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High Expression Levels of the Genes *cyclin-A2* and *glucocorticoid receptor* Are Associated with High-Quality Embryos in Gilthead Sea Bream (*Sparus aurata* L.)

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Abstract: Identifying early egg-quality predictors is a major challenge in finfish hatcheries, and relevant research is now focused on the development of molecular markers. In our study, we examined whether fertilization rates and early morphological abnormalities in sea bream egg batches of high (HQ) and low quality (LQ) are associated with mRNA levels of *cathepsin D*, *cathepsin Z*, *cyclin-A2*, and *glucocorticoid receptor*. Additionally, we examined whether these early quality descriptors were associated with the development of skeletal abnormalities during the larval period. HQ egg batches were characterized by significantly higher rates of normal embryos (95.8 ± 2.3%) and lower rates of unfertilized (2.8 ± 1.0%) and abnormal eggs (1.3 ± 1.4%), compared to LQ (84.2 ± 0.8% normal embryos, 12.3 ± 12.3 unfertilized eggs, and 3.5 ± 1.4% abnormal eggs) ($p < 0.05$, Mann–Whitney U test). Relative expression of *cyclin-A2* and *glucocorticoid receptor* was found to be significantly higher in HQ embryos compared to those of LQ (respectively, $p < 0.01$ and $p < 0.05$, Mann–Whitney U test). No statistically significant differences were observed in the mRNA transcripts of *cathepsin D* and *cathepsin Z* ($p > 0.05$, Mann–Whitney U test). Differences in the rate of skeletal abnormalities between the two quality groups of larvae were not significant ($p > 0.05$, G-test), indicating that *cyclin-A2* and *glucocorticoid receptor* may serve as reliable molecular markers for early prediction of fish egg quality but not for later larval stages.

Keywords: egg quality; molecular marker; genes expression; skeletal abnormalities; finfish; aquaculture

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1. Introduction

In finfish hatcheries, morphological egg quality is mainly assessed by monitoring various parameters during embryonic development. Fertilization rate, embryonic and larval mortalities [1–3], egg size [4], the buoyancy of the pelagic eggs [5–7], distribution of lipid vesicles [8] and their shape, yolk uniformity [9,10], early cleavage patterns and blastomere morphology [2,11–13], as well as macroscopically evident malformations during early ontogeny, have been considered as appropriate morphological descriptors of the quality of fish eggs and embryos. Furthermore, the need for early, precise, reliable, and simple to use biomarkers applicable to the industry has increased interest in molecular components that can be considered key role players in regulating egg quality parameters [13–18]. Although these studies relate molecular mechanisms with the early developmental potential of the fertilized egg, there is still the need to study and identify egg quality biomarkers. As Bobe [19] has noted, different factors affect the quality of eggs through various molecular pathways, resulting in differential mRNA and protein expression. On the other hand, molecular markers specific for certain developmental landmarks, such as bone formation,

may have the potential to be used as egg quality indices, as long as their expression provides reliable descriptive and predictive information for the developmental potential of a fish batch [19–23].

The purpose of this study was to examine whether morphological indicators of egg quality are linked with the transcript levels of four genes (*cathepsin D*, *cathepsin Z*, *cyclin-A2*, and *glucocorticoid receptor*) and with cortisol levels in gilthead sea bream embryos. In addition, the purpose was to examine whether these morphological indicators of egg quality may be related to the presence of skeletal abnormalities in the late-stage larvae of gilthead sea bream. The selection of the above genes was based on their important role in the development of fertilized eggs and their correlation with egg quality, as previously reviewed [5,14]. Cortisol was included in our study because of its significant role in the early ontogeny of fish. In the unfertilized teleost eggs and early embryos, cortisol appears to be of maternal origin [24,25], with endogenous production starting at later stages [24,26,27]. A number of studies have stated that maternally derived cortisol is essential for early fish development and may shape the quality of gametes [28] and/or offspring's phenotype [29–31]. The targeted species is an important fish in European aquaculture with a well-characterized ontogeny [32–34].

