosts and their life-history patterns may hinder the development of a standardized staging system beyond the family level (Ditty et al., 2003). Moreover, the diversity of conditions in aquaculture settings may lead to different developmental rates and probably contribute to variations in the transcriptome, even between populations of the same species. The primary goal of this study was to integrate the transcriptome profiles of gilthead seabream larvae collected from diverse aquaculture hatcheries, and so samples encompassed variations in broodstock, installations, production scale, and hatchery management. Subsequently, the aim was to identify common differential transcriptome responses occurring during metamorphosis, irrespective of the origin of the larvae. To assess if a set of conserved core gene expression patterns underlie metamorphosis irrespective of species or ecology (i.e. pelagic or benthic), transcriptomes of larval gilthead seabream and Senegalese sole (*Solea senegalensis*) were compared. The knowledge generated contributes to a better understanding of metamorphosis and generates candidate stage and quality-related gene markers for commercial hatcheries.

2. Material and methods

2.1. Larvae

Gilthead seabream larvae were collected from three aquaculture sites located in France (Fr), Italy (It) and Greece (Gr), from March-2018 to December-2018. A summarized version of the rearing parameters of the participating hatcheries has previously been reported but due to the commercial sensitivity of the hatchery production data only an overview with the average and standard deviation across the participating hatcheries is available (Kourkouta et al., 2022). The data collected covers broodstock to pre-growing phases and demonstrates the wide variability in the production regime of the hatcheries from which the larval samples used in the present study were obtained (e.g., nutrition, abiotic conditions, tank characteristics, specific procedure such as tank/egg disinfection etc).

For collection the larvae were anesthetized in MS-222 (Sigma-Aldrich), preserved in RNAlater™ and stored at −20 °C until RNA extraction. The larvae came from different production batches, and they were collected before metamorphosis at the flexion stage (23–25 days post hatch, dph), at early, mid and late-metamorphosis (43–60 dph, Fig. 1). These two stages were chosen since they are easy to identify, which favoured standardization of collected samples across hatcheries. Data about age, weight, and length of larvae samples used in this study are detailed in Najafpour et al. DIB - Unpublished results. Based on sample availability, two separate RNA-seq runs included 11 libraries of gilthead seabream larvae from sites Gr (flexion, mid-metamorphosis stages) and Fr (mid-metamorphosis stage), and 14 libraries from sites It (early, mid and late-metamorphosis stages) and Fr (mid-metamorphosis stage). A pool of gilthead seabream eggs, that contained embryos 24 h before hatch, was sampled from Gr hatchery and fixed in RNAlater™ and stored at −20 °C until RNA extraction.

The primary approach to determine larval stage used hatchery-based classification criteria, which included larval standard length, changes in external morphology and behaviour. More specifically the flexion stage included larvae with a total length of 7–10 mm and mid-metamorphosis larvae had a total length of 15–20 mm. On receipt of samples by CCMAR the weight and length of each larva was recorded and the stage assessed using consensus criteria reported in the literature, feeding (4 dph), swim bladder inflation (10 dph, ~ 4 mm standard length), flexion (23–24 dph, ~ 7 mm), pre-metamorphic stages (30–45 dph, ~ 8 mm standard length) and juvenile stage (60–90 dph) (Çoban et al., 2009; Morais et al., 2017; Mhalhel et al., 2023). Ultimately, Principal Component Analysis (PCA) served as the basis to refine the definition of larval stage, utilizing the whole transcriptome profile of each larva. We observed that larval clustering was generally linked with their weight, length, and age, and permitted robust identification of outlier samples and larvae with divergent transcriptome profiles and stages were excluded from subsequent analysis. Consequently, each cluster of individual samples was

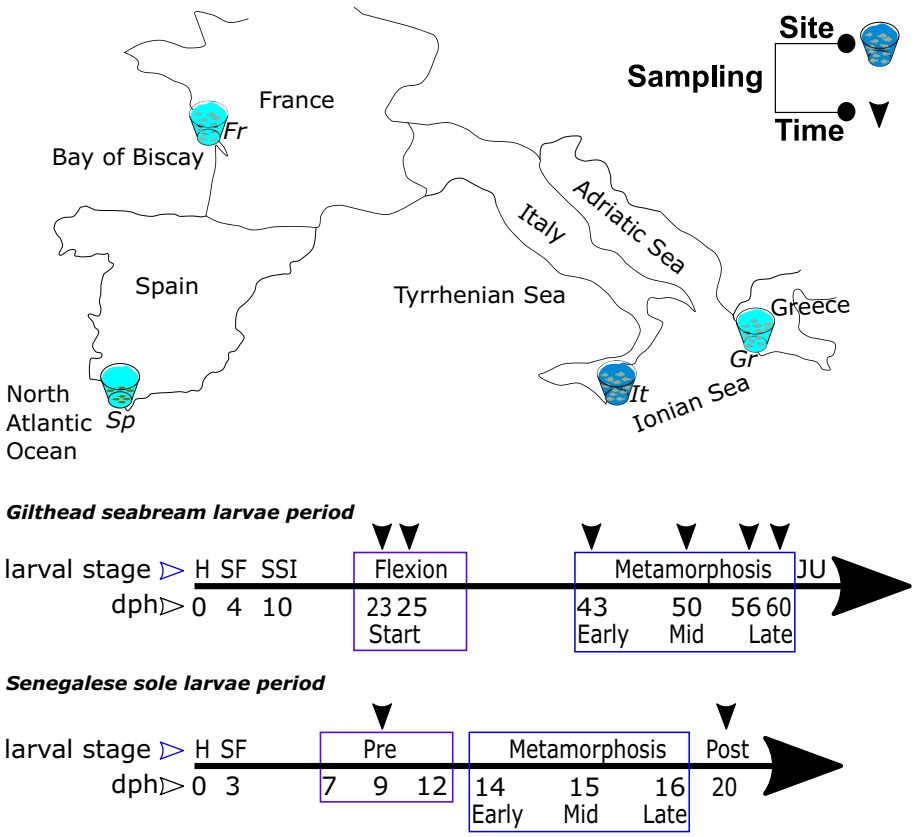


Fig. 1. The sampling scheme of gilthead seabream and Senegalese sole during larval development from different hatcheries. H = hatching; SF = start of feeding; SSI = start of swim bladder inflation. The gilthead seabream samples were collected from form three hatcheries (Fr, Gr, It) at flexion and metamorphosis, including early, mid- and late metamorphic stages. The Senegalese sole sample was collected from a hatchery in Spain (Sp) in pre- and post-metamorphic stages. Arrows show the sampling time points.

considered a group of larvae at a similar developmental stage. Further details of the PCA analysis are provided in Section 4.3.

The larval period of the Senegalese sole (approximately 30 days) is shorter than the gilthead seabream (~ 60 days) and it is subdivided into pre-metamorphosis (7–12 dph, metamorphic stage 0), metamorphosis (13–14 dph, Stage 1/early-metamorphosis, 14–15 dph, Stage 2/mid-metamorphosis and 15–16 dph Stage 3/late-metamorphosis) and post-metamorphosis (20 dph, Stage 4). Metamorphic stages of Senegalese sole were defined according to Fernández-Díaz et al. (2001) and larvae were cultivated under optimum rearing conditions as previously reported (Manchado et al., 2008; Hachero-Cruzado et al., 2014; Roman-Padilla et al., 2016). Although early stages of gilthead seabream and Senegalese sole larvae are pelagic, at the onset of metamorphosis in S3 (15–16 dph) Senegalese sole the eye starts to migrate, the body axis starts to tilt and when the eye is fully migrated around 20 dph (post-metamorphosis) it becomes a bottom dwelling asymmetric flatfish. Senegalese sole larvae were collected at 9 dph (pre-metamorphosis) before the shift in the body axis and 20 dph (post-metamorphosis) from a wild broodstock held at IFAPA El Toruño facilities in Spain. All samples were euthanized using MS-222 (Sigma-Aldrich; 200 ppm), washed using diethylpyrocarbonated (DEPC) water, frozen in liquid nitrogen, and stored at −80 °C until analysis. All procedures were performed in accordance with national and European Union legislation for animal care and experimentation (Directive 86\609\EU) and were authorized by the IFAPA Bioethics and Animal Welfare Committee with registration nr. 26–11–15-374 from the Spanish authorities.

2.2. RNA extraction

For RNA extraction, gilthead seabream samples comprised a pool of 3 larvae 23–25 dph or 1–2 larvae 43–60 dph (Najafpour et al., n.d.). For Senegalese sole, the two pools of larvae 9 dph (Stage 0) or 20 dph (Stage 4) contained 5 specimens ($n = 3$ replicates per stage). For extraction of RNA from gilthead seabream eggs, a pool of 10 eggs (10 mg) was used. Gilthead seabream larvae and eggs were processed at CCMAR by defrosting them in lysis buffer and then homogenizing them by mechanical disruption with two iron beads (5 mm) in a Tissue lyser II (Qiagen, Germany) using 3 cycles (30 Hz) of 30 s at room temperature. Total RNA from whole larvae homogenates was extracted using an E.Z.N.A. Total RNA Kit I (VWR, USA) according to the manufacturer's instructions. Genomic DNA was removed by DNAse I treatment performed directly on the column during RNA isolation, following the manufacturer's instructions. Senegalese sole larvae were processed at IFAPA, and were homogenised using a Fast-prep FG120 instrument (Bio101) and Lysing Matrix D (Q- Bio-Gene) for 40 s at speed setting 6. Total RNA was isolated using a RNeasy Mini Kit (Qiagen) following the manufacturer's indications and treated twice with DNase (RNase-Free DNase kit, Qiagen) for 30 min.

2.3. Library preparation and sequencing

The preparation and sequencing of the gilthead sea bream sequencing libraries were done by Novogene (Shanghai, China). Before library preparation, RNA quality was checked using a 2100 Bioanalyser (Agilent Technologies, Inc., Santa Clara CA, USA) and only high-quality RNA samples (RIN $\geq$ 8.0) were used. The 25 gilthead sea bream RNA sequencing libraries, from two RNA-seq runs, were prepared using a TruSeq™ RNA sample preparation Kit (Illumina, San Diego, CA) and were sequenced in paired-end mode (2 × 150 bp read length) using an Illumina HiSeq Xten (more details in Najafpour et al. DIB - Unpublished

results). The Senegalese sole Illumina libraries were constructed at the Centre Nacional d'Anàlisi Genòmica (Barcelona, Spain) using a TruSeq RNA Sample Preparation Kit v2, following the manufacturer's instructions. Each library was sequenced using a TruSeq SBS Kit v3-HS, in paired end mode, 2 × 76 bp (*Solea senegalensis*) on a HiSeq2000 sequencing system (Illumina, Inc) following the manufacturer's protocol (more details in Benzekri et al., 2014). Raw data have been deposited in the GenBank SRA repository bioproject PRJNA956882 (gilthead sea bream) and PRJNA241068 for pre-metamorphic 9 dph and PRJNA261151 for Senegalese sole 20 dph post-metamorphic larvae.

2.4. Quality control and differential gene expression

Quality-checks of the raw reads were done with FASTQC using default parameters in GALAXY (Andrews, 2010; Afgan et al., 2018). After quality checks, the reads were mapped to the gilthead sea bream (NCBI assembly name: fSpa Aur1.1, RefSeq assembly accession: GCF_900880675.1) and Senegalese sole (Male_LA_Total, Claros et al., 2020) reference genomes using the Bioconductor package Rsubread (Liao et al., 2013). The number of reads mapped per gene was counted using the "*featureCounts*" function in R with default parameters and GTF.attrType = "gene transcript".

