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Apoptosis and autophagy-related gene transcription during ovarian follicular atresia in European hake (*Merluccius merluccius*)

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ABSTRACT

Follicular atresia is an energy-saving oocyte resorption process that can allow the survival of female fish when environmental conditions are unfavourable and at the expense of fecundity. This study investigated the transcription levels of apoptosis and autophagy-related genes during atresia in the European hake that can show episodes of increased follicular atresia throughout the reproductive cycle. 169 female individuals were collected from the Bay of Biscay, and the ovaries were analysed using histological and molecular methods. Different levels of atresia were histologically detected in 73.7% of the ovaries analysed and the TUNEL assay identified apoptotic nuclei in follicles from both previtellogenic and vitellogenic stages. Transcripts of *beclin-1* and *ptenb* were up-regulated in the ovaries containing atretic follicles, whereas *p53*, *caspase-3*, *cathepsin D* and *dapk1* were up-regulated only in ovaries presenting vitellogenic atretic follicles. Our results indicate different implications of apoptotic vs autophagic processes leading to atresia during oocyte development, vitellogenesis being the moment of maximal apoptotic and autophagic activity in atretic hakes. The analysed genes could provide early warning biomarkers to identify follicular atresia in fish and evaluate fecundity in fish stocks.

1. Introduction

The reproductive potential of most female fishes establishes the capacity of wild populations to sustain their numbers and to face increased mortality associated with natural factors (food deprivation, disease, predation, competition, ageing, etc.) or unsustainable fishing activity (Cadima, 2003; Jørgensen et al., 2008; Jørgensen and Holt, 2013; McBride et al., 2015). As part of the policy to improve fisheries management, the assignment of maturity and estimation of fecundity of fish populations are key exercises in the assessment of fish stocks. Many fisheries managers calculate maturity through the macroscopic evaluation of the gonads and assigning proportions of mature individuals, hence estimating the proportion of mature females in fish stocks (Vitale et al., 2005). Fecundity in fish is very much limited by the capacity of females to produce sufficient and good-quality oocytes (McBride et al., 2015) and environmental stressors might interfere with oocyte development. Food deprivation, contaminant exposure, unfavourable temperature or photoperiod regimes and suboptimal water quality may disrupt the endocrine system and induce follicular atresia, a degenerative process in which the oocytes and their follicles are reabsorbed (Corriero et al., 2021; Kjesbu, 2009; Saidapur, 1978; Serrat et al., 2019).

During each reproductive cycle, fish species tend to recruit more oocytes into vitellogenesis than those that will finally be spawned, irrespective of the reproductive strategy they show (Kjesbu, 2009). In some circumstances, fish oocytes in vitellogenesis destined for ovulation cannot be spawned so they are diverted to atresia and follicles are resorbed to facilitate the redistribution of energy-rich yolk materials (Janz and van der Kraak, 1997; Miranda et al., 1999; Wood and van der Kraak, 2003, 2001). This allows the fish to interrupt its normal reproductive cycle by skipping a batch of oocytes during their recruitment, thereby adjusting the number of eggs produced to an affordable amount of stored energy (Kennedy et al., 2009). This can have a strong impact on fecundity calculations if the reduction in the number of secondary follicles by atresia is not considered during fish stock analyses.

Follicular atresia is an evolutionarily conserved process that is essential for the maintenance of ovarian homeostasis in vertebrates

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including fish (Bhardwaj and Sharma, 2012; Corriero et al., 2021; Guraya, 1986; Krysko et al., 2008; Saidapur, 1978). In birds and mammals, most of the oocytes recruited during ovarian development lose their integrity and are eliminated before ovulation by follicular atresia (Bhardwaj and Sharma, 2012; Krysko et al., 2008; Saidapur, 1978). In teleost fishes, follicular atresia is frequently associated with environmental stress or changes in hormonal levels (Habibi and Andreu-Vieyra, 2007) and has been observed irrespective of the differentiation stage between previtellogenesis and final maturation in the follicles (Guraya, 1986; Janz and van der Kraak, 1997; Miranda et al., 1999; Rizzo and Bazzoli, 1995; Wood and van der Kraak, 2001).