2. Materials and Methods

Egg samples were taken on four different dates between 20 March and 20 April, during the spawning period of one single gilthead sea bream broodstock. The rearing of gilthead sea bream embryos, larvae, and juveniles was performed under routine production conditions at Andromeda S.A. located in western Greece. At the stage of blastodisc formation to epiboly onset (8 to 12 hours post-fertilization at 18.5 °C), floating eggs were collected from the spawning tank. Breeders were kept in the commercial hatchery, under natural photoperiod and controlled temperature conditions, in an 18 m^3 tank, with a sex ratio of 2 females to 1 male and a 6 kg/m^3 stocking density. Water renewal for each tank was 30% per hour. The minimum size of the breeders was 2 kg, while the minimum age was 5 years old. They were fed ad libitum four times a week with commercial pellets (Lansy Breed, INVE Technologies, Belgium) and frozen squid. Eggs were collected through the water overflow using a planktonic collector. The tanks were supplied with borehole seawater. During the spawning period, the water temperature was 19.0–20.5 °C, salinity was 35 g/L, and oxygen saturation was 90–95%. Larval rearing was performed with the standard methodology for the intensive larval rearing of gilthead seabream. In short, larvae were reared in the presence of background phytoplankton (*Chlorella* sp.), with initial feeding on enriched rotifers (5–30 dph), followed by the gradual provision of Artemia instar I (19–24 dph), enriched instar II nauplii (22–45 dph), and finally, of inert commercial diets (>30 dph).

A total of 20 egg batches were examined microscopically in order to classify them as high- or low-quality (HQ and LQ, respectively). From each eggbatch, a random sample of 100 eggs was used for this purpose. A total of 6 high-quality and 6 low-quality spawns were used to conduct this study. During egg morphological examination, the number of unfertilized, abnormal, and normal fertilized eggs was recorded. The discrimination of the unfertilized from the fertilized eggs was based on the lack of embryonic development, as well as on the opaque yolk and the narrow perivitelline space in the former (Figure S1A,B) [35]. To separate the abnormal from the normal embryos, yolk homogeneity, the number of lipid globules, and embryo morphology were examined. Following Divanach [35], normal sea bream embryos are characterized by a single lipid globule and a homogeneous and transparent yolk (Figure S1A,A'). During the studied ontogenetic period (blastodisc formation to epiboly onset), embryos develop on the animal pole and are symmetric when viewed from the top. Embryos with non-homogenous yolk, irregularities of the periphery of the animal pole, and small globules inside the yolk (Figure S1C,C') were considered abnormal.

Following the microscopical examination, eggs from 6 high-quality and 6 low-quality spawns were analyzed for mRNA levels of *cathepsin D*, *cathepsin Z*, *cyclin-A2,* and *glucocorticoid receptor* genes (1 pool of 50 eggs per spawn), as well as for cortisol content (1 pool of 40 eggs per spawn). For transcriptome analysis, pooled eggs were stabilized in RNA Stabilization Reagent (Qiagen, Hilden, Germany) and kept at −20 °C until RNA extraction. For cortisol analysis, pooled eggs were frozen (−20 °C) until cortisol extraction. The 2 groups of egg batches of distinct quality were incubated and reared up to 35 days post-hatching (dph) and examined for the presence of skeletal abnormalities (in a random sample of 100 specimens per larval population) [36].

