

Article

Evidence for the Functional Relevance of *vgl3* and *six6* on Developmental Stages of Commercially Important Fish Species: Gilthead Seabream (*Sparus aurata* Linnaeus) and European Seabass (*Dicentrarchus labrax* Linnaeus)

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Abstract: The *six6* and *vgl3* genes play evolutionarily conserved roles in developmental processes and life history traits across species, including teleosts. Notable differences in genotype and allele frequencies of these genes have been observed between farmed and wild populations of European seabass and gilthead seabream, suggesting potential roles in traits associated with domestication. Here, we hypothesized that genetic variations in the *six6* and *vgl3* are associated with distinct expression profiles that underlie domestication-related traits in the two species. Using quantitative PCR (qPCR), we examined the expression profiles of these genes in early developmental stages across genotypes linked to domestication. Our results confirmed that *vgl3* and *six6* genotypes significantly influenced their expression in gilthead seabream, with statistically significant differences between genotypes. In European seabass, *six6* expression did not significantly differ among genotypes, although heterozygous larvae showed higher variability that decreased at the juvenile stage. Meanwhile, no genetic variation was observed in *vgl3*, precluding genotype-specific expression analysis. Altogether, our findings provide the first evidence confirming that prior DNA-based associations of these genes with domestication traits are reflected at the mRNA level, particularly in *vgl3* in gilthead seabream. This highlights the potential functional relevance of these genetic variations in shaping expression profiles linked to domestication in the species.

Keywords: developmental process; gene expression profiling; genetic variation; marine teleost; quantitative PCR (qPCR)

Key Contribution: This study provides novel evidence that genetic variations in *six6* and *vgl3* influence their expression profiles during early developmental stages in gilthead seabream and European seabass. These findings, particularly the genotype-linked expression differences in *vgl3*, underscore the functional relevance of these genes in the domestication process.



Academic Editor: Eric Hallerman

Received: 22 January 2025

Revised: 17 February 2025

Accepted: 22 February 2025

Published: 25 February 2025

Citation: Moulistanos, A.; Kaitetzidou, E.; Minoudi, S.; Gkagkavouzis, K.; Kallimanis, A.; Antonopoulou, E.; Triantafyllidis, A.; Papakostas, S. Evidence for the Functional Relevance of *vgl3* and *six6* on Developmental Stages of Commercially Important Fish Species: Gilthead Seabream (*Sparus aurata* Linnaeus) and European Seabass (*Dicentrarchus labrax* Linnaeus). *Fishes* **2025**, *10*, 96. <https://doi.org/10.3390/fishes10030096>

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1. Introduction

The SIX homeobox 6 (*six6*) and vestigial-like family member 3 (*vgll3*) genes function in an evolutionarily conserved manner in developmental processes and life-history traits across a wide range of taxa, including mammals and teleosts [1–8]. The *six6* gene encodes a transcription factor involved in sexual maturation [3,7–9]. A locus near the *six6* gene has been linked to age at maturity and spawning ecotype in different salmonid species [7,8]. Additionally, *six6* has been associated with maturation in mammals, specifically, age-at-menarche in humans [2] and puberty timing in beef cattle [3]. The *vgll3* gene encodes a transcriptional cofactor involved in adipogenesis and the Hippo signaling pathway [9–11] that has been linked to pubertal timing and growth in humans [1]. In Atlantic salmon (*Salmo salar* Linnaeus), a single nucleotide polymorphism (SNP) in the *vgll3* gene correlates with maturation timing [5,6], with different genotypes of this SNP influencing the expression profiles of reproductive axis genes in juvenile males [12]. It was further demonstrated that *vgll3* influences the body condition of the species [13,14]. Both *six6* and *vgll3* are expressed during early development in salmon and are associated with adipogenesis and gametogenesis [9]. This growing body of evidence highlights that *six6* and *vgll3* play key roles not only in reproductive traits but also in growth and growth-related processes. In aquaculture, growth rate is a critical trait for productivity, and understanding how these genes regulate developmental processes may provide insights into improving growth performance in commercially farmed species [15–17]. By leveraging candidate genes identified in one species and assuming their evolutionarily conserved roles, we can investigate their potential for similar effects in other species [8]. It remains an open question whether *six6* and *vgll3* also influence developmental processes and life-history traits in other economically important teleost fish.