Apoptosis is an evolutionarily conserved process of programmed cell death characterized by biochemical and morphological changes that remodel, differentiate and degenerate tissues to maintain the number of cells (Steller, 1995; Tilly, 1996a). In teleost fish, apoptosis is involved in selecting and recruiting follicles for vitellogenesis (Janz and van der Kraak, 1997) and postovulatory regression after spawning (Santos et al., 2008; Thomé et al., 2006; Wood and van der Kraak, 2001). The first morphological signs of apoptosis-mediated atresia are the disintegration of the oocyte nucleus and other cytoplasmic organelles such as the mitochondria, cortical alveoli and annulate lamellae, followed by the fragmentation of the zona pellucida and hypertrophy of the follicle cells (Janz and van der Kraak, 1997; Miranda et al., 1999; Saidapur, 1978; Wood and van der Kraak, 2001). The follicle cells accompany the process by incorporating and digesting the proteins that the dying oocyte had previously accumulated (Lubzens et al., 2010). However, increasing evidence suggests that apoptosis is not the exclusive mechanism and that autophagy represents an alternative non-apoptotic process that contributes to the efficient elimination of granulosa cells and oocytes (Cassel et al., 2017; Corriero et al., 2021; Morais et al., 2012, 2016; Sales et al., 2019; Santos et al., 2008; Thomé et al., 2009). Recently, a review paper by Bhardwaj et al. (2022) has reported the implication of autophagy marker genes also in the process of atresia in mammals and involving all cell types forming the follicle. Autophagy recycles nutrients from degraded cell organelles in the follicle, and hence could provide the energy that may be required for future oocyte and egg production in vertebrates (Bhardwaj et al., 2022). Whereas in mammals, apoptosis in cells that form the ovarian follicles is considered the main cellular mechanism mediating early ovarian atresia (Hsueh et al., 1994; Matsuda et al., 2012; Tilly, 1996a, 1996b; Tiwari et al., 2015), in fish, apoptosis is accompanied by autophagy at least during the late stages of follicular atresia (Miranda et al., 1999; Morais et al., 2012); thus suggesting the existence of another novel pathway during follicular atresia (Yang et al., 2022).

While follicular atresia is a common degenerative process in fish ovaries, it has been mainly described histologically, and much of its molecular and cellular mechanisms are poorly understood. A recent literature review identified a list of 20 different genes that play a potential role in follicular atresia in teleost fish (González-Kother et al., 2020). Although some of these genes participate in processes such as lipid or oxidative metabolism, most of them are usual suspects that participate in regulating apoptotic and autophagic mechanisms (González-Kother et al., 2020). For instance, the sequential activation of caspases essential for proteolytic cleavage and apoptosis-mediated cell death (Andreu-Vieyra and Habibi, 2000) has been demonstrated during follicular atresia in fasted coho salmon (Yamamoto et al., 2016). In the freshwater fishes, *Astyanax bimaculatus, Leporium obtusidens* and *Prochilodus argenteus* overexpression of apoptotic and autophagy marker genes c*aspase-3*, Bcl2 protein family genes *bcl2* and *bax*, *cathepsin D* and *beclin*-1 has been demonstrated in follicular and theca cells during early and advanced ovarian regression (Morais et al., 2012). In the Japanese flounder *Paralichthys olivaceus*, the phosphatase and tensin homolog B (*ptenb*) has been implicated in the process of follicular atresia through its relationship with Beclin-1 and the initiation of autophagy and apoptosis (Li et al., 2020).