RNA was extracted using Tripure Isolation Reagent (Roche Applied Science, Germany), according to the manufacturer's instructions. RNA quality was evaluated by 0.9% agarose gel electrophoresis, with the concentration measured with a Q5000 UV-Vis Spectrophotometer (Quawell Technology Inc, San Jose, CA, USA), according to the manufacturer's instructions. RNA quality was evaluated by 0.9% agarose gelelectrophoresis, with the concentration measured with a Quawell Q5000. All primers, except for *cathepsin D*, were designed using the NCBI Primer Blast tool (URL: https://www.ncbi.nlm.nih.gov/tools/primer-blast/, access date: 15 July 2014) with parameters set to create a product between 120 and 300 bp that spans an exon–exon junction. Primers for *cathepsin D* were taken from the study of Carnevali et al. [16]. A Two-Step qRT-PCR was performed with the use of 500ng of total RNA as a template for cDNA synthesis (PrimeScript RT-PCR Kit, Takara Bio USA, Inc., San Jose, CA, USA) with the MJ Research PTC-200 Gradient Thermal Cycler (Bio-Rad/MJ Research, Waltham, MA, USA). cDNA amplification was performed on Roche Light Cycler® 1.5 Instrument (Roche Diagnostics GmbH, Manheim, Germany) with SYBR Green fluorescent dye (Roche Diagnostics, KAPA SYBR® FAST Universal Kit, Kapa Biosystems, Inc., Wilmington, MA, USA). The relative transcript abundance of each gene was normalized to *beta-actin* expression, and the analysis of the qPCR results was carried out under the proposed methods of Schmittgen and Livak [37], using the $2^{-\Delta Ct}$ method. Cortisol was extracted by partial thawing of samples (pool of 40 fish) on ice and homogenization in 250 µL of ice-cold phosphate-buffered saline (PBS, pH 7.2) with a Mini Cordless Grinder (Nippon Genetics, Europe) based on the protocol reported by De Jesus et al. [38]. Quantification of cortisol was performed with a Cortisol ELISA analysis kit (Cayman Chemical, Michigan). Cortisol was normalized to the protein content of the homogenates, which was determined with the Bio-rad protein assay, which is based on the Bradford dye-binding method. This method is based on the color change of Coomassie brilliant blue G-250 dye in response to various concentrations of protein. A standard curve was developed using a series of Bovine Serum Albumin (BSA) standards in the 0.1 ug/mL to 10 ug/mL range [39].

3. Results

Statistical analysis verified that, compared to the LQ group of egg batches, the HQ group was characterized by a significantly higher rate of normal embryos (95.8 ± 2.3 vs. 84.2 ± 0.8, mean ± SD) and lower rates of unfertilized and abnormal eggs (G-test, [40]) (Table 1). Differences in the mRNA expression values, as well as in the normalized values of cortisol concentration between HQ and LQ egg samples, were tested by the Mann–Whitney U test. Compared to the LQ group, HQ egg batches presented significantly higher transcript levels of *cyclin-A2* and *glucocorticoid receptor* genes (Figure 1A,B), while no significant differences were detected in the transcript levels of *cathepsin D* and *cathepsin Z* genes between the two quality groups (Figure 1C,D). Similarly, the egg cortisol contents were not significantly different between the two groups of eggs (Figure 1E). Finally, differences in the morphological abnormalities of larvae at 35 dph between the HQ and LQ groups of fish were tested with a G-test [40].

Table 1. Percentage (%) of unfertilized eggs (n-Fert), malformed embryos (Abn), and normal fertilized eggs in the different eggbatches. Depending on the frequency of the normal fertilized eggs, egg batches were categorized as high (HQ) or low (LQ) quality. Different superscripts indicate significant differences in the mean frequencies (±SE) between the 2 quality groups ($p < 0.05$, Mann–Whitney U test). Eggs were stocked in 12 larval rearing tanks (T1–T12).

Tank	n-Fert	Abn	Normal	Group	Mean n-Fert	Mean Abn	Mean Normal
T1	3	4	93	HQ	1.3 ± 1.4^{a}	2.8 ± 1.0^{a}	95.8 ± 2.3^{a}
T2	3	4	93				
T3	1	2	97				
T4	1	3	96				
T5	0	2	98				
T6	0	2	98				
T7	3	13	84	LQ	3.5 ± 1.4^{b}	12.3 ± 1.6^{b}	84.2 ± 0.8^{b}
T8	6	10	84				
T9	4	11	85				
T10	3	12	85				
T11	3	14	83				
T12	2	14	84				