Genes with very low expression were removed using the *cpm* function in edgeR (Robinson et al., 2009) and only transcripts with at least one count-per-million reads (cpm) across four samples were retained. The distance between the global transcriptome profile of all the analysed samples was assessed by principal component analysis (PCA) using the *MDSplot* function in *R*. The *MDSplot* was built using normalized data with the *voom* function in the limma package. Larval transcriptomes that clustered together had the same stage classification and were considered to represent samples at the same developmental stage. To track significant changes in gene expression and their functional profiles, differentially expressed genes (DEGs) were identified between the independent clusters of the larval transcriptomes generated by PCA.

DEGs (adjusted *p*-value (BH) < 0.05) were identified using normalized read counts and computing moderated t-statistics by empirical Bayes moderation of the standard errors in the limma package (Ritchie et al., 2015). Based on the clustering of the PCA analysis, which generally confirmed the hatchery stage classification, and clustering linked to age and weight, two comparisons (named I and II) were made using the gilthead sea bream larval transcriptomes and included data from the two RNA-seq runs. In comparison I, the transcriptomes at the flexion stage of gilthead sea bream larvae (age = 24 ± 0.7 dph; weight = 4.4 ± 1.8 mg) were compared to the transcriptomes of the mid-metamorphosis stage (age = 51 ± 0.5 dph; weight = 47.1 ± 9.7 mg). In comparison II, the transcriptomes of the larvae at early and mid-metamorphosis stages (age avg. = 46 ± 1.7 dph; weight avg. = 16.5 ± 4.4 mg) were compared to the transcriptomes of larvae at mid and late-metamorphosis stages (age avg. = 54 ± 1.6 dph; weight avg. = 49.9 ± 10.6 mg). To minimise bias, comparisons were made using only samples with a similar range of read numbers in each of the comparisons, and samples that did not cluster in the PCA were excluded; this resulted in 7 samples for comparison I and 13 samples for comparison II. For the Senegalese sole, DEGs were identified by comparing the transcriptomes of 9 dph (Stage 0, $n = 3$) and 20 dph (Stage 4, $n = 3$) larvae (comparison III).

A library of 258 endocrine genes coding 464 transcripts was extracted from the NCBI Refseq database and was used to analyze modifications in the endocrine repertoire of the gilthead sea bream during development.

The common DEGs associated with metamorphosis in the gilthead sea bream and Senegalese sole were extracted from comparisons I and III and were used to identify genes with similar expression patterns (up-regulated or down-regulated). Orthologues of the identified DEGs were extracted from the zebrafish genome.

2.5. Functional analysis

DEGs were annotated by local BLAST against the Ensembl zebrafish translated proteins (Danio_rerio.GRCz11.pep.all.fa.gz) and their orthologues were extracted. The cut-off accepted for orthologue annotation was an expected *p*-value $>1e^{-5}$ and identity >40%.

The R clusterProfile package was used to carry out the Gene Ontology (GO) over-representation analysis using the *enrichGO* function and the gene set enrichment analysis (GSEA) using the *gseGO* function for each of the comparisons I-III (Yu et al., 2012). Redundant GO terms were removed, and GO terms were considered significantly enriched if $p < 0.05$ and the Benjamini-Hochberg (BH) adjusted $p < 0.05$. The Bioconductor release v 3.14 of the genome annotation for Zebrafish (org.Dr.eg.db.3.14.0) was used in R as the background for *gse*A (Carlson, 2019). The over-represented GOs were visualized based on the relative percentage of the gene ratio of each GO using the R package plotrix v 3.8–2 and *pie* function (Lemon, 2006).

KEGG Gene Set Enrichment was analysed using the *gseKEGG* function of the R package clusterProfile v 4.2.2 (Yu et al., 2012) with the same statistical criteria as applied to GSEA (with and without a BH adjusted *p*-value). The classification of DEGs involved in specific processes (e.g., immune response) was further determined by manual searches of the UniProt (www.uniprot.org) and PANTHER (www.pantherdb.org) databases (Mi et al., 2019).

Weighted gene co-expression network analysis (WGCNA) was used to identify co-expressed gene modules in comparisons I and II above (Langfelder and Horvath, 2008). WGCNA analysis was run independently for the two groups of samples (each group included samples used in the separate comparisons I and II). WGCNA I included larvae at flexion (avg. age of 24 dph) and mid-metamorphosis avg. age of 51 dph) and WGCNA II included larvae at early (avg. age of 43 dph), mid (avg. age of 51 dph) and late-metamorphosis avg. age of 60 dph). Briefly, absent or low abundance genes were filtered out (based on rowSums ≥50 for WGCNA I and ≥ 200 for WGCNA II), and data were normalized and transformed using the *vst* function in the DESeq2 R package. The power soft threshold for WGCNA was determined using the output of *sft* object (*sft* = 16 for WGCNA I and *sft* = 24 for WGCNA II), and $R^2 > 0.85$ was applied. The gene network was constructed, and modules were identified using the *blockwiseModules* function in the WGCNA R package. Modules that were significantly associated with age and weight traits were identified by correlating each module (applying the eigengene concept) with the traits of interest (Pearson correlation method). The quantitative measure of module membership (MM) was also defined as the correlation of the module eigengene and the gene expression profile using default parameters.

WGCNA modules that had the highest association with weight and age were selected and functional information mapped. The module associated gene sets were mapped to gilthead seabream functional data through the g:Profile web server for functional enrichment analysis (https://biit.cs.ut.ee/gprofiler/gost). Gene ontology enrichment results were visualized using the python package GO-Figure! that provided a comprehensive summarized list of GO terms (Reijnders and Waterhouse, 2020).

2.6. Real-time quantitative RT-PCR

Real-time quantitative RT-PCR of six candidate genes (*hbae5*, *map4*, *lct*, *chia*, *rh50*, and *tcnba*) selected among the DEGs was carried out in a 10 ul final volume using KAPA SYBR® FAST qPCR Master Mix (2×) Kit (Kapa Biosystems) and an appropriate concentration of each transcript specific set of primers. Primers were designed using Primer3 (v.0.4.0, Untergasser et al., 2012) and Beacon Designer software and all primers were within the coding sequence of the transcripts. The thermocycle was: 5 min at 95 °C, followed by 40 cycles of 95 °C for 20 s and 60 °C for 20 s, followed by a dissociation curve step to verify single reaction products were obtained. Each reaction was performed in duplicate. A

Table 1
The selected candidate genes and primers used in qPCR reactions.

Gene name	Gene symbol	Primer sequence	Product size	Primer concentration	Primer efficiency	Refseq Accession
Hemoglobin, alpha embryonic 5	*hbae5*	*Fw_CAGCGTCTATCCCCAAACCA* *Rv_ GGTCGTCGATCTTGGACACA*	131	300 nM	100.85	XM_030399324
Microfibril-associated glycoprotein 4-like	*map4*	*Fv_CTCCGAGAGCAGAGAGAGTTTT* *Rv_TGCCTTTGGCAAGAACTGTG*	122	250 nM	99.18	XM_030413465
Lactase-phlorizin hydrolase-like	*lct*	*Fw_GCAGAAGACGATACTCCCCA* *Rv_TGGTGCCAAATACACTTCCCT*	157	200 nM	100.50	XM_030427745
Rh50-like protein	*rh50*	*Fw_TGTGTTGGTCGTCACCCTTT* *Rv_GTTTGCCGCCTACTTTGGTC*	185	300 nM	103.77	XM_030420128
Transcobalamin beta a	*tcnba*	*Fw_GAGGCCATGAGTCTCTTCAGG* *Rv_TGCTTCCAGGGACATTGACT*	267	300 nM	104.88	XM_030405178
Acidic mammalian chitinase-like	*chia*	*Fw_TGCTACTTCACCAACTGGGC* *Rv_TTCAGGCCATTGAAGGTCGT*	179	200 nM	96.22	XM_030420597

standard curve using a dilution series (1:5, 1:10, 1:20, 1:50, 1:100) from pooled cDNA was prepared to estimate amplification efficiency (Table 1). Three genes, *ef1*, *rpl13a,* and *rpl19*, were chosen as the reference genes based on geNorm rating (Vandesompele et al., 2002). From the Ct values, the starting content of the target sequence (R0) was calculated as R0 = Threshold/(1 + efficiency)^{ct} normalized to the geometric mean of the three reference genes.

3. Results

3.1. RNA-seq libraries and statistics

The average number of gilthead sea bream paired-end reads (R1 + R2) per sequencing library was 12,143,070 (11 samples) in the first RNA-seq run and 46,420,480 (14 samples) in the second RNA-seq run. Four samples were excluded from subsequent data analysis either because they failed to cluster in PCA analysis or had a very discrepant read number from other samples (Supplementary Table 1). The average number of merged reads that mapped to the gilthead seabream reference genome was 5,810,781 in the first RNA-seq run and 22,018,717 in the second RNA-seq run, which corresponds to 95.1% of reads mapped to the reference genome (Najafpour et al. DIB - for details). The average number of Senegalese sole paired-end reads per sequencing library was 56,451,220 (R1 + R2) yielding 26,369,914 merged reads of which 93.3% mapped to the reference genome.

3.2. Clustering and differential gene expression

The PCA of the gene expression profiles of the 25-gilthead sea bream sequencing libraries originated three main clusters (Fig. 2, Supplementary Table 1). As expected, the single egg sample from site Gr used in the analysis was an outlier to the larvae samples (Fig. 2). Overall, the clustering was driven by age/stage and weight rather than aquaculture site (Fig. 2). One cluster contained larvae with an average age and weight of 24 dph and 5.9 mg (larvae at flexion, PCA coordinates: x-axis ~ 0.8, y-axis ~ 0.4) the second cluster contained larvae of 48 dph and 38.8 mg (larvae at early and mid-metamorphosis, PCA coordinates: x-axis ~ −0.01, y-axis ~ 0.2) and the third cluster contained larvae of 54 dph and 43.3 mg (larvae at mid and late-metamorphosis, PCA coordinates: x-axis ~ −0.7, y-axis ~ −0.3, Fig. 2).

Comparison I (flexion vs mid-metamorphosis) yielded 2243 DEGs of which 1083 were up-regulated, and 1160 were down-regulated (Fig. 3). Comparison II (early and mid-metamorphosis vs mid and late-metamorphosis) resulted in 2299 DEGs of which 1153 were up-regulated and 1146 were down-regulated (Fig. 3). There were 684 common DEGs between the two comparisons, 1559 specific to comparison I and 1615 specific to comparison II (Fig. 3, Supplementary Tables 2 and 3).

The Senegalese sole comparison III (pre-metamorphosis vs post metamorphosis) yielded 14,473 unique DEGs of which 7054 were up-regulated and 7419 were down-regulated. Common and unique Senegalese sole DEGs were identified when the assigned zebrafish gene orthologues were compared to those assigned to the DEGs obtained from comparison I and II in the gilthead sea bream (Supplementary table 4).