p53, a main regulator of both apoptotic and autophagic pathways, has not received enough attention in relation to the phenomenon of follicular atresia in teleosts. Its role in controlling the fate of ovarian follicles of fish is envisaged to be very relevant as demonstrated by the process of zebrafish sex differentiation (Rodríguez-Marí et al., 2010). All zebrafish after hatching develop a juvenile ovary that in males is degenerated to allow the formation of testis in substitution. This process is regulated mainly through p53 activation that triggers the resorption of immature oocytes (Rodríguez-Marí et al., 2010). In this sense, it has been reported that during previtellogenesis oocytes in all teleost fish produce immense amounts of 5S ribosomal RNA (5S rRNA) in preparation for ribosomal assembly during early embryonic development (Diaz De Cerio et al., 2012; Rojo-Bartolomé et al., 2016). In mammalian cells under ribosomal stress, 5S rRNA in conjunction with some ribosomal proteins is known to regulate p53 activity and trigger apoptosis (Deisenroth and Zhang, 2010; Donati et al., 2013; Sloan et al., 2013) and in ovaries this could make of 5S rRNA and p53 a regulatory switch deciding between oocyte survival and death.

The present study evaluated the role that apoptosis and autophagy-related genes may play in regulating follicular atresia in the European hake (*Merluccius merluccius*). This is a commercially important fish species with indeterminate fecundity and asynchronous oocyte development which experiences periods of a high incidence of follicular atresia linked to environmental changes as it was for instance in the Prestige oil spill in 2002 (Díez et al., 2011; Murua and Motos, 2006). Since the European hake northern stock assessment considers maturity and not fecundity, developed early oocyte atresia molecular markers could be applied in potentially useful fishery-independent methods such as the daily egg production method proposed (Murua et al., 2010) for the European hake.

2. Materials and methods

2.1. Study area, fish and tissue sample collection

The Biscay Bay is a gulf of the northeast Atlantic Ocean located south of the Celtic Sea (44° 14′ 23″ N°, 4° 52′ 45.9″ W) defined as major fishing area 27.8 by the International Council for the Exploration of the Sea (ICES). Samples were fished in subarea 8 (divisions 8b and 8c). In total, 169 adult European hakes (*M. merluccius*) were obtained from landings of commercial longline fishing vessels in the Bay of Biscay between March and July (2016–2018). From these, 76 mature female individuals were selected and studied (Mean fork length = 58.41 ± 3.78 cm; Mean body weight = 1863.61 ± 360.24 g). The ovaries were analysed using histological and molecular biology techniques. Ovaries were dissected and weighed (to the nearest 0.1 g) within 24 h of landing. One portion of the ovarian tissue of each individual was fixed in 10% neutral buffered formalin (NBF) containing 1% glutaraldehyde at 4 °C. Another portion of the same ovarian tissue was placed in RNAlater® Stabilization Solution (Ambion™, ThermoFisher Scientific, Waltham, Massachusetts, USA) for 5–10 min, frozen in liquid nitrogen and stored at −80 °C until further analysis. All chemicals were of analytical grade and were obtained from Sigma-Aldrich (St. Louis, Missouri, USA) unless otherwise specified. Ethical approval from the Ethics Committee for Animal Experimentation of the University of the Basque Country (UPV/EHU) was not necessary because the fish samples were obtained from landings of commercial fishing vessels. Genetic material was collected and utilized according to the Access and Benefit Sharing Legislation in place in Spain and under the Internationally Recognized Certificate of Compliance (ABSCH-IRCC-ES-258968-1).