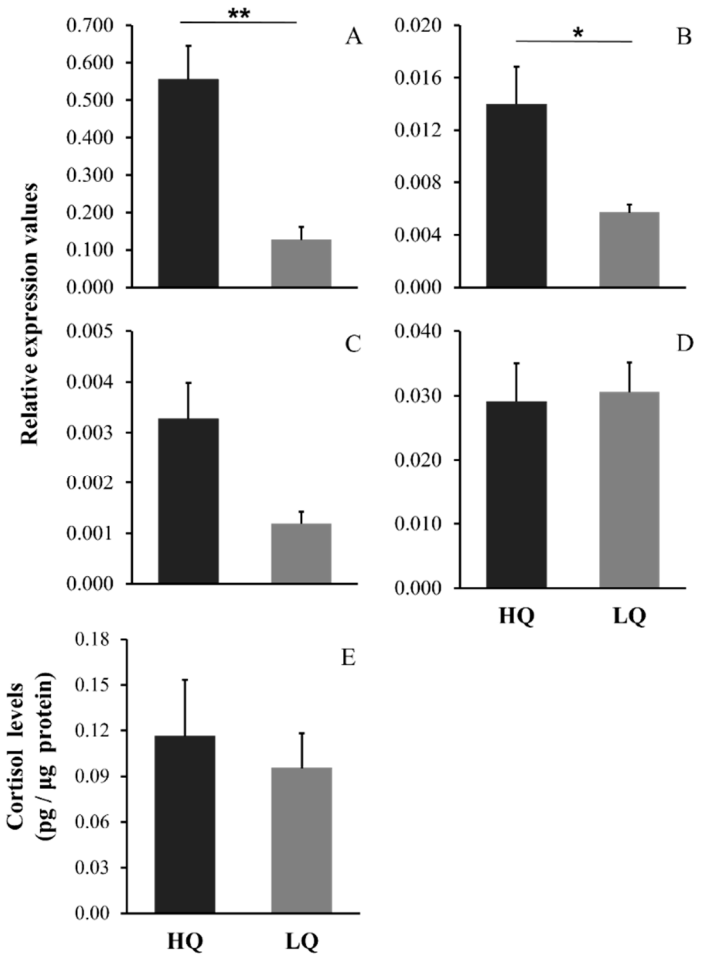

Figure 1. mRNA expression levels (mean + SE) of (**A**) *cyclin-A2*, (**B**) *glucocorticoid receptor*, (**C**) *cathepsin D*, (**D**) *cathepsin Z* genes, and (**E**) cortisol levels in fertilized eggs of HQ (black column) and LQ (grey column). A total of 6 samples (mRNA expression levels: pools of 50 eggs per sample, cortisol levels: pools of 40 eggs per sample) were used for the HQ group and 6 for the LQ group, respectively. (*) Asterisk represents statistically significant differences between the expression levels of the two groups (mRNA expression levels: Mann–Whitney U test, (*) $p < 0.05$, (**) $p < 0.01$).

4. Discussion

The expression of *cyclin-A2* in rainbow trout (*Oncorhynchus mykiss)* oocytes aged in vivo was studied by Aegerter et al. [14], who indicated that the high abundance of *cyclin-A2* mRNA was positively associated with oocyte maturation and increased egg viability/embryonic survival. In the study of Fernandez et al. [41], the observed expression pattern of *cyclin-A2* in gilthead sea bream during early development, up until epiboly onset, indicated the possible function of *cyclin-A2* as a maternal gene. Genes of maternal origin, whose role in early development is critical and, at the same time, their mRNA abundance, which is associated with differences in egg quality, have been suggested as possible reliable biomarkers for fish egg quality [17,42,43]. *Cyclin-A2* belongs to this group of genes [41], and the results of the present study relate its expression to the quality of gilthead sea bream eggs. In our study, each spawn (and in each egg sample) was a mixture of multiple spawning events at the same time with the contribution of many females and included a range of developmental stages extending from blastodisc formation to epiboly onset when, according to literature, zygotic transcription of *cyclin-A2* initiates [41]. Therefore, it is possible to suggest that we have measured maternal and zygotic transcripts of *cyclin-A2*. Since our experiments were conducted under realistic conditions, in a commercial hatchery, it was not possible to collect samples at a specific developmental stage. It should also be noted that the random distribution of the developmental stages included in each spawn (and in each sample), as well as the fact that the stages were equally distributed among the HQ and LQ groups, enhances the validity of the observed differences between the two egg quality groups.