3.3. Functional analysis

Over-represented and informative molecular functions (MF) and biological process (BP) related to each gilthead sea bream DEG comparison are summarized in Fig. 4 and details of the analysis is provided in Supplementary Table 5. In comparison I, the BPs showing the highest gene ratio were 'monocarboxylic acid metabolic process', visual perception, 'extracellular matrix organization', 'fatty acid biosynthetic process' and 'apical junction assembly' (Fig. 4). For comparison II, they were 'carbohydrate metabolic process', 'anion transport', 'glycoprotein biosynthetic process', 'protein glycosylation', 'lipid transport' and 'apical junction assembly' (Fig. 4). In Senegalese sole, comparison III yielded 'axon development', 'skeletal system development', 'ribonucleoprotein complex biogenesis', 'retina development in camera-type eye', and 'muscle cell differentiation' as the top BPs (Fig. 4).

GSEA identified the BPs 'regulation of immune response', 'antigen processing and presentation', and 'proteolysis' as down-regulated at the gilthead seabream flexion stage (avg. age of 24 dph) and Senegalese sole pre-metamorphosis stage (9 dph, Fig. 4). 'Enteroendocrine cell differentiation' and 'lipid homeostasis' BPs were up-regulated in early and mid-metamorphosis (avg. age of 46 dph) gilthead seabream (Fig. 4).

The KEGG pathways associated with 'pathogen recognition receptor' (PRR), and 'inflammatory response' (e.g., 'NOD-like receptor signalling pathway', 'Cytokine-cytokine receptor interaction'), 'lysosome', and 'phagosome' were suppressed in the younger stages of larvae compared to gilthead seabream at mid and late-metamorphosis or post metamorphic Senegalese sole larvae (Fig. 4). The detailed outcome of GSEA and KEGG pathway enrichment and the full list of enriched gene sets identified are presented in Supplementary Fig. 1–5 and Najafpour et al. (co-submitted DIB).

3.4. Common gilthead seabream and Senegalese sole larvae transcriptome profiles

The functional profile of common DEGs was obtained by comparing flexion versus mid-metamorphic larvae of gilthead seabream and pre- versus post-metamorphic larvae of Senegalese sole (Supplementary table 4). 'Endopeptidase activity', 'peptidase inhibitor activity', 'multicellular organism development', 'bicellular tight junction assembly', and 'lysosome' were common enriched GO terms and included genes with similar expression patterns, such as the down-regulated *iodothyronine deiodinase type I (dio1)*, *iodothyronine deiodinase II* (*dio2*), *proteolipid protein 1b* (*plp1b*), *crystallin gamma M2b* (*crygm2b*), and the up-regulated *POU class 4 homeobox 2* (*pou4f2*), *gastrulation brain homeobox 2* (*gbx2*), and *actinodin 2* (*and2*, Fig. 5, Supplementary table 4).

Overall, genes coding for proteins with endopeptidase activity (e.g., *proteasome 20S subunit beta 9a psmb9a*; *serine protease 59*, *prss59.2*;

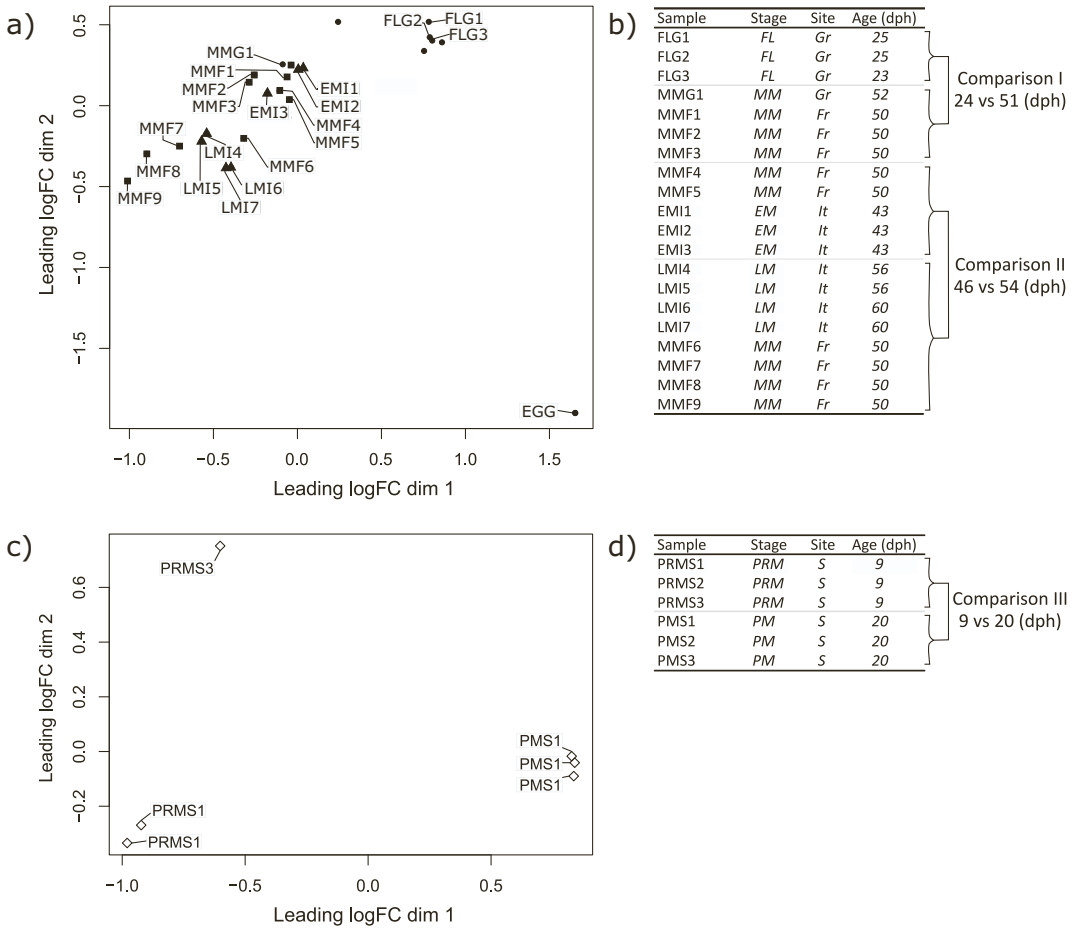


Fig. 2. PCA and identification of the samples used for the analysis of differential gene expression (DEGs). a) Multidimensional Scaling Plot of Distances (Euclidean distance) between transcriptome profiles of 25 gilthead seabream larvae samples that differed by age and weight. The different shaped symbols represent different aquaculture sites (circle = site A; triangle = site P; rectangle = site F). Three main clusters were found and their associations with age and weight was identified. The three clusters comprised larvae with an average age and weight of i) 24 dph and 5.9 mg (cluster included larvae at the flexion stage with an approximate average position: x-axis ≅ 0.8, y-axis ≅ 0.4); ii) 48 dph and 38.8 mg (cluster included larvae at early and mid-metamorphosis with an approximate position: x-axis ≅ −0.01, y-axis ≅ 0.2); and iii) 54 dph and 43.3 mg (cluster included larvae at mid and late-metamorphosis with an approximate position: x-axis ≅ −0.7, y-axis ≅ −0.3). b) the details of the samples that were selected for the two differential gene expression analyses (I & II). Seven samples with a similar range of sequencing reads were selected to perform comparison I (24 vs 51 dph) between larvae at flexion (FL) and larvae at mid-metamorphic stage (MM). In comparison II (46 vs 54 dph), 13 samples with a similar range of sequencing reads were chosen, including larvae at early (EM), mid (MM), and late- (LM) metamorphosis. c) Multidimensional Scaling Plot of Distances (Euclidean distance) between transcriptome profiles of 6 Senegalese sole larvae at pre-metamorphosis (PRM) and post metamorphosis (PM) stages. A clear separation was observed between Senegalese sole larvae at different stages. The samples were collected from a single site in Spain (S). d) the details of the samples that were used for the differential gene expression analyses III between larvae at 9 dph and 20 dph.

chymotrypsin like elastase family member 1, *cela1.6*; *trypsin I − P1 − like, AL954146.1*; *complement factor I*, *cfi*) and peptidase inhibitor activity (e.g., *serpin peptidase inhibitor clade A*, *serpina1*; *complement component c3a*, *complement 4B*, *c4b*) were less expressed ($p < 0.05$) in younger larvae before metamorphosis (24-day gilthead seabream and 9-day Senegalese sole) compared to older larvae at mid- or post-metamorphosis (51-day gilthead seabream and 20-day Senegalese sole, Fig. 5). 'Bicellular tight junction assembly' included several claudin genes for example *claudin a* (*cldna*), *claudin e* (*cldne*), and *claudin k* (*cldnk*) in low abundance during early developmental stages in both species ($p < 0.05$, Fig. 5). Several lysosome component genes (e.g., *cathepsin S, ctss2.1*; *hexosaminidase B, hexb*; *N − acetylgalactosaminidase, naga*) were down-regulated in early developmental stages of both species ($p < 0.05$, Fig. 5). Phototransduction was a common KEGG pathway enriched in both species (Supplementary table 4C), and an inverse expression pattern was identified for melanin biosynthesis and visual phototransduction genes.

3.5. Immune-related gene repertoire during the larval-juvenile transition

In general, immune-associated genes involved in innate and adaptive immunity were of lower abundance in younger gilthead seabream larvae (~ 24–46 dph) compared to mid and late-metamorphic larvae (~ 51–54 dph). A heatmap of 100 immune-associated DEGs with the highest levels of expression indicated that immune-related factors, including innate and adaptive -related genes, were up-regulated at mid and late-metamorphosis (avg. age of 51 and 54 dph) relative to flexion or early and mid-metamorphic stages (avg. age of 24 dph and 46 dph, Fig. 6). Some immune gene transcripts of the major histocompatibility complex (e.g., *major histocompatibility complex class I-related gene protein-like*, XM_030411748), complement (e.g., *complement C1q-like protein 4*, XM_030431421) and lectin (e.g., *galactose-specific lectin nattectin-like*, XM_030422915) were absent at flexion (~ 24 dph) compared to mid-metamorphosis (~ 50 dph).