2.2. Histological analysis, ovary development staging and atresia identification

After 24 h, the fixed ovarian tissue samples were dehydrated in a graded ethanol series (80, 96 and 100%) and embedded in paraffin using the Leica ASP300 S Automated Vacuum Tissue Processor (Leica

Biosystems, Nussloch, Germany). For sectioning, paraffin blocks were cooled rapidly on a PF100 cooling plate (Bio-Optica Milano, Milano, Italy) and cut into 5 μm tissue sections on a Leica RM 2125RT Rotary Microtome (Leica Biosystems, Germany). Sections were mounted onto albumin-coated 90° frosted-end microscope slides (BPB019 RS France, Wissous, France). Haematoxylin-Eosin (HE) staining (Gamble, 2008) was performed using a Leica Autostainer XL (Leica Biosystems, Germany). An Olympus BX50 light microscope (Olympus Corporation, Tokyo, Japan) was used to analyse the tissue sections. The gametogenic stages of each individual were determined according to Murua and Motos (2006) and Korta et al. (2010) and as explained in Table 1. The presence of a single oocyte in the most advanced stage of development was used to label the ovary as being in that exact phase of development, without taking into consideration the prevalence of the oocyte developmental stage in the ovary (West, 1990). Ovaries of each individual that had at least one oocyte in a state of cellular structural disorganization were classified for our study as atretic (Hunter and Macewicz, 1985). Although different stages (alpha, beta, gamma and delta) of atresia have been described (Hunter and Macewicz, 1985), the alpha stage being an earlier stage at which the oocyte begins to become irregular in shape and granulosa cells phagocytize the oocyte yolk, and the delta stage is the last stage when granulosa cells reduce in numbers and are no longer surrounded by thecal cells and blood vessels (Corriero et al., 2021; Hunter and Macewicz, 1985), no distinction among the different atretic stages was done. High-resolution histological images were captured with a Nikon DS-Fi2 digital camera (Nikon Instruments Inc., Tokyo, Japan).

2.3. In-situ TUNEL assay and apoptosis

Apoptotic cells in hake ovaries were detected using a DeadEnd Colorimetric *in-situ* terminal deoxynucleotide transferase (TdT) dUTP nick-end labelling (TUNEL) assay kit (Promega, Madison, WI, USA) following the manufacturers' instructions. Tissue sections, 5 μm thick, were mounted on silanized microscope slides. The slides were deparaffinised with xylene, rehydrated with a series of ethanol dilutions and then washed at room temperature in 0.85% NaCl (5 min), phosphate buffer solution (PBS) (5 min), followed by a fixation step in 4% paraformaldehyde (15 min). After the slides were washed twice in PBS (5 min each), 100 μL of proteinase K solution was added to each slide and incubated for 20 min. Slides were washed with PBS (5 min) and re-fixed in 4% paraformaldehyde (5 min). Sections were treated with TdT equilibration buffer at room temperature for 10 min before being incubated with rTdT enzyme in a humidified chamber for 1 h at 37 °C equilibration buffer for 10 min. TdT was removed and sections were stained at room temperature for 10 min with 10% DAB staining solution (1X) after being rinsed in PBS. Slides were rinsed several times in deionized water and mounted in glycerol (Kaiser's glycerol gelatine – for microscopy; Merck KgaA, Darmstadt, Germany). The sections were observed under an Olympus BX50 light microscope, and the images were captured with a Nikon DS-Fi2 digital camera.

Table 1
Oocyte developmental staging in European hake ovaries during histological analysis (adapted from Murua and Motos (2006) and Korta et al. (2010)).

Ovary phase	Developmental stage	Main histological characteristic
Previtellogenic	Previtellogenesis	Lightened basophilic cytoplasm with no cytoplasmic inclusions
	Cortical alveoli	Presence of cortical alveoli vesicles in the cytoplasm with lipid droplets accumulating in the cytoplasm
Vitellogenic	Early vitellogenic	Yolk proteins arranged in the periphery of the ooplasm with more lipid droplets present in the cytoplasm
	Vitellogenic	Yolk proteins present in the cytoplasm with an equal amount of lipid droplets
	Late vitellogenic	Further accumulation of yolk proteins in the cytoplasm with fewer lipid droplets present
Maturation	Maturing	Nuclear migration, fusion of lipids droplets and yolk proteins, germinal vesicle breakdown and hydration