The gilthead seabream (*Sparus aurata* Linnaeus) and the European seabass (*Dicentrarchus labrax* Linnaeus) are among the most economically important fish species in the Mediterranean region. A recent study on genetic differentiation between farmed and wild populations of these species investigated how the domestication process impacts the genetic diversity of the *six6* and *vgll3* genes [18]. Significant differences in genotype and allele frequencies were observed in an exonic SNP on *vgll3* and in two intronic SNPs on *six6* between farmed and wild populations of both species [18]. Given this evidence and the high level of functional conservation of *vgll3* and *six6*, further investigations, including gene expression profiling across various developmental stages in these species, could yield highly informative insights. Such studies would be particularly valuable for the aquaculture sector and future selective breeding programs targeting maturation and growth performance [8,18,19]. Research in this direction could also enhance our understanding of the genotype–phenotype link and shed light on the genomic architecture of domestication.

To investigate the functional roles of *six6* and *vgll3* in the developmental processes of European seabass and gilthead seabream, we performed a gene expression analysis using quantitative real-time PCR (qPCR) in early developmental stages. Gene expression studies are essential for understanding the functional impact of genetic variants and for bridging the gap between genotype and phenotype [20,21]. By examining *six6* expression across genotypes and developmental stages (larvae and juveniles) in European seabass, and *vgll3* and *six6* expression patterns across genotypes in gilthead seabream juveniles, we aimed to explore the association of previously identified domestication-related genes with their gene expression patterns [18]. This study sought to provide deeper insights into the roles of these genes in the developmental processes of European seabass and gilthead seabream, as well as their potential evolutionarily conserved functions.

2. Materials and Methods

2.1. Sampling

Fifty larvae at 34 days post-hatch (dph) and 50 juveniles at 71 dph of European seabass, along with 50 juveniles at 69 dph of gilthead seabream, were collected from a fish farm. These stages were selected as critical time points in the developmental process of fish [22–24]. European seabass individuals were sampled at both stages, while only juvenile gilthead seabream were available due to constraints at the collaborating fish farm. All individuals were reared under the same controlled conditions, which included a temperature of 19 °C, a salinity of 40‰, a pH range of from 7.6 to 7.8, and O₂ saturation levels of 6–7 ppm. Samples were preserved in RNAlater stabilization solution (Invitrogen, Waltham, MA, USA) before being frozen at −20 °C.

2.2. Genotyping

DNA extractions from each individual were carried out using the TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) [25]. The primer sequences, amplicon sizes, and annealing temperatures for each studied genomic region are listed in Table 1 [18]. For PCR, we used a total reaction volume of 25 µL, which included 100 ng of genomic DNA as the template, 0.05 units of Qiagen Taq polymerase, 2 mM dNTPs, 0.25 µL of each primer (100 µM), and 2.5 µL of 10× Reaction Buffer (Qiagen, Hilden, Germany). The PCR products were analysed by electrophoresis in 1.5% (*w/v*) agarose gels. Enzymatic cleanup and Sanger sequencing were outsourced to Genewiz (Leipzig, Germany). SNP genotyping was performed by aligning the resulting sequences with the corresponding reference sequences from GenBank (assemblies *S. aurata*: GCA_900880675.1; *D. labrax*: GCA_000689215.1) using the Geneious v.10.2.6 program [26].

Table 1. Primer sequences, amplicon sizes, genomic coordinates (assemblies *S. aurata*: GCA_900880675.1; *D. labrax*: GCA_000689215.1), and annealing temperature for each studied genomic region.