Among the pattern recognition (PRR) genes, there was generally lower abundance in larvae at 24 and 46 dph compared to 51 and 54 dph, which was a common pattern for most immune-associated genes (Fig. 7,

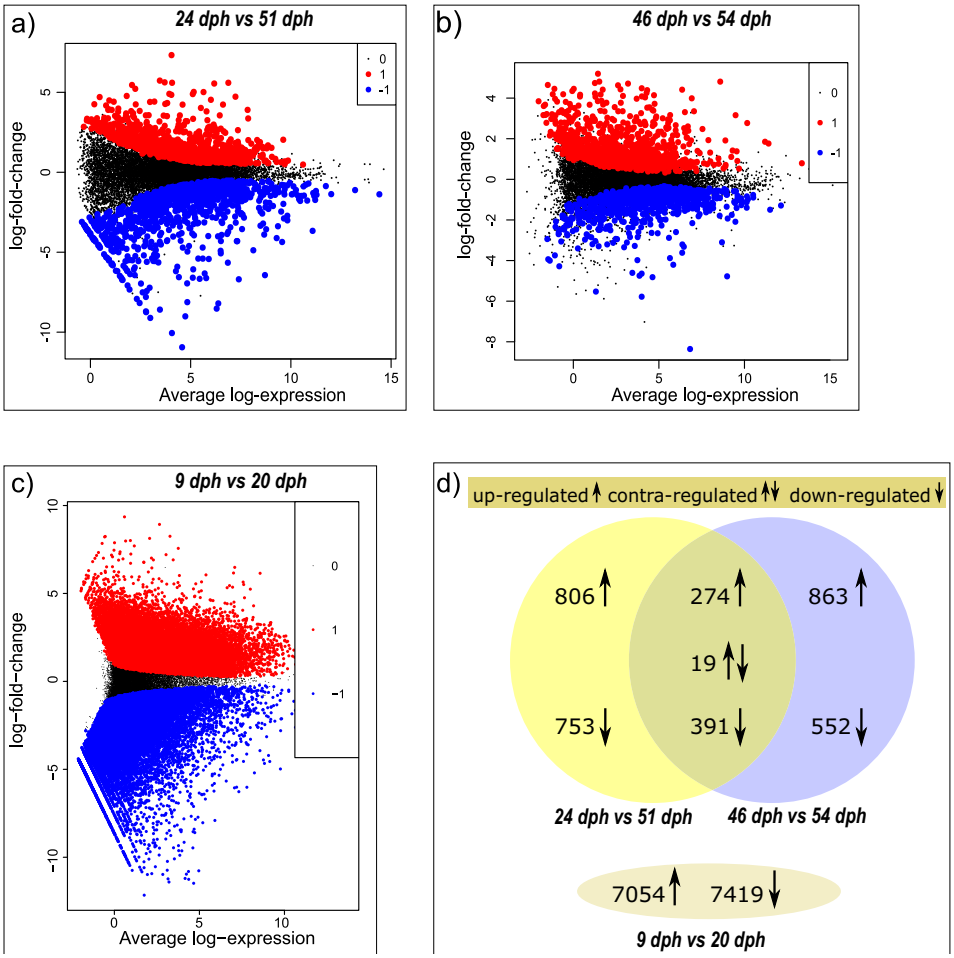


Fig. 3. The results of the comparisons I, II, and III to identify differentially expressed genes (DEG) in gilthead seabream and Senegalese sole larvae samples (details in Fig. 2). a) MD plot of the log-fold change of gene transcripts in the comparison I between 24 dph (avg. weight = 4.6 mg) vs 51 dph (avg. weight = 43.8 mg) gilthead seabream larvae, b) MD plot of the log-fold change of gene transcripts in comparison II between 46 dph (avg. weight = 16.54 mg) vs 54 dph (avg. weight = 41.8 mg) gilthead seabream larvae. c) MD plot of the log-fold change of gene transcripts in comparison III between 9 dph vs 20 dph Senegalese sole larvae. Significantly up- and down-regulated DEG transcripts are shown in red and blue, respectively. d) VennPlex diagram showing the number of shared DEGs and up-, down- and contra-regulated DEGs in the comparisons I and II in gilthead seabream larvae. The number of up- and down-regulated DEGs from comparison III in Senegalese sole larvae are presented in lower panel of part d. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

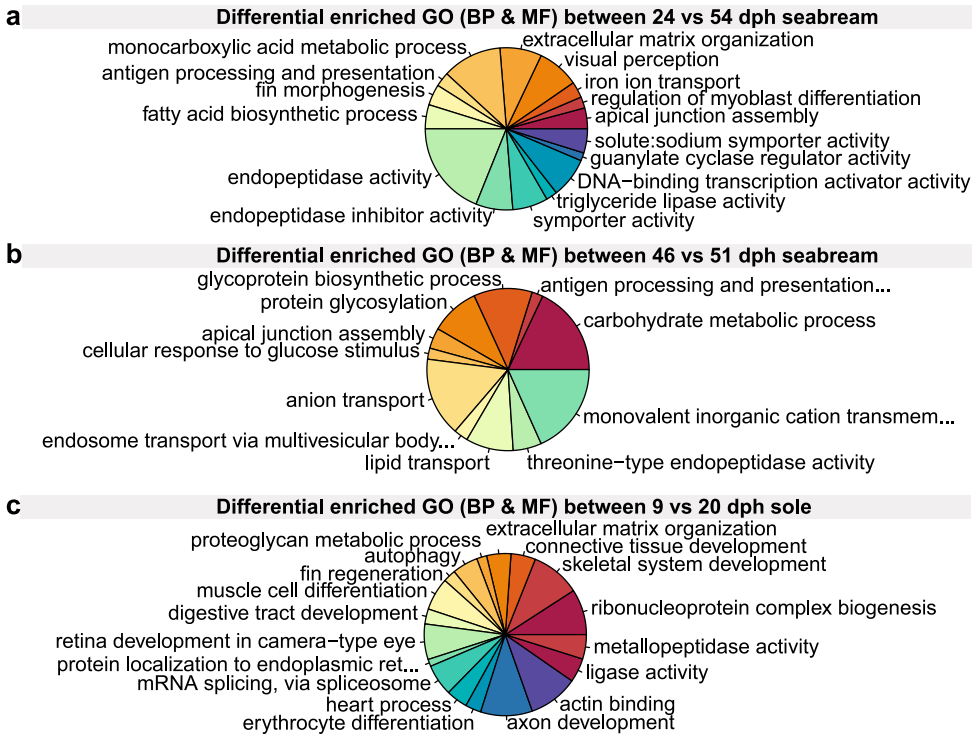
Supplementary Table 6 A, B). Overall, during gilthead seabream larval development, members of the Leucine rich repeat protein (LLR) genes had the highest level of expression at all stages, followed by NOD-like receptors (NLR), and Toll-like receptors (TLR). Of the 16 unique TLR genes identified, only TLR5 was significantly modified between flexion and mid-metamorphosis (Supplementary Table 6 A). Nineteen (19) *nlr*-like genes were significantly changed during metamorphosis in comparisons I and II and included representatives of NLR containing CARD domain and NLR containing PYD domain sub-families (Supplementary Table 6 A). Eight *nlr*-like genes were down-regulated at flexion compared to mid-metamorphosis (Supplementary Table 6 A). Of the 167 unique LLR genes, 39 were significantly modified (Supplementary Table 6 A). Most of the lectin genes that changed significantly were low abundance in younger larvae at 24 dph compared to older larvae at 51 dph, and in larvae at 46 dph compared to 54 dph (Supplementary Table 6 A).

3.6. Transcriptome profile of the endocrine repertoire during larval development

Analysis of the global transcriptomes of gilthead seabream larvae at flexion and mid-metamorphosis (n = 20), identified 181 endocrine transcripts that were expressed in >50% of the samples (Supplementary Table 7 A-B). Of the 100 endocrine genes (Supplementary Table 6) with the highest level of expression during flexion and mid-metamorphosis, few (16) were DEGs in comparisons I and II (Fig. 8).

The endocrine related genes differentially expressed between stages belonged to the GH/IGF axis and thyroid axis. Among the members of the GH/IGF axis, somatostatin-2-like (*sst2-l*) was low abundance and somatostatin receptor type 2-like (*sstr2-l*) was more abundant in larvae at 24 dph compared to larvae at 50 dph (Fig. 8). Several igf related genes - *igfbp1a*, *igfbp1b*, *igf1ra*, *igf2b* - and *ghrb* were significantly more abundant in larvae at flexion compared to larvae at mid-metamorphosis (Fig. 8). In comparison II, *igfbp7* was down-regulated and *igfbp4* was up-regulated (Fig. 8). Overall, most of the IGFBPs (including *igfbp2a*, *igfbp2b*, *igfbp3a*, *igfbp3b*, *igfbp5a*, *igfbp5b*, *igfbp6a*, *igfbp6b*) had a constant expression pattern in early development from flexion to late-metamorphosis, but for duplicated genes one of the isoforms always had a higher expression level (*igfbp2a*, *igfbp5b*, *igfbp3a*, *igfbp6b*) during ontogeny. The IGFBPs with the highest level of expression was *igfbp5b* and *igfbp5a*, and the least expressed genes were *igfbp6a* and *igbp3b*.

In relation to the thyroid axis thyroxine 5-deiodinase-like (*Dio3*) was significantly up-regulated while iodothyronine deiodinase 1 (*Dio 1*) and iodothyronine deiodinase 2 (*Dio 2*) were low abundance in larvae at flexion (24 dph) compared to larvae at mid-metamorphosis (Fig. 8). The expression of thyroid hormone receptor-associated protein 3a (*thrap3a*)



d

Enriched Biological Process	GO	9 d *Ss*	24 d *Sa*	46 d *Sa*
regulation of immune response	GO:0050776	↓	↓	-
immune system process	GO:0002376	↓	-	↓
antigen processing and presentation	GO:0019882	↓	↓	-
inflammatory response	GO:0006954	↓	-	↓
gland development	GO:0048732		-	↑ -
enteroendocrine cell differentiation	GO:0035883		-	↑ -
proteolysis	GO:0006508	↓	↓	-
lipid homeostasis	GO:0055088	↓	-	↑
Enriched pathway	**KEGG**			
Lysosome	dre04142	↓	↓	-
NOD-like receptor signaling pathway	dre04621	↓	-	↓
C-type lectin receptor signaling pathway	dre04625	↓	-	-
RIG-I-like receptor signaling pathway	dre04622	↓	-	-
Toll-like receptor signaling pathway	dre04620	↓	-	-
Cytokine-cytokine receptor interaction	dre04060	↓	-	-
Phagosome	dre04145	↓	-	↓

Fig. 4. Identification of enriched Gene Ontology and KEGG pathways during gilthead seabream and Senegalese sole larval development. Pie charts represent the relative percentage of the enriched biological process (BP) and molecular function (MF) GO terms related to the DEGs between 24 vs 54 dph gilthead seabream (a), 46 vs 51 dph gilthead seabream (b), and 9 vs 20 dph Senegalese sole (c). Part d provides a summary of GO terms and KEGG pathways with enriched gene sets in less developed gilthead seabream (*Sa*) and Senegalese sole (*Ss*) larvae. The arrows pointing upwards and downwards represent up- and down-regulated gene sets related to each enriched GO term or KEGG pathway, respectively.