2.4. Total RNA extraction, quantification and 5S/18S rRNA ratio calculation

Total RNA was extracted from the remaining portion of the same ovarian tissue taken for histology using TRIzol Reagent® Solution (Ambion®, ThermoFisher Scientific, Massachusetts, USA) following the manufacturer's instructions. After histological analysis, oocyte developmental staging and atresia identification, 46 ovaries showing extensive follicular atresia (21 previtellogenic, 25 vitellogenic) and 22 ovaries without signs of atresia (11 previtellogenic, 11 vitellogenic) were selected. RNA yield of each sample was assessed using a cuvette photometer (Biophotometer plus, Eppendorf, Hamburg, Germany) using a 1:50 dilution (or appropriate dilution factor) (Rojo-Bartolomé et al., 2016).

The quantity and quality of the RNA were then determined by capillary electrophoresis using the Agilent RNA 6000 Nano Kits Assay Protocol (Agilent Technologies, Santa Clara, California, USA). The Bioanalyzer (Agilent 2100 Bioanalyzer; Agilent Technologies) electropherograms were used to quantify the concentration of the bands corresponding to 5S and 18S ribosomal RNAs (rRNAs) in each sample. The Time-Corrected-Area of each peak was used to calculate the 5S/18S rRNA ratio and the Log_2 of this value was used to develop a 5S/18S rRNA ratio, in order to rank ovaries according to their different developmental phases (Rojo-Bartolomé et al., 2016.

2.5. Gene transcription analyses by qPCR

First-Strand cDNA was synthesised using Affinity Script Multiple Temperature cDNA Synthesis Kit (Agilent Technologies) in a 2720 Applied Biosystems Thermal Cycler (Applied Biosystems, Foster City, California, USA). Total RNA was reverse transcribed to cDNA using random primers following the manufacturer's instructions (2 μg total RNA in a reaction volume of 20 μL for a final theoretical cDNA concentration of 100 ng μL^{-1}). The cDNA was stored at −40 °C for subsequent PCR amplification and gene expression analysis. Single-strand cDNA (ssDNA) concentrations were quantified by measuring fluorescence in a Synergy HT Multi-Mode Microplate Reader (Biotek, Winoosky, USA) using Quant-iT™ OliGreen® Kit (Life Technologies™, ThermoFisher Scientific, Waltham, Massachusetts, USA). The quantification was run in triplicate, in a reaction volume of 100 μL with a theoretical cDNA concentration of 0.2 ng μL^{-1} (Rojo-Bartolomé et al., 2017). The fluorescence was measured at standard fluorescein excitation and emission wavelengths of 480 nm and 520 nm respectively. Real cDNA concentration was calculated using a five-point standard curve according to the manufacturer's instructions and the exact amount of cDNA loaded in qPCR reactions was calculated, adjusting dilutions used for each gene (Rojo-Bartolomé et al., 2017).

Gene sequences for European hake *p53* (DQ146942), *beclin-1* (KY771082), *caspase-3 (caps3)* (KY771084), *fshr* (KY178270.1) and *lhr* (KY178271.1) were obtained from Genbank in the NCBI database. The sequence for other target genes (*mdm2, rpl11, rpl5, bcl2, ptenb, ctsd* and *dapk1*) were obtained from querying the draft genome assembly produced for *M. merluccius* in a broad comparative genomic study of gadoid fish species published in the Dryad Digital Repository (Malmstrøm et al., 2016a, 2016b). To identify the coding domain sequences (cds) of each target gene, a BLAST (BlastN) against ortholog sequences of *Danio rerio* and *Gadus morhua* available in the NCBI database was run. Scaffolds with the best hits were selected and a local BLAST (BlastN and BlastX) ran. Primer pairs for each target gene were designed using Eurofins online tools and their specificity was verified by conventional PCR

amplification on at least two independent cDNA samples of each tissue (Table 2). The PCR programme for each target gene was as follows: 94 °C for 2 min, denaturation at 94 °C for 30 s, annealing step (temperature for each primer set in Table 2) for 30 s, and elongation at 72 °C for 30 s. PCR was finalised at 72 °C for 8 min (2720 thermal cycler, Applied Biosystems, USA). All PCR products/amplicons were visualized in 1.5% (w/v) agarose gels stained with ethidium bromide and sequenced at the Sequencing and Genotyping Service of the University of the Basque Country (SGIker-UPV/EHU).