Species	Gene	Genomic Coordinates	Annealing Temperature	Primer Sequence (5'-3')	Amplicon Size (bp)
<i>D. labrax</i>	<i>vgll3</i>	9203305–9203951	63 °C	TACCCTCCCCGATACCTGG TGTGTGGACAGTGCAGGAC	646
	<i>six6</i>	11590632–11591488	63 °C	GGCTACAGGACTTACACCCA AAGTACCACAGCAAGATCGC	856
<i>S. aurata</i>	<i>vgll3</i>	24911798–24912208	63 °C	AACGTCTATCACCTCACCC ACCAAAGTACGCTCTTTGCT	410
	<i>six6</i>	14406022–14406799	63 °C	AACCGCAGACAAAGAGACAG ACCCCTTATTAAACAACAAGCAC	777

2.3. RNA Extraction and cDNA Synthesis

Total RNA was extracted from whole European seabass and gilthead seabream individuals using the TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) [25]. In total, 27 European seabass individuals (nine biological replicates per SNP genotype) and 24 gilthead seabream individuals (eight biological replicates per SNP genotype) were selected for analysis. The quality and quantity of RNA were assessed using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and by electrophoresis on 1.5% (*w/v*) agarose gels. The cDNA synthesis was performed using the PrimeScript RT reagent Kit with the gDNA Eraser (TaKaRa, Shiga, Japan) following the manufacturer's instructions.

2.4. Quantitative Real-Time PCR

We selected the ribosomal protein L13 (*rpl13*), FAU ubiquitin-like and ribosomal protein S30 fusion (*fau*), and elongation factor 1-alpha 1 (*ef1α*) as candidate reference genes for both species [27]. Primers and probes for the reference and target genes were designed using the qPCR Primer & Probe Design Tool (Eurofins, Luxembourg City, Luxembourg) (Table 2). Gene locations and intron/exon information were retrieved from annotations deposited in Ensembl for both species (assemblies *S. aurata*: GCA_900880675.1; *D. labrax*: GCA_000689215.1). Gene expression profiles were quantified using quantitative real-time PCR with the KAPA[®] PROBE FAST Universal reagent (Kapabiosystems, Cape Town, South Africa) in three technical replicates per sample, performed on the ABI StepOne[™] platform (Applied Biosystems, Foster City, CA, USA).

Table 2. Primers and probe sequence, amplicon sizes, and annealing temperatures for the reference and target genes.

Species	Gene	Annealing Temperature	Primers and Probe Sequence (5'-3') (*Probe Sequence)	Amplicon Size (bp)
<i>D. labrax</i>	<i>six6</i>	60 °C	GCCTTTCACACCGGCAATTTC TCCGCACCCTGTATTTGTCCAC	169
			TGGAGAACCACAAGTTCACCAAAGAGTCGCAC*	
	<i>rpl13</i>	61 °C	GACACAAAGTGGTGGTGGTGAG AAGTGGTAAGGTCCACGAGAGG	130
			AGGCATCAACATCTCCGGCAACTTCTATCGCA*	
	<i>fau</i>	61 °C	TCGGCCCTTAAACAGCCCTTTC GCAACAGCACCTGATCCTCAAC	190
			CGAGCATCAAGATGCAGCTCTTCTTGCGTGCC*	
<i>S. aurata</i>	<i>ef1α</i>	61 °C	AGCAGACAACCTTCAACGCCC TCAAGCTTCTTGCCAGAACGAC	156
			CCACACAGCCCACATCGCCTGCAAGTTC*	
	<i>vgl3</i>	56 °C	TCGATTCAAGCCGGAGATCCAC GCCAATGTCGCCTTGGAAGTAG	119
			TCCGATTCAGAGCTGAAGGACGGCACTCA*	
	<i>six6</i>	56 °C	CCGCGAACTTTACCACATCCTG TCCGCACCCTGTATTTGTCCAC	149
			ACCACAAGTTCACCAAAGAGTCGCACACGAAGCTG*	
	<i>rpl13</i>	58 °C	GTTCAACCAGCCAGCCAGAAAG TGGTGGGACATCTGACTTGTGG	116
			CGTCAGGCTAAGGCCCGTCGCATTGCTC*	
	<i>fau</i>	56 °C	TGTCCCATGCGGCTAGTTATGC AGTGTTCTGGGCACGCAAGAAG	108
			AGCTCCCGGCACGTTGCTGTCCTC*	
	<i>ef1α</i>	56 °C	ACTCCACCGAGCCAACTACAG CCGAAATGGGCACAAAGGCAAC	117
			AGTGAGCACCTACATCAAGAAGATCGGCTACAATCC*	