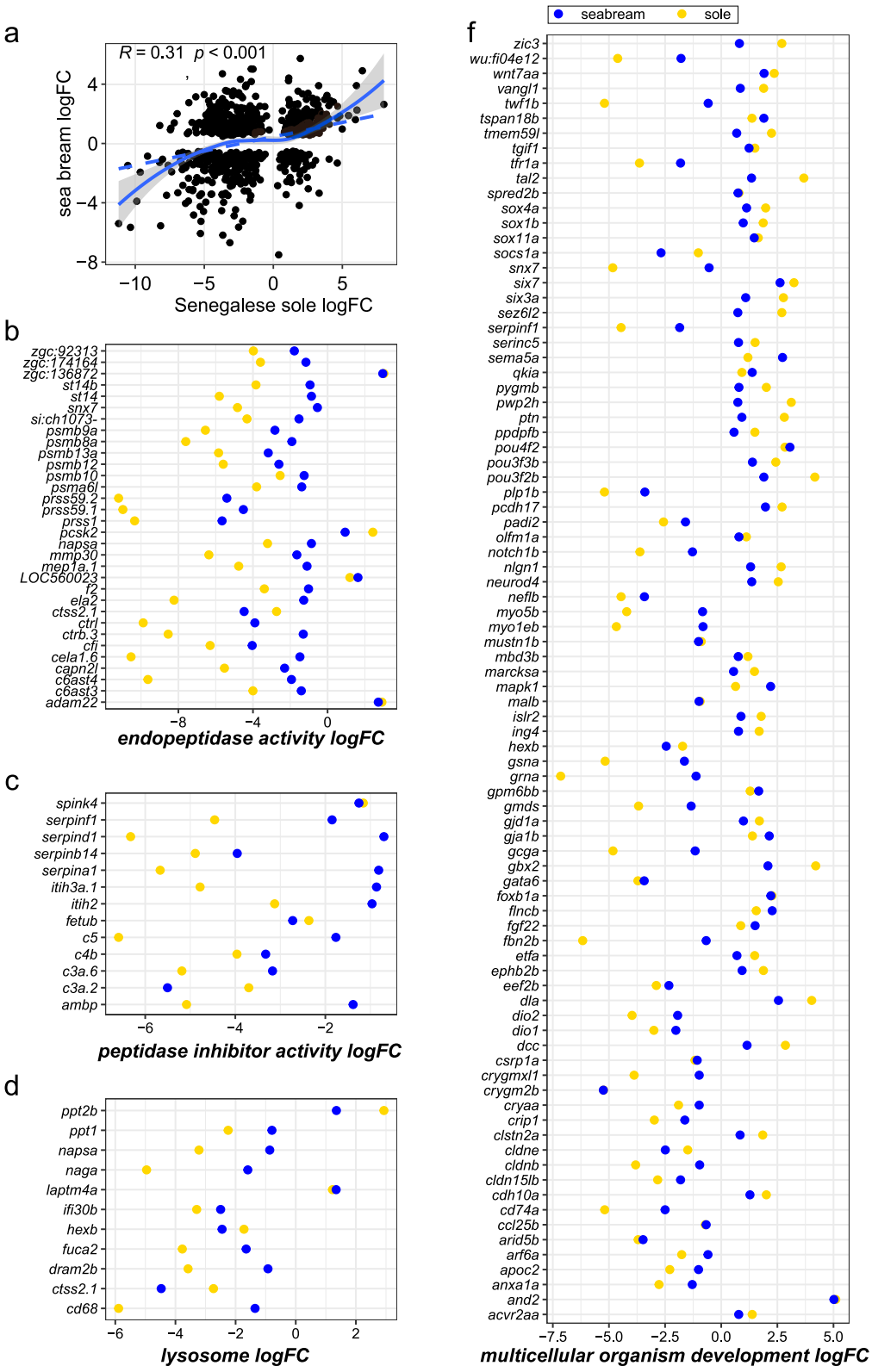


Fig. 5. Comparative analysis of DEGs and the expression pattern of common DEGs in gilthead sea bream and Senegalese sole. a) the correlation between DEGs in the gilthead seabream and Senegalese sole, b) endopeptidase activity, c) peptidase inhibitor activity, d) lysosome, and f) multicellular organism development, in gilthead seabream and Senegalese sole during metamorphosis. The DEGs were identified in similar developmental stages of gilthead seabream and Senegalese sole. In the case of gilthead seabream, the transcriptome at flexion (avg. age 24 dph) and early to mid-metamorphic (avg. age 46 dph) larvae were compared and in the case of Senegalese sole the transcriptome of pre-metamorphic (9 dph) and post-metamorphic (20 dph) larvae were compared. The identified DEGs from gilthead seabream and Senegalese sole that were common and had a similar expression pattern are included in the graphical representation.

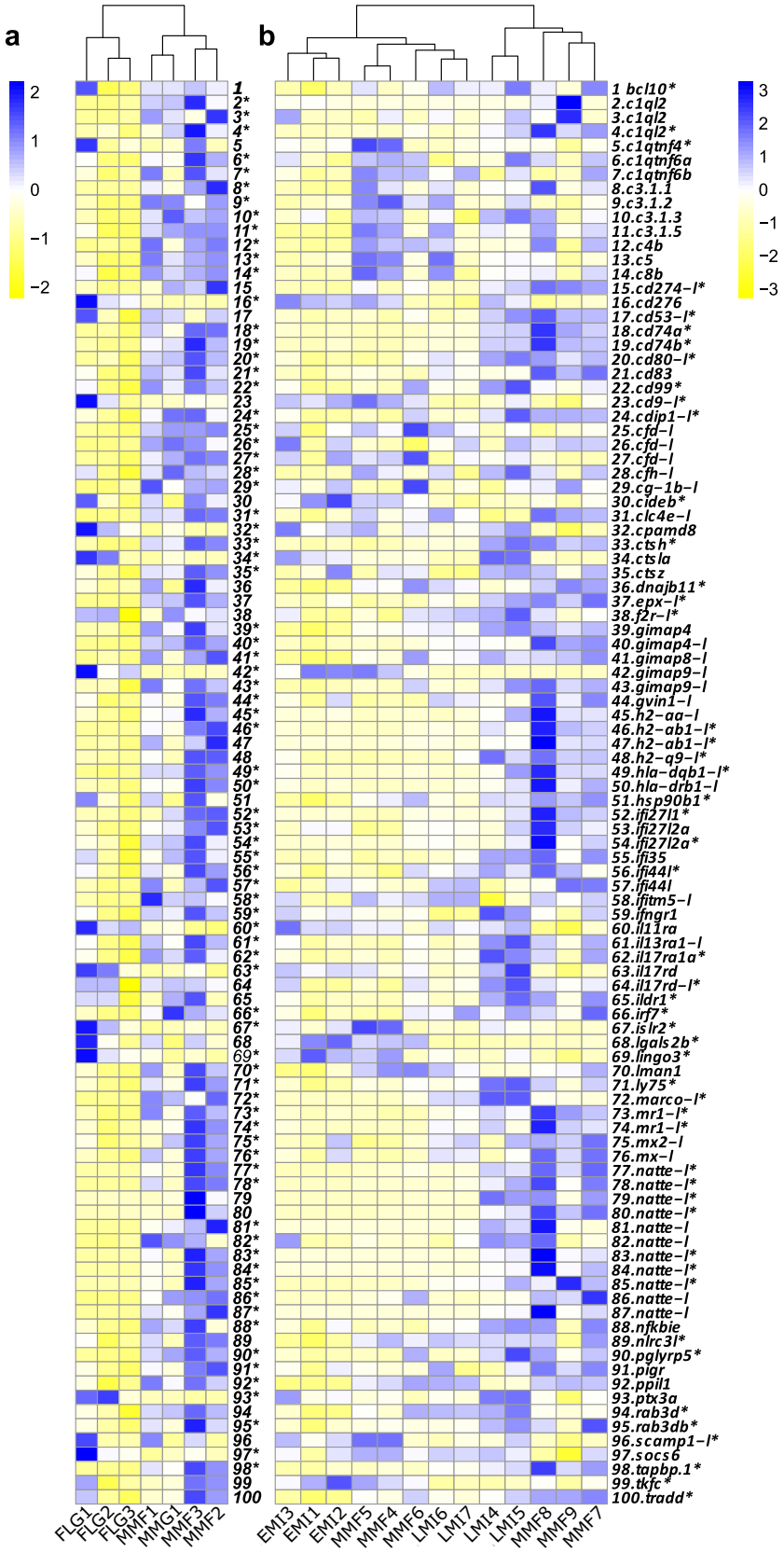


Fig. 6. Heatmap of immune-associated gene transcripts identified as DE in the comparisons I and II in gilthead seabream larvae. a) comparison I (24 vs 51 dph gilthead seabream larvae) DEGs were identified by comparing the transcriptomes of 3 larvae at flexion (FL) from site Gr and 4 larvae at mid-metamorphosis (MM) from site Gr and Fr (Fig. 2). b) comparison II (46 vs 54 dph gilthead seabream larvae) DEGs were identified by analysing thirteen larval samples at different metamorphic stages, including five larvae samples at early-metamorphosis (EM) to mid-metamorphosis (from site Fr and It) versus eight larvae at mid to late (LM)-metamorphosis (from site Fr and It). The Refseq accession of the gene transcripts used in the heatmap are presented in Supplementary Table 6. Asterisk (*) marks the genes that were significantly modified in each comparison.

was higher in more developed larvae at the mid and late-metamorphic stages.

Genes involved in neuroendocrine differentiation such as insulinoma-associated 1a (*insm1a*) and insulinoma-associated 1b (*insm1b*) were significantly more abundant in larvae at flexion (24 dph) compared to larvae at mid-metamorphosis (51 dph, Fig. 8). *Insm1a* was also significantly more abundant in larvae at 46 dph compared to larvae at 54 dph (comparison II, Fig. 8).

3.6.1. *Association of co-expressed gene modules with age and weight*

WGCNA I and II generated 18 gene modules. The genes with high gene significance (GS) or correlation with age and weight were analysed in more detail (Supplementary Fig. 6 and Supplementary Table 8). The green module (Megreen) of WGCNA for comparison I showed the highest positive correlation with age ($r = 0.86$, $p = 0.01$), and blue modules (Meblue) of WGCNA comparison I and WGCNA comparison II showed the highest positive correlation with weight ($r = 0.88$, $p = 0.01$; $r = 0.81$, $p =$ 9e-04). The functional profile of genes in the most correlated modules with age and weight were further analysed (Megreen and Meblue of WGCNA I, Meblue of WGCNA II). The ontology of gene sets in each module was specified and GO terms associated with age and weight were identified (Fig. 9). The results showed that biological processes like skeletal muscle, organ development, and complement activation were strongly associated with age (Fig. 9 a). Biological processes like immune response, antigen processing and presentation, oxygen transport, peptide metabolic process, respiratory electron transport chain, and cellular respiration were associated with weight (Fig. 9 b).

Fig. 7. Differential expression pattern of pathogen recognition receptors (PRRs) in gilthead seabream larvae at different stages. Sum of the log-fold change of PRR gene transcripts that were significantly down-regulated or up-regulated in a) larvae at 24 dph (compared to larvae at 51 dph and b) in larvae at 46 dph (compared to larvae at 54 dph).