The relative transcription levels for the target genes in all the atretic and non-atretic individual samples were determined by real-time quantitative PCR (qPCR) amplifications performed in a final volume of 20 μL with 10 μL FastStart Universal SYBR Green Master (Rox) (Roche Diagnostics, Mannheim, Germany), 2 μL appropriately diluted cDNA, 7.94 μL or 7.88 μL of nuclease-free water and 0.03 μL (6.25 pmol μl^{-1}) or 0.06 μL (12.5 pmol μl^{-1}) of primer pair respectively (Rojo-Bartolomé et al., 2016). Optimal concentrations of primers and samples were used for each gene (Table 2). Samples were run in triplicate on 96-well reaction plates using the 7300 PCR thermal cycler (Applied Biosystems, Forster City, California, USA). A no-template control (NTC) was also run in triplicate in each plate using the same reaction conditions. The amplification protocol was set up to follow an initial denaturation and activation at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and an annealing step of 60 s at the appropriate primer pair temperature (Table 2). To obtain a dissociation curve, the amplification reaction was followed by a dissociation step carried out at 95 °C for 15 s, 60 °C for 15 s and 95 °C for 15 s. The reaction efficiencies for each plate were estimated by generating a standard curve for each primer pair from a 2-fold serial dilution of a pool of first-strand cDNA templates from all samples as described (Rojo-Bartolomé et al., 2017):

$$E = (10^{(-1/m)}) - 1$$

Where E = amplification efficiency of the qPCR reaction and m = slope of the standard curve of the qPCR reaction. The standard curve represented the cycle threshold (C_t) of the sample as a function of the logarithm of the number of copies generated.

The delta C_t (ΔC_t) method adapted from the delta-delta C_t ($\Delta\Delta C_t$) normalization method (ΔC_t = C_t sample – C_t Intercept) was used to normalize (Log2) the relative quantity (RQ) of all gene transcription levels to the amount of cDNA in nanograms per sample used in the qPCR, with the C_t intercept of the standard curve when the logarithm of the number of copies generated is zero (Rojo-Bartolomé et al., 2016):

$$RQ = \mathrm{Log}_2\,[(1 + E)^{-\Delta Ct}/\text{ng cDNA}]$$

Where RQ = relative quantity of each gene transcription level in the sample, E = amplification efficiency of the qPCR reaction, ΔC_t = difference between the C_t values of the gene of interest and the C_t value of the standard curve when the logarithm of the number of copies generated is zero in the sample and ng cDNA = amount of cDNA in nanograms per sample used in the qPCR.

No reference or housekeeping gene was used to normalize the target gene transcription and instead, the total amount of input cDNA was used (Libus and Štorchová, 2006; Mittelholzer et al., 2007; Rojo-Bartolomé et al., 2016; Valencia et al., 2020). Considering that fish ovaries undergo profound changes in terms of cell composition, physiological status, hydration levels or meiotic stage, their mRNA content varies enormously throughout growth and maturation. In these circumstances, it is hard to concieve the existence of any valid reference gene.

2.6. Statistical analysis

Statistical analysis was performed on R using the base R stats package (R Core Team, 2020) and the packages "Tidyverse" (Wickham et al., 2019) and "YaRrr" (Phillips, 2017) with all data expressed as the median with upper and lower limits and 95% confidence interval. First and second-order correlations (linear-quadratic regressions) were performed, and a two-way ANOVA test was applied for multiple comparisons in all the data (Zar, 2010). Relative quantification values for each gene in each individual were plotted against the 5S/18S rRNA ratio either on a regression curve for the statistically significant coefficient of correlations or in a constant representing the median of the total values. The statistically significant difference was set at $p < 0.05$.