2.5. Analysis of Gene Expression Data

The Ct values were converted to quantities using the comparative Ct method. The most stable reference genes were identified using the NormFinder and geNorm algorithms, which ranked the genes based on their expression stability [28,29]. The normalization factor was calculated as the geometric mean of the two most stable reference genes for each species (*rpl13* and *fau* in European seabass; *rpl13* and *ef1α* in gilthead seabream). The normalized expression levels of the target genes were determined by dividing the raw target gene quantities for each sample by the corresponding normalization factor. The Kruskal–Wallis test was used to compare the normalized expression levels of the target genes among the different SNP genotypes [30]. Additionally, the distribution of the expression data was assessed using the Shapiro–Wilk normality test, followed by the Bartlett’s test for homogeneity of variances [31,32].

3. Results

3.1. *six6* and *vgll3* Genotypes

We analyzed independent datasets from two developmental stages (larvae at 34 dph and juveniles at 71 dph) in European seabass and one developmental stage (juveniles at 69 dph) in gilthead seabream. For both species, we genotyped two intronic SNPs of *six6*, previously associated with the domestication process, and selected the SNPs with sufficient sample sizes for each genotype (*S. aurata* 16:14406442 and *D. labrax* 12:11591053) [18]. No genetic variation in *vgll3* was observed in the studied European seabass individuals; thus, the expression profile per genotype was not examined. In contrast, all genotypes for the *vgll3* SNP (*S. aurata* 9:24911884) were identified in the studied juveniles of gilthead seabream [18]. The sample sizes for each studied genotyped SNP are shown in Table 3.

Table 3. Number of individuals genotyped for each SNP of the *six6* and *vgll3* genes across developmental stages in European seabass and gilthead seabream.

Species	Developmental Stage	Gene	Number of Individuals for Each Genotype		
<i>D. labrax</i>	Larvae	<i>six6</i>	AA (10)	AT (31)	TT (9)
	Juveniles	<i>six6</i>	AA (9)	AT (23)	TT (17)
<i>S. aurata</i>	Juveniles	<i>six6</i>	CC (8)	CT (17)	TT (25)
	Juveniles	<i>vgll3</i>	AA (8)	AG (30)	GG (12)

3.2. Expression Patterns of *vgll3* and *six6* per Genotype in Gilthead Seabream

In gilthead seabream, *six6* and *vgll3* expression levels were analyzed in juveniles (69 dph). For *vgll3*, the expression levels were highest in the AA genotype, followed by AG, with the GG genotype showing the lowest expression levels (Figure 1a). The differential expression across genotypes was statistically significant ($p = 0.040$), and pairwise comparisons revealed that the AA genotype was significantly different from the other two genotypes (AA-AG: $p = 0.030$; AA-GG: $p = 0.027$). Variance differences were also significant across genotypes ($p = 0.002$), as well as between AA-AG ($p = 0.016$) and AA-GG ($p = 0.003$). A similar trend was observed for *six6*, where the CC genotype exhibited the highest expression levels, followed by CT, with the TT genotype displaying the lowest (Figure 1b). A significant differential expression was detected between the CT and TT genotypes ($p = 0.027$), but no significant differences in variability were observed.