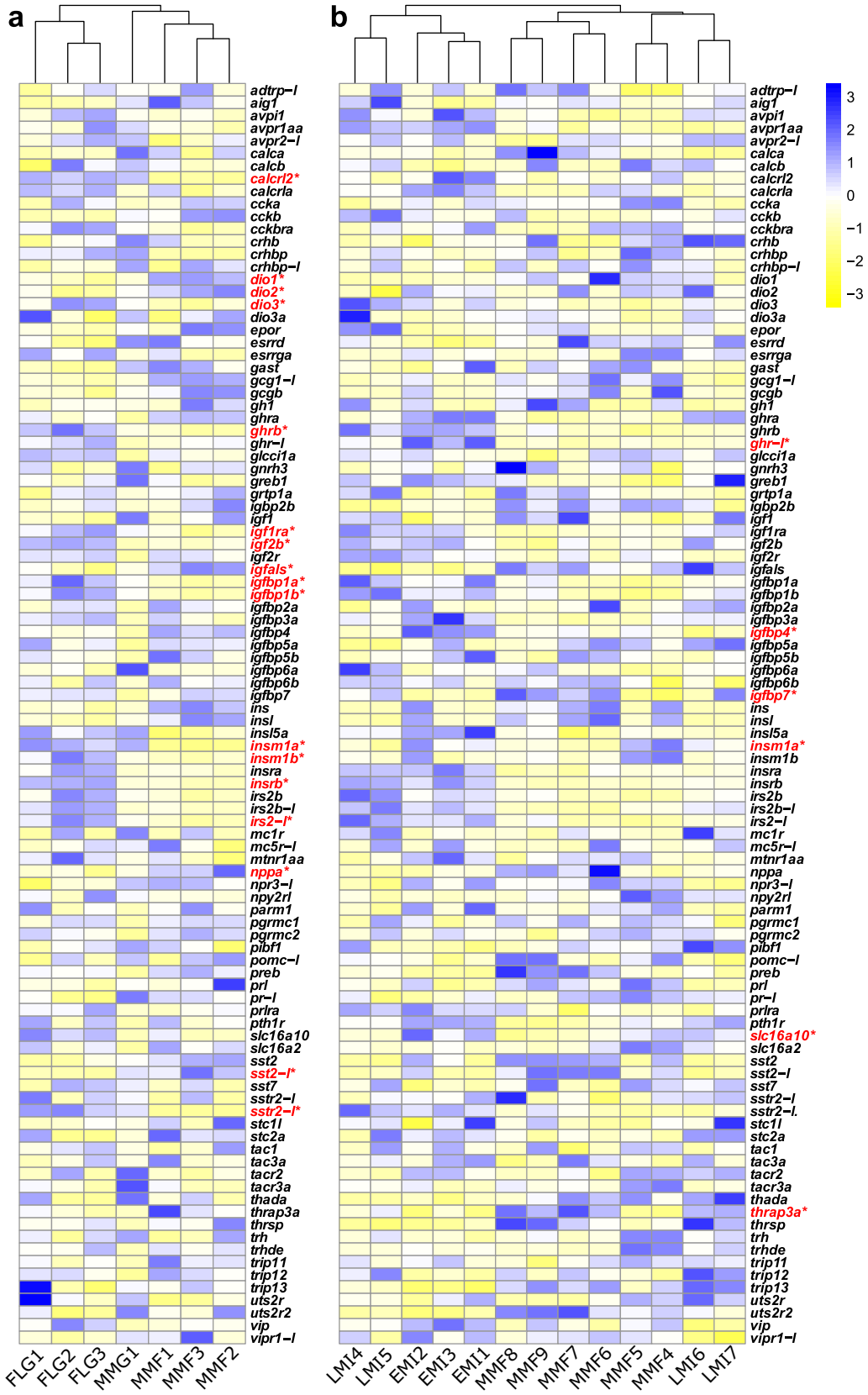


Fig. 8. Heatmap of the 100 most represented endocrine-associated genes identified in gilthead seabream transcriptomes of larvae during metamorphosis. a) the gene transcripts that were significantly modified based on comparison I between larvae at flexion (FL, avg. 24 dph) compared to larvae at mid-metamorphosis (MM, avg. 51 dph) are indicated in red and marked with an asterisk (*), and b) the gene transcripts that were significantly modified based on comparison II between larvae at early (EM) to mid-metamorphosis (avg. 46 dph) and larvae at mid to late (LM)-metamorphosis (avg. 56 dph) are indicated in red and marked with an asterisk (*). The colour shading (blue and yellow) represents the fold-change in gene expression. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.7. QPCR validation of transcriptome data

The results for qPCR amplicons of six genes (*hbae5, map4, lct, chia, rh50, and tcnba)* selected from the DEGs with a high log-fold change ($n = 20$ samples) had a significant positive correlation with the normalized RNA-seq read counts and qPCR amplicons (Fig. 10). Similar gene expression patterns (up- or down-regulation) of DEGs (comparisons I and II) and qPCR amplification of the candidate genes were identified, e.g. increased expression of *hbae5*, *map4*, *tcnba*, and decreased expression of *lct* and *chia* with age (Supplementary Fig. 7).

4. Discussion

By integrating the transcriptomes obtained for larvae from different commercial hatcheries, it was found that the larval developmental stage, whether determined by age or weight, was the primary factor driving the transcriptional response during metamorphosis. Consequently, a group of common modifications in the transcriptional responses of larvae were identified, regardless of their hatchery of origin or the species.

The shift from larvae to juveniles (metamorphosis) involves a multitude of alterations in physiology, morphology, behaviour, and metabolism in fish. This critical developmental stage encompasses the maturation of various vital systems such as the digestive tract, immune response, neuro-muscular skeletal system, skin and pigmentation, visual capabilities, and sexual maturation, ultimately leading to the establishment of a fully functional and independent organism (Manchado et al., 2016). Consistent with prior observations, our analysis revealed an enrichment of differentially expressed gene (DEG) sets associated with diverse biological processes, including development, growth, detoxification, the nervous system, the ubiquitin-proteasome system, metabolism, and phototransduction. Notably, we propose specific enrichments are indicative of stage-specific developmental processes, such as the abundance of genes related to the nervous system or the FoxO signaling pathway during flexion, and the enhanced immune response during mid-metamorphosis. The expression of the majority of endocrine genes analysed remained stable, but notable modifications of some endocrine-associated genes, e.g., *dio1*, *dio2*, *cldn1*, *ing4*, *Pou3f4*, and *fgf22*, underscore their significant role in metamorphosis. These consensus findings from larvae of different commercial hatcheries support and reinforce existing knowledge and unveil cornerstone molecular and biological processes and stage-specific gene expression dynamics during development and metamorphosis.

Previous transcriptome studies of fish development have tended to be based on homogeneous samples from small-scale or pilot scale production with low sample numbers and a single captive broodstock. Such studies reported an abundance of specific-gene sets, such as genes encoding myogenic proteins in gilthead seabream at hatch (Sarropoulou et al., 2016), homeobox genes in early stages of Atlantic bonito (*Sarda sarda*, Sarropoulou et al., 2014), genes of extracellular matrix/ECM components (e.g., *col1a1*, *col1a2*, *col5a2*, *ddr1*, *mmp13*, *mmp2* and *metalloproteinase inhibitor 3*) in flatfish (Alves et al., 2016), and genes of fat digestion and absorption and antigen processing and presentation pathways in the zebrafish mouth-opening stage (Xu et al., 2017). The extensive sampling in the present study from different commercial hatcheries and of two crucial stages of gilthead seabream larvae identified conserved genetic programs that promote metamorphosis and are not perturbed by management regimes or broodstock. Furthermore, from an applied perspective gene sets and processes were identified that demonstrated enrichment in specific larval stages and were consistently observed across multiple commercial hatcheries and production regimes. The results hold great promise for the identification of potential biomarkers for monitoring both the developmental stage and production quality, since origin or management regime does not perturb them.

4.1. Stage-specific modification of gene sets and pathways crucial for morphogenesis and adaptation in larvae

Major changes in gilthead seabream morphology occur between 40 and 123 dph, as the head region becomes shorter, the trunk grows, and the orientation of the mouth changes and becomes more ventral (Russo et al., 2007). In gilthead seabream, metamorphosis occurs at approximately 45–55 dph (Manchado et al., 2016). The changes in morphology at metamorphosis are orchestrated by the activation/suppression of specific gene sets or pathways with a well described role in vertebrate development and adaptation such as focal adhesion, ECM receptor interactions (Martins-Green and Bissell, 1995; Kjær, 2004;), Wnt signaling (Logan and Nusse, 2004; Komiya and Habas, 2008), the phototransduction pathways (Shimmura et al., 2017), and homeobox genes (Hobert and Westphal, 2000; Duverger and Morasso, 2008;). For example, in early stages (flexion) of both gilthead seabream and Senegalese sole DEGs included those of focal adhesion such as *mpk1*, *thbs4b*, *col9a2*, *col9a3*, *arhgap35b*, *itga8*, *thbs1a*, ECM receptor interactions (*frem2a*, *frem2b*, *thbs1a*, *thbs1b*, *itga8*) and biological processes involved in cellular development, fusion and interaction. Changes in these genes are indicative of active cell differentiation, adhesion and migration, all of which are important for the development and remodeling of tissue, hallmarks of early development. DEGs encoding cadherins (*cdh17,* LI cadherin and *cdh15*, M-cadherin), which are important in development of the liver, intestine, and skeletal muscle in mouse and humans (Baumgartner, 2013; Esteves de Lima et al., 2021) were highly expressed in early stages of gilthead seabream larvae (24 and 46 dph). The high expression of homeobox genes in early stages of gilthead seabream larvae (24 and/or 46 dph) is in line with their known role as major regulators of anatomical development, including the hox genes (e.g., *hoxd4b*, *hoxa10*, *arxa*, *six1*), LIM genes (e.g., *lhx4*, *lhx1*, *lhx5*) and POU genes (e.g., *pou3f2a*, *pou3f4*, *pou4f1*, *pou4f2*), which function in brain and sensory structure development and neural patterning (Hobert and Westphal, 2000; Seufert et al., 2005; Kitambi and Chandrasekar, 2021).

Feeding regime is crucial and determines hatchery production quality so it needs to be adapted to the morphology, physiology, and nutritional requirements of the larvae. The results from the transcriptome and histology are indicative of high lipid metabolism (e.g., *mgat*, *dgat*, *agpat*, *gpat1*) and this may be due to the feeding regime of live and artificial feeds rich in fats as has previously been reported (Fontagné et al., 1998; Morais et al., 2007). Functional profiling of the genes involved in lipid metabolism revealed they were mostly up regulated at early and mid-metamorphosis in larvae with a lower growth rate and this has previously been linked to reduced digestibility and absorption of high lipid diets in larval gilthead seabream as well as to reduced growth performance of other marine fish (Izquierdo et al., 2000; Olsen et al., 2000; Gawlicka et al., 2002; Morais et al., 2007; Bonaldo et al., 2010). The upregulation of genes involved in carbohydrate metabolism (*lct*, *pfkm*, and *pygm*) in 24 dph and 46 dph gilthead seabream larvae indicate carbohydrates are an important source of metabolic energy for metamorphosis. This is further supported by the high enrichment of the 'insulin-like growth factor receptor signaling pathway' and 'cellular response to insulin stimulus' in 24 dph larvae together with the higher expression of genes such as *insrb* and *ghrb*, that are both involved in glucose hemostasis. The greater dependence of early-stages of gilthead seabream larvae on carbohydrates may be linked to their reduced capacity to digest protein (Fig. 11) as has previously been reported in other fish larvae (Douglas et al., 2008; Hilerio-Ruiz et al., 2021; Najafpour et al., 2021). An increased capacity for protein digestion after metamorphosis is common in carnivorous fish species, such as white seabass (Galaviz et al., 2011), spotted rose snapper (Galaviz et al., 2012), the rainbow trout (*Oncorhynchus mykiss*) and the Caspian brown trout (*Salmo trutta caspius*, Najafpour et al., 2021). The appearance of a stomach and the up-regulation of pepsin, pepsinogen and trypsin (Fig. 11) after metamorphosis is consistent with carnivorous preferences after metamorphosis (Mata-Sotres et al., 2016).