In the model fish, zebrafish (*Danio rerio)*, knockdown maternal *glucocorticoid receptor* generated disruption in mesodermal formation and muscle development [44]. Our results demonstrate significantly higher *glucocorticoid receptor* mRNA transcripts in HQ embryos compared to LQ, raising the question of the possible use of *glucocorticoid receptor* as a molecular biomarker for egg quality. It should be noted that in zebrafish, the transition from maternal to zygotic transcription of *glucocorticoid receptor* has been placed in the gastrula stage [45], in the shield stage [46], or after hatching [24]. Based on these studies, it can be assumed that the *glucocorticoid receptor* mRNA levels in our study are related to transcripts of maternal origin. However, further relevant studies need to be conducted on gilthead sea bream in order to have a clear picture of *glucocorticoid receptor* expression during early ontogeny.

At the end of the larval rearing phase (35 dph), all the examined larval populations presented light abnormalities of the upper jaw (Figure S2A) and inside folding of the gillcover (Figure S2B). Differences in the abnormality rates between the HQ and LQ groups of fish were not significant ($p > 0.05$, G-test, [40]) for both upper jaw ($5.0 \pm 2.5\%$ and $4.3 \pm 1.8\%$ for the HQ and LQ group, respectively) and gillcover ($9.3 \pm 4.5\%$ and $10.0 \pm 3.6\%$ for the HQ and LQ group, respectively). This result cannot be considered an outcome of high larval mortality since there was no difference in the survival rate between the two egg quality groups. The lack of a correlation between poor egg quality and the rate of skeletal deformities at the end of the larval rearing phase could be explained by the high variability in the development of skeletal malformations in marine finfish hatcheries, as well as a large number of factors (nutritional, abiotic, genetic) that have been shown to be involved in the development of skeletal deformities [47,48] which eventually may mask the effect, if any, of egg quality on larval traits. Especially in the case of a species such as sea bream, with a relatively small size and undifferentiated skeleton during hatching [32,49].

To conclude, in the present study, we found a significant differentiation of the embryonic mRNA levels between egg batches of different levels of morphological quality. Despite the significance of the results, more research effort is required to explain this differentiation and decide whether these molecular indices could be used by commercial hatcheries as routine quality descriptors or predictors.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/aquacj2020005/s1. Figure S1. Embryos and unfertilized eggs of gilthead sea bream. A, A', lateral and top view of a normal embryo at the epiboly onset. B, B'. lateral and top view of an unfertilized egg. C, C', embryo at the stage of blastodisc formation presenting an additional small droplet near the lipid globule (arrow in C'). Scale bars equal to 0.25 mm. Figure S2. Main skeletal abnormalities developed in the experimental populations of gilthead sea bream. (A) Light fusion between maxillary and pre-maxillary bones. (B) Inside folding of the gill cover. Samples were stained for bone and cartilage. Scale bars equal to 1 mm. Table S1. Complete name (1st column of the table, Genes), GenBank accession number, amplicon length (Amp.), annealing temperature (T), and primers sequences (forward and reverse sequence) used for real-time qPCR of total RNA obtained from the eggs of gilthead sea bream. Reference [50] are cited in the supplementary materials.

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Institutional Review Board Statement: Rearing of gilthead sea bream embryos and larvae was performed under routine production conditions at Andromeda S.A. This company is registered (registration number GGN 5200700699992) for aquaculture production in Greece and has secured a GLOBAL G.A.P quality certification, which requires a certified Veterinary Doctor to periodically verify fish health and welfare. Animal sampling followed routine procedures, and samples were collected by a qualified member of staff from standard production cycles. The legislation and measures implemented by the commercial producer complied with existing Greek (PD 56/2013) and EU (Directive 63/2010) legislation (protection of animals kept for farming). Production and sampling by an experienced worker were optimized to avoid unnecessary pain, suffering, or injury and to maximize larval survival.

Data Availability Statement: The data that support the findings of this study are included in the text and in the tables. Raw data of Figure 1 are available from the corresponding author upon reasonable request.

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

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