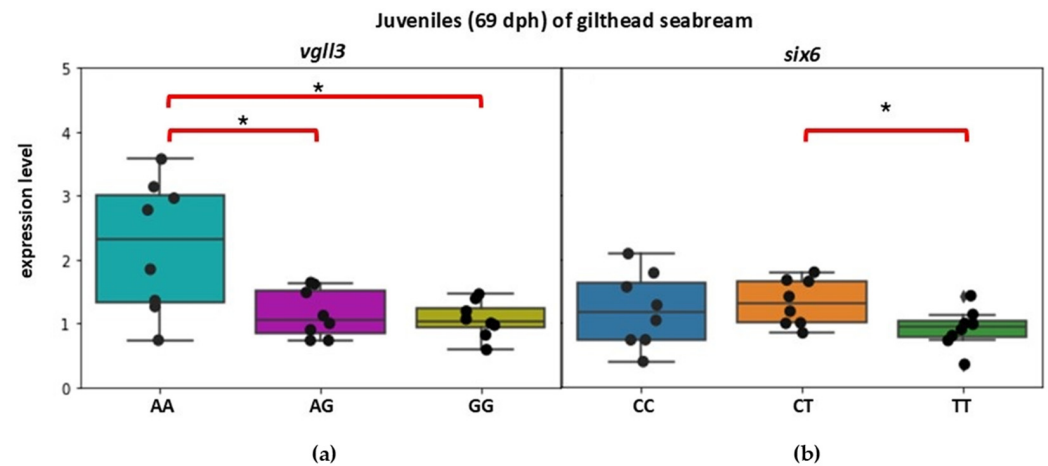


Figure 1. Boxplots showing the expression levels of *vgl3* and *six6* genes in gilthead seabream juveniles at 69 dph. Panel (a) illustrates the expression levels of the *vgl3* gene across AA, AG, and GG genotypes, with red brackets and asterisks indicating significant differences between the AA and AG genotypes and the AA and GG genotypes (* $p < 0.05$). Panel (b) depicts the expression levels of the *six6* gene across the CC, CT, and TT genotypes, with red brackets and asterisks highlighting significant differences between the CT and TT genotypes (* $p < 0.05$).

3.3. Expression Patterns of *six6* per Genotype and Developmental Stage in European Seabass

All observed trends in expression levels among genotypes at both developmental stages were not statistically significant. However, in larvae at 34 dph, the heterozygous genotype (AT) exhibited high variability compared with homozygous genotypes (AT-AA: $p = 0.040$; AT-TT: $p = 0.046$; Figure 2a). In the 71 dph juveniles, this pattern had disappeared, with all genotypes exhibiting a similar range of expression levels (Figure 2b). Overall, the variations in *six6* expression for the heterozygous genotype showed a decreasing trend between larvae and juveniles ($p = 0.030$).

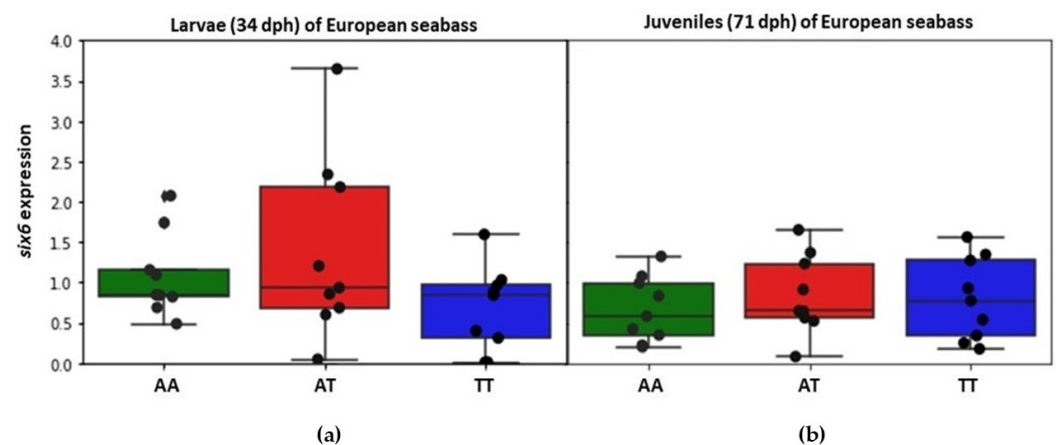


Figure 2. Boxplots showing the expression levels of the *six6* gene in larvae (34 dph) and juveniles (71 dph) of European seabass. Panels (a,b) depict the expression levels of the *six6* gene across AA, AT, and TT genotypes in larvae and juveniles, respectively.

4. Discussion

In this study, we investigated the gene expression profiles of *vgl3* and *six6* in the early developmental stages of two commercially important fish species, the gilthead seabream and the European seabass. We aimed to provide the first evidence that prior SNP associations of these genes with domestication [18] are also reflected at the mRNA level. Gene expression profiling was performed using quantitative real-time PCR (qPCR) with

probes due to its high accuracy, low background signal [33], and efficiency [34]. In gilthead seabream, we identified differences in both the means and variances of mRNA expression across the genotypes of *vgll3* and *six6* at the examined developmental stage (69 dph juveniles). Additionally, in European seabass, significant variability in *six6* expression was observed in the heterozygous genotype during the larval stage (34 dph), which was reduced in juveniles (71 dph). These observations provide initial evidence that *vgll3* and *six6* may play functional roles in domestication and developmental processes in these species. Given the suggested roles of these genes in growth and growth-related processes [13,35–37], our findings further underscore their potential relevance to the aquaculture sector. These insights open up new opportunities to implement gene-based management practices aimed at enhancing desirable traits in cultured populations of the species, ultimately benefiting the aquaculture industry [38,39].