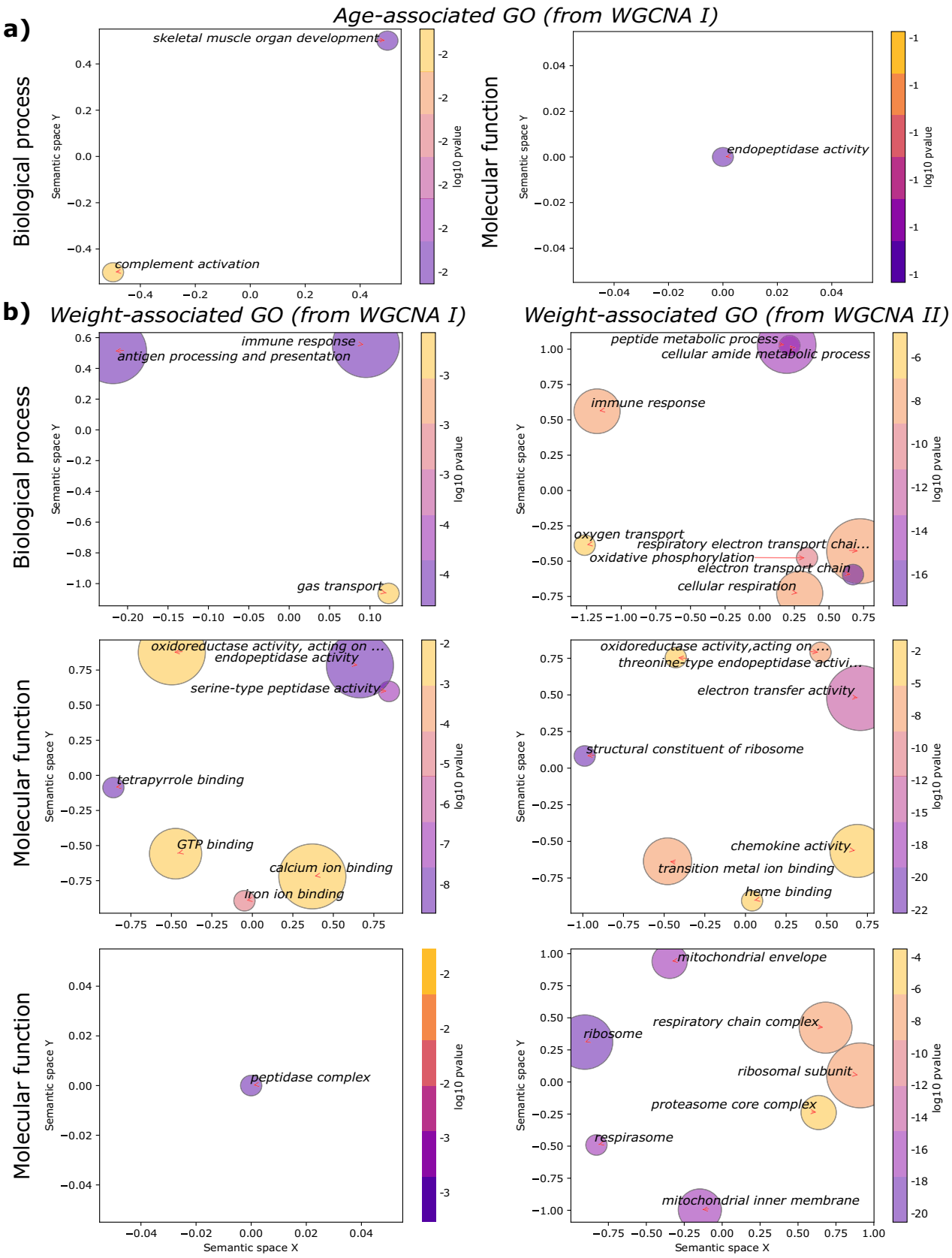


Fig. 9. Semantic similarity scatterplots summarizing a list of enriched Gene Ontology (GO) terms related to highly correlated gene modules with age (a) and weight (b) in Weighted gene co-expression network analysis (WGCNA). The gene set of the green modulewith the highest correlation with age in WGCNA I and the gene sets of blue modules with the highest correlation with weight in WGCNA I and WGCNA II were used in the functional enrichment analysis (see Supplementary Table 8). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

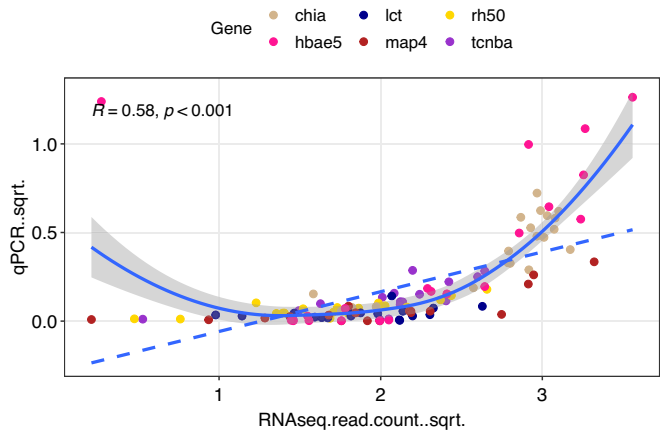


Fig. 10. Validation of RNA-seq data using a correlation analysis between qPCR amplification and RNA-seq read counts of seven genes. The square root (sqrt) of the normalized qPCR and RNA-seq results were used in the analysis. The scatter plots were generated using the "Spearman" method in an R environment. A positive correlation was observed ($P < 0.05$).

4.2. The immune-related gene repertoire during the larval-juvenile transition

Overall, the immune repertoire of teleost fish is more complex than other vertebrates because of the acquisition and retention of additional gene copies as a consequence of the teleost specific whole genome duplication (TSWGD, Glasauer and Neuhauss, 2014) and lineage and species-specific evolution of immune-related genes (e.g., the complement genes, Najafpour et al., 2020). Studies of immune-related molecules during larval development have generally been based on a candidate gene approach, and relatively little information is available about the acquisition of immune capacity in fish larvae. For example, in the Atlantic cod a study of IgM revealed it was absent until 8–10 weeks post-hatch (Schrøder et al., 1998), which suggests the immune repertoire and immune response differs between larvae, juvenile and adult fish. In zebrafish, the alternative pathway of the complement system was proposed to be activated in response to LPS in larval stages (Wang et al., 2008). The general notion that teleost larvae have a less-developed adaptive immune response compared to the innate immune response (Vadstein et al., 2013; Castro et al., 2015; DeWitte-Orr et al., 2019) means studies have focused primarily on innate immunity in larvae. Additionally many studies and experimental strategies in larvae have targeted immune training in intensive-aquaculture fish larvae using immunostimulants or molecules simulating pathogen-associated molecular patterns (PAMPs) such as ß-glucans, bacterial products, and plant constituents (Bricknell and Dalmo, 2005; Citarasu, 2010; Abarike et al., 2019; Zhang et al., 2019).

The detailed analysis of immune-related genes in the present transcriptome study contributes to improving understanding of the ontogeny of immunity in developing fish larvae. Many innate and adaptive immune-associated genes (e.g., complement, MHC, interferons and interleukins) were down-regulated in larvae at flexion and early to mid-metamorphosis (24 and 46 dph) compared to larvae at mid-metamorphosis and mid- to late metamorphosis (51–54 dph). The significant changes in immune-related gene transcripts between larvae at flexion and mid-metamorphosis were coherent with a profound change in the immune capacity of gilthead seabream larvae during development (Fig. 6). The down-regulation of complement associated gene transcripts (e.g., *c1qa*, *c1qb*, *c1qc*, *mr1*) detected in gilthead seabream at flexion mirrors what happens to the complement system genes (*c3*, *c1r/s*, *c4*, *bf*, *mbl* and *masp*) in zebrafish larvae after hatching (Wang et al., 2008). A low mean antibody titre up to 3-weeks post-hatch was also considered to indicate low immune competence in the Indian major carp, *Labeo rohita* (Swain et al., 2006). The onset of immune gene expression in Atlantic cod (*Gadus morhua*) was identified at hatch and first feeding (Seppola et al., 2009). A marked increase was observed in transcriptional levels of the immune genes such as hepcidin, cathelicidin, LGP2, heavy chain C-regions of IgD and sIgM prior to and during metamorphosis in Atlantic cod (Seppola et al., 2009). Overall, our transcriptome data from gilthead seabream larvae agrees with previous observations in the cod since there was low abundance of many gene transcripts related to the immune system at flexion, although it remains to be established whether this is synonymous with the functional capacity of the system. The generally low expression of immune related gene transcripts in early larval stages of gilthead seabream is corroborated by histological studies of hemopoietic tissue and lymphoid organs, which develop from flexion to mid-metamorphosis (Jósefsson and Tatner, 1993). A similar situation occurs in the rock bream (*Oplegnathus fasciatus*) with the pronephric kidney (10 dph) developing first and then the thymus (15 dph) and spleen (21 dph, Xiao et al., 2013). The results of our gene transcription study were coherent with the histological observations of previous studies and indicated that immune-related gene expression achieved significantly higher expression at mid- and late-metamorphosis compared to earlier stages, and suggested comprehensive immune competence was only acquired in late larval stages of gilthead seabream.

An interesting facet of the immune-related DEGs was the significant change in expression of Pattern Recognition Receptor (PRR) genes. PRR proteins permit recognition of molecules associated with pathogens (Pathogen-Associated Molecular Patterns-PAMPs, Amarante-Mendes et al., 2018). The results of the gilthead seabream transcriptome analysis with detection of strong down regulation of PRRs at flexion and up regulation at mid- to late-metamorphosis suggests that contrary to the accepted viewpoint, the innate immune repertoire underwent maturation from poorly developed in early larvae to well-developed in older larvae. Although numerous PRRs were identified in the larval stages analysed they were much less abundant in larvae at flexion compared to larvae at metamorphosis. Although the abundance of toll-like receptors (TLR) was not significantly changed during larval development, lectin-like PRRs and galactose-specific lectin nattectin-like, involved in neutrophil mobilization in mice (Lopes-Ferreira et al., 2011), were more abundant in metamorphic stages. Further work will be required to better characterize innate immune maturation and immunocompetence in fish larvae and the potential regulatory factors that influence immune system development and the immune response.

4.3. Common molecular mechanisms during symmetric and asymmetric fish metamorphosis

The meta-analysis of transcriptomes from metamorphosing flatfish (Senegalese sole) and round fish (gilthead seabream) uncovered a shared set of conserved molecular modifications, despite the substantial differences in their external morphology and ecological adaptations, these included significant modifications in 'endopeptidase activity', 'peptidase inhibitor activity', 'multicellular organism development', and 'lysosomes'. This suggests the existence of fundamental regulatory processes that underlie metamorphosis across diverse fish species, transcending their apparent morphological and ecological disparities. Furthermore, the comparison of the Senegalese sole and gilthead seabream transcriptomes during metamorphosis suggested common molecular mechanisms underlie organ and tissue development and maturation during metamorphosis in these two species. Many genes involved in nervous system development (e.g., neuron differentiation and development) were active in both species, especially in gilthead seabream at flexion and early to mid-metamorphic stages (compared to mid to late metamorphic stages) and in Senegalese sole at the pre-metamorphic stage (compared to post-metamorphic stage).