Our findings indicate that gene expression profiles are influenced by both genetic variation (Figure 1) [40–42] and developmental stage (Figure 2) [9,22,43]. Specifically, we report a statistically significant difference in expression levels between the two homozygous genotypes (AA and GG) of *vgll3* in gilthead seabream (Figure 1a). The AA genotype exhibited significantly higher expression levels and variance compared to the GG genotype, which was associated with domestication in previous DNA-based analysis [18]. A similar pattern was observed in juveniles of Atlantic salmon, Verta et al. (2020) [40] previously found that the late-maturing *vgll3* genotype exhibited 14% higher expression and variation within the testis compared to the early-maturing genotype [40]. Beyond their association with maturation, the *vgll3* genotypes influenced body condition, with the early-maturing genotype exhibiting higher body condition [13,14] and seasonally increasing lipid storage in immature Atlantic salmon [41]. The consistency in *vgll3* expression patterns among genotypes between juvenile gilthead seabream and Atlantic salmon, underscores the gene's likely evolutionary conservation and raises critical questions about its role in the physiological processes of gilthead seabream.

The *six6* differential expression was statistically significant between the CT and TT genotypes in gilthead seabream, with the TT genotype exhibiting the lowest expression levels compared to the other two genotypes (Figure 1b). Notably, the TT genotype was associated with domestication in a previous DNA-based analysis comparing farmed and wild populations [18]. Additionally, in Atlantic salmon, *six6* expression patterns have been associated with the early developmental process, particularly in the sensory, musculoskeletal, and cardiovascular systems [9,37]. However, there is a lack of literature on the effects of *six6* genotypes on expression levels and whole transcriptome profiles. The observed differential expression among genotypes in this study suggests a potential role of *six6* in the juvenile stage in gilthead seabream, warranting further investigation to clarify its specific functions and regulatory mechanisms.

Observations of *six6* expression in European seabass also indicate its potential significance in this species, with the variation in expression levels for heterozygous genotype in larvae being higher than those observed in homozygous genotypes. The expression variance may suggest an association with the molecular basis of complex traits, diseases [44,45], and environmental adaptation mechanisms [46]. Early development is a highly dynamic period in an organism's life, during which constant changes in gene expression are crucial for regulating processes that govern growth, cell differentiation, organogenesis, and the physiological adaptations of each stage [9,22,43]. The marked reduction in *six6* expression variability for the heterozygous genotype by 71 dph under controlled farming conditions suggests that ontogenetic processes primarily regulate this change, with minimal impact from environmental factors such as water temperature or dissolved oxygen [47]. A decreasing trend in *six6* expression was also observed from the embryonic stages to the fry stage in

Atlantic salmon, highlighting the importance of the gene's role during the early stages for the hypothalamic–pituitary–gonadal (HPG) axis and eye development [9]. These findings collectively underscore the potential role of *six6* in early developmental processes across multiple fish species, warranting further investigation to uncover its specific roles in them.

It remains unclear how the studied SNPs are associated with the observed patterns in expression levels of targeted genes. The *vgll3* SNP in gilthead seabream is a synonymous mutation and the *six6* SNPs are intronic in both species [18]. The literature provides evidence that synonymous and intronic mutations can alter the regulation of gene expression and potentially play a role in fish domestication and traits linked to aquaculture [48–50]. Synonymous mutations do not alter the protein sequence, but they may affect mRNA stability, translation efficiency, and protein folding [51,52]. Intronic variation can also affect gene expression levels and phenotype by modifying splice site activity, altering *cis*-regulatory elements like enhancers and silencers, and changing the formation of chromatin loops [53–56]. Furthermore, it is recognized that alternative splicing contributes to transcriptional diversity in genes associated with developmental processes [57], such as those under study. However, the presence of these SNPs does not necessarily mean they are the causative variants affecting gene expression, as they may be in linkage disequilibrium with the actual causative variant. Further studies are needed to determine if the SNPs have a functional effect or are merely markers for a linked variant.

Our study focused on the expression of targeted genes at specific developmental stages (larvae and juveniles), providing important insights into the roles of *vgll3* and *six6* in gilthead seabream and European seabass. Notably, research on Atlantic salmon has identified these stages as relevant for studying the expression of these genes [9]. Future research should include additional developmental stages and omic-wide approaches, such as genomic and transcriptomic analyses, to confirm our current observations. Additionally, functional assays (e.g., knockdown or overexpression) are needed to establish direct mechanistic links between *vgll3* and *six6* expression and their contributions to developmental and growth-related processes. Moreover, incorporating phenotypic data, which were absent in this study, will be crucial for linking gene expression patterns to specific growth-related traits and physiological processes. Such efforts will provide a more comprehensive understanding of the functional relevance of these genes in aquaculture.

5. Conclusions

In conclusion, this study provides new insights into the expression profiles of *six6* and *vgll3* in developmental stages in gilthead seabream and European seabass, linking these gene expression patterns to domestication-associated SNPs previously identified in DNA-based studies. In gilthead seabream, we observed significant genotype-dependent differences in the mRNA expression of *vgll3* and *six6* at the juvenile (69 dph) stage. In European seabass, *six6* expression exhibited significantly higher variability in heterozygous larvae (34 dph) compared to homozygous genotypes. These findings, combined with the evolutionarily conserved functions of these genes and their involvement in developmental processes and life history traits across vertebrates, including teleosts, highlight the potential roles of these genes in growth, development, and adaptation to domesticated environments. Particularly, the expression differences observed in *vgll3* and *six6* among gilthead seabream genotypes, as well as the variance of *six6* expression between the larval and juvenile stages in European seabass, suggest a potential involvement in growth-related processes and physiological traits relevant to aquaculture. Given the absence of phenotypic measurements and direct functional validation, these differential expression patterns warrant further investigation. Future studies that include additional developmental stages and incorporate transcriptome-wide approaches and phenotypic data could validate these observations

and enhance our understanding of the genetic underpinnings of domestication and growth in cultured fish, ultimately contributing to improved breeding strategies in aquaculture.

Author Contributions: Conceptualization, A.T. and S.P.; methodology, A.M., E.K., S.M., K.G., A.K., E.A., A.T. and S.P.; validation, E.K. and S.P.; formal analysis, A.M., E.K., S.M. and S.P.; investigation, A.M., E.A., A.T. and S.P.; resources, S.P.; data curation, A.M., E.K., S.M. and S.P.; writing—original draft preparation, A.M., E.K., S.M. and S.P.; writing—review and editing, K.G., A.K., E.A. and A.T.; visualization, A.M.; supervision, E.A., A.T. and S.P.; project administration, K.G., A.T. and S.P.; funding acquisition, A.T. and S.P. All authors have read and agreed to the published version of the manuscript.

Funding: This study was conducted under the project “SEaLIFT: SystEms Biology Modelling of Key LIfe History Traits for Sustainable Aquaculture Production in the Mediterranean Region,” funded by the Hellenic Foundation for Research & Innovation (H.F.R.I.) Grant Number 00414.

Institutional Review Board Statement: The “Principles & Procedures Governing the Operation of the Research Ethics Committee” of the University in which the research was conducted (Aristotle University of Thessaloniki). This regulation is drafted by the provisions of Article 68, Law 4485/2017, and Articles 21–27, Law 4521/2018 of Greece, an EU member country. Article 25 of the regulation (“Research Involving the Use of Animals”) states that the implementation of our experimental protocols did not use endangered species or wildlife animals (i.e., aquaculture-reared fish) and is part of routine practice in aquaculture practice (Article 25, section 5.d); such procedures do not require approval.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding authors.

Acknowledgments: We thank Nikoleta Karaïskou for her invaluable guidance throughout this work.

Conflicts of Interest: The authors declare no conflicts of interest.

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