This comparative analysis of the transcriptomes of two species revealed common gene sets activated or suppressed with age in both species. Examples of activated genes and processes were the development of the liver and hepatobiliary system (e.g., *gata6*, *arf6b*, *cldn15lb*,

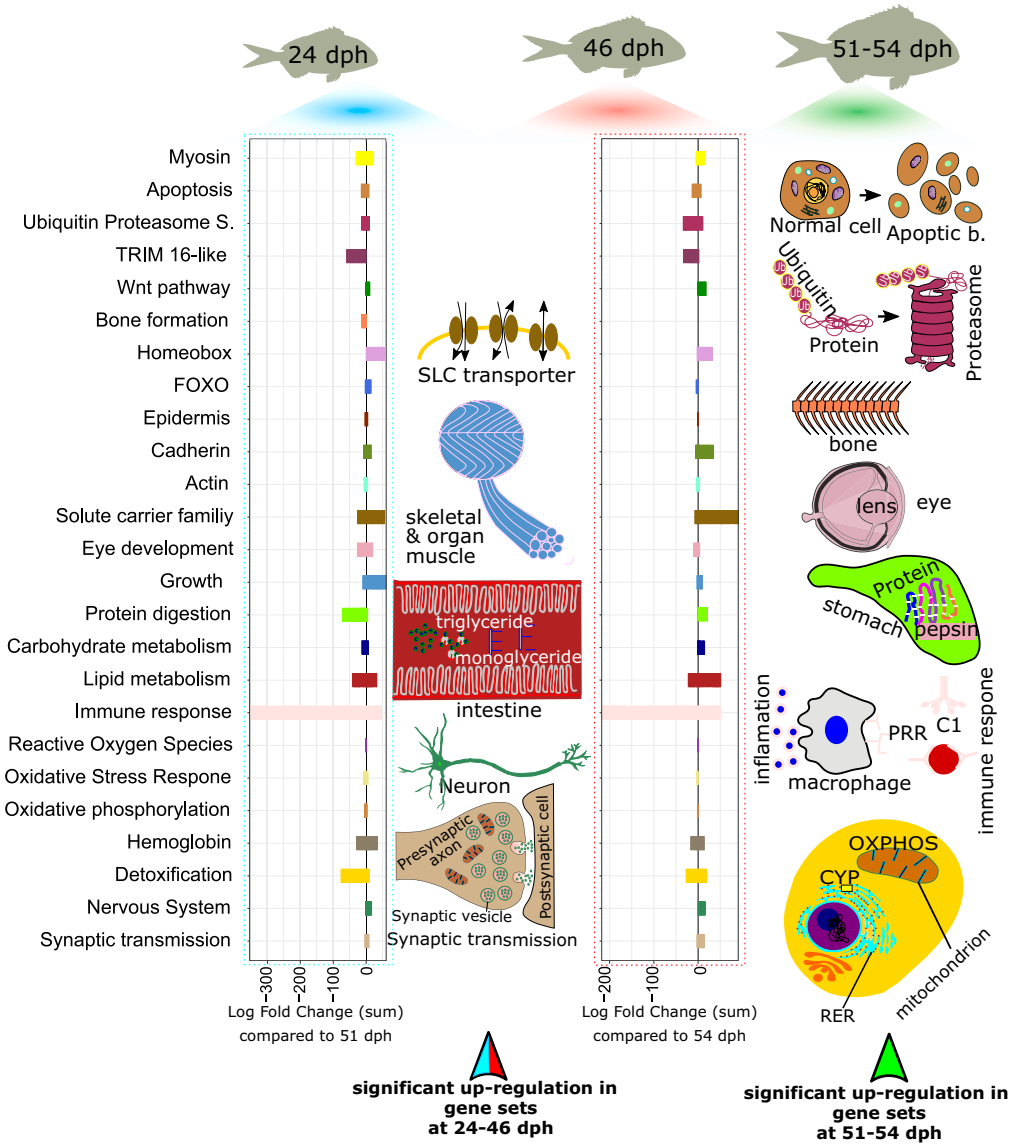


Fig. 11. Pictogram relating transcriptional changes with biological function during the ontogeny of gilthead seabream larvae. The sum of log fold change of the gene sets with diverse biological roles that were up- and down-regulated at 24 versus 46 dph compared to older larval stages, 51 versus 54 dph are represented. In the case of DEGs for the 24 versus 46 dph comparison the gene sets more activated compared to older larvae were involved in skeletal and muscle development and nervous system development, while for the DEGs for the 51 versus 54 dph larvae gene sets involved in the immune response were more activated than in younger larvae.

anxa1a), circulatory system and vasculature (e.g., *gata6*, *fbn2b*, *myo5b*, *crip1*, *notch1b*, *padi2*), muscle hyperplasia (e.g., *mustn1b*, *myo5b*, *tgif1*), immune system (*ccl25b*, *notch1b*), thyroid hormone metabolic processes (e.g., *dio1*, *dio2*), and bicellular tight junctions (e.g., *cldn15lb*, *cldne*, *cldnb*, *cldna*, *cldnk*). Suppressed genes and processes were mostly involved in brain and eye development (*gbx2*, *six3a*, *pou3f2*, *pou3f3b*, *foxb1a*, *sox11a*, *six7*, *pou4f2*, *tgif1*, *tal2*, *sox4a*, *olfm1a*) and overall transcription factor activity declined significantly from 9 to 24 dph in Senegalese sole and 20 and 51 dph in the gilthead seabream. However, it should be noted that analysis based on whole larvae or pools of larvae has limited resolution and likely masks important transcriptional changes of some genes since it does not consider, the developmental status and relative contribution of specific organs and tissues to the whole-body transcriptome. To establish the timing of important developmental processes and their responsiveness to hormones it will be important in the future to conduct tissue or even cell specific gene transcript studies.

The current study focused on common DEGs with similar expression patterns in the Senegalese sole and gilthead seabream and identified core and essential developmental changes shared in these fish larvae. Another group of genes were identified with an inverse expression pattern between Senegalese sole and gilthead seabream, which we hypothesize are linked to the unique characteristics of flatfish metamorphosis (asymmetry and ecology driven) since they included gene sets involved in melanin biosynthesis (e.g., *tyrp1b*, *qdpra*, *dct*) and visual phototransduction (*gnb1b*, *pde6gb*, *gnat1*, *bco2b*, *cnga1b*, *rpe65a*), both of which profoundly change during asymmetry acquisition in flatfish (Bolker and Hill, 2000; Shao et al., 2017; Zhang et al., 2022).

4.4. Modulation of the larval – juvenile transition by the endocrine repertoire

Overall, a stable expression pattern of most of the endocrine-associated genes was identified during gilthead seabream development. This is unsurprising when the dynamic nature of endocrine systems and the associated gene expression is considered along with the relatively small contribution endocrine-related gene transcripts make to whole larvae transcriptomes. Nonetheless, several genes encoding

endocrine factors were well-correlated with weight (WGCNA analysis) and specific gene isoforms of the GH-IGF and thyroid axis were associated with the larval developmental status such as activation of *igfbp7*, *igfals*, *dio1* in more developed larvae and *igfbp1a*, *dio3*, *igfbp4* in less developed larvae. The variation in the expression level of endocrine-associated genes such as IGFBP isoforms may reflect their tissue-specific function in growth and development. In line with our results we propose, that the high expression levels of *igfbp5b* may result from its expression in several different organs, and a broad tissue distribution of *igfbp5b* has previously been reported in fish (e.g., zebrafish), whereas *igfbp5a* had a more specific tissue distribution (e.g., high levels in brain and gill of zebrafish, Dai et al., 2010; de la Serrana and Macqueen, 2018). Interestingly, we found that genes of the GH-IGF system relative to other endocrine systems were most significantly changed during metamorphosis, and this observation merits further attention in the future. The *igf1ra* expression patterns (up-regulated at flexion compared to mid-metamorphic gilthead seabream larvae) is another example that suggests the GH - IGF I system is important during gilthead seabream larvae metamorphosis, especially since the other associated gene (*igf1rb*) was absent or expressed at a low level. In line with our results, the significant changes in gene expression involved in the GH–IGF-I pathway (*igf1r* and *ghr* transcripts) during Atlantic halibut metamorphosis further emphasize the substantial role in teleost fish of the GH-IGF I system for successful development (Hildahl et al., 2007, 2008). Furthermore, the role of the GH/IGF axis in immune system development should be investigated since it has already been shown to play a role in immune cell proliferation and differentiation in adult fish (Kajita et al., 1992; Calduch-Giner et al., 1995; Yada, 2007; Franz et al., 2016).

The significant change in expression of genes belonging to the iodothyronine deiodinase family (*dio1*, *dio2*, *dio3*), in gilthead seabream during flexion to the mid-metamorphosis transition, echoes what is already established in flatfish metamorphosis and highlights the importance of the thyroid axis and deiodinases in fish metamorphosis in general (Isorna et al., 2009). Interestingly in mammals and some fish (e.g., zebrafish) *DIO3*, which regulates thyroid hormone inactivation is suggested to delay growth and is highly expressed in early stages (Heijlen et al., 2014; Luongo et al., 2019). Similarly, in gilthead seabream at flexion (24 dph) *dio3* was significantly up-regulated, while expression of *dio1* and *dio2* were significantly increased at mid-metamorphosis (51 dph), suggesting a higher rate of thyroid hormone activation by conversion of T4 to T3 at mid-metamorphosis. The current results corroborate the outcome of a previous candidate gene study of deiodinase (*dio1* and *dio2*) expression using RT-PCR in gilthead seabream (Campinho et al., 2010).

5. Summary

Principal component analysis (PCA) using larval samples from different commercial hatcheries and the designed differential expression analyses based on clustering and the distance between larvae samples provided unbiased gene expression profiles at each developmental stage. In addition, the association of co-expressed gene modules with age and weight uncovered putative age and quality-related gene markers. The approach taken for transcriptome analysis using larvae from several different hatchery sites and regimes allowed the identification of core conserved pathways of fundamental importance for larval development and survival. This approach eliminated the "noise" from variability of DEG profiles between samples since only those common across all the hatcheries were identified as significantly modified.

The study provided insight into core transcriptome modifications of gilthead seabream larvae from hatchery production during development from flexion to late-metamorphosis. The most abundant and common differentially expressed genes during larval ontogeny were involved in development, growth, detoxification, the nervous system, the ubiquitin-proteasome system, metabolism, phototransduction, and the immune system (Fig. 11). The consensus stage-specific activation and suppression of gene sets in larvae from several different hatchery sites were identified and provides robust markers indicative of morphogenesis and adaptation to hatchery conditions. Gene set enrichment analysis identified the main important processes through GOs at each stage, including nervous system development at the flexion stage and early to mid-metamorphosis (avg. age 46 dph) or muscle system process, immune system process, and proteasomal ubiquitin-independent protein catabolic process at mid to late-metamorphosis (avg. age 51–54 dph). Functional profiling of the genes involved in lipid metabolism revealed they were mostly up-regulated at early and mid-metamorphosis in larvae with lower growth rates and highlights the importance of balanced feeding regimes and the identified gene cluster may be interesting candidate markers of larval quality. Gene transcript analysis indicated that carbohydrate metabolism was the predominant source of metabolic energy for gilthead seabream metamorphosis. This was supported by the high enrichment of the 'insulin-like growth factor receptor signaling pathway' and 'cellular response to insulin stimulus' in 24 dph larvae.

The comparison of the Senegalese sole (an asymmetric and benthic species) and gilthead seabream (a symmetric and pelagic species) transcriptomes during metamorphosis suggested common molecular mechanisms underlying organ and tissue development and maturation during metamorphosis. In contrast, genes determining body pigmentation and eye symmetry had an inverse expression pattern in gilthead seabream and sole and these differences along with others we presume reflects gene sets important for the unique metamorphosis of flatfish.

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Author statement

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CRediT authorship contribution statement

Babak Najafpour: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Soraia Santos:** Methodology. **Manuel Manchado:** Writing – review & editing. **Aurora Vidal:** Investigation. **Andreas Tsipourlianos:** Investigation. **Adelino V.M. Canário:** Writing – review & editing. **Katerina A. Moutou:** Writing – review & editing. **Deborah M. Power:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

The raw data have been submitted to NCBI, and the related BioProject accession number to each library has been shared in the attached file.

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