Table 2
Nucleotide sequences of forward (F) and reverse (R) primer pairs with the temperatures (T_A = annealing temperature) used for the qPCR. GenBank accession numbers are available in the International Nucleotide Sequence Database and the scaffold numbers in the draft genome assembly produced for *M. merluccius* in the Dryad Digital Repository (Malmstrøm et al., 2016a, 2016b).

Target gene	GenBank Accession No./Scaffold No.	Primer Sequence (5' – 3')	T_A (^{0}C)	Primer concentration (pmol.μl^{-1})	Sample dilution factor
p53	DQ146942	F:GAGCCAGAGGGTCCAGT	58.0	12.5	1:40
		R:CATGAGCTGTTGCACATG	58.0		
mdm2	Scf718000323709	F:GAAGAGGAGGCGSTCTGATAG	59.0	6.25	1:200
		R:ACCTGGTCRTCCCCAGATARC	59.0		
rpl11	Scf7180003655120	F:CTGGAGAAGGGACTCAAGGT	55.0	12.5	1:50
		R:CCAGAACCACCACGTAGAAG	55.0		
rpl5	Scf7180005283183	F:CAAGAGGTACAGGTCAAGTTC	55.0	6.25	1:200
		R:GCRTCCAGGTAGCAKGTGA	55.0		
caspase-3	KY771084	F:CGGTGCGTCATCATCAAC	59.0	12.5	1:200
		R:GAACGGCTGTGATCTTCCATC	59.0		
bcl2	Scf7180005228352	F:AGCTTCGAGARCGTGATGGAY	55.0	6.25	1:200
		R:CASGGSTSRATGGTTGTCCAG	55.0		
beclin-1	KY771082	F:GAGGAGGAGATGCTGGT	59.0	6.25	1:200
		R:TCCAGCTCCAGCTGCTGC	59.0		
ptenb	Scf7180003609668	F:GCATGTGGAGAGGCTGGAAA	59.0	12.5	1:50
		R:GAGAGCACCAAGAAGTCCCG	59.0		
ctsd	Scf7180003655488	F:GGTGAGCTGTGACAAGATCC	57.0	6.25	1:200
		R:TGGCCAATGAATACATCTCC	57.0		
dapk1	Scf7180003611438	F:CTCTGTGAGCATCTCCAACC	57.0	6.25	1:200
		R:CACCGTTGATGTTGGTGTTC	57.0		
fshr	KY178270.1	F:GCATGGCCGTGCTCATCTTC	58.0	6.25	1:40 & 1:50
		R:GCGTACAGGAAGGGGTTGG	58.0		
Lhr	KY178271.1	F:GTCAGCGAGTTGGACATGGA	56.0	6.25	1:10
		R:ATGACCCAGGTGAGAAAGCG	56.0		

3. Results

3.1. Histological analysis, gonad developmental staging and atresia identification

Histological analysis of the ovaries of European hakes obtained from landings of commercial longline fishing vessels fishing the Bay of Biscay between March and July (2016–2018) showed different ovarian developmental stages ranging from previtellogenesis to maturation that were ranked according to Korta et al. (2010) (Table 1). While we were able to examine non-atretic oocytes in some of the ovaries (Fig. 1A – D), atresia was identified in oocytes in any stage of development and was characterized by some level of disorganization of the cellular structure of the oocyte (Fig. 1E – H) according to Hunter and Macewicz (1985). The presence of atresia was identified in 73.7% of mature females analysed, with atresia being most prevalent in vitellogenic oocytes in comparison to previtellogenic ones or oocytes transitioning from cortical alveoli to early vitellogenesis. Hereby, we did not attempt to study the prevalence of atresia in the hake population analysed, nor compare the situation between studied years. This was concieved as a mechanistic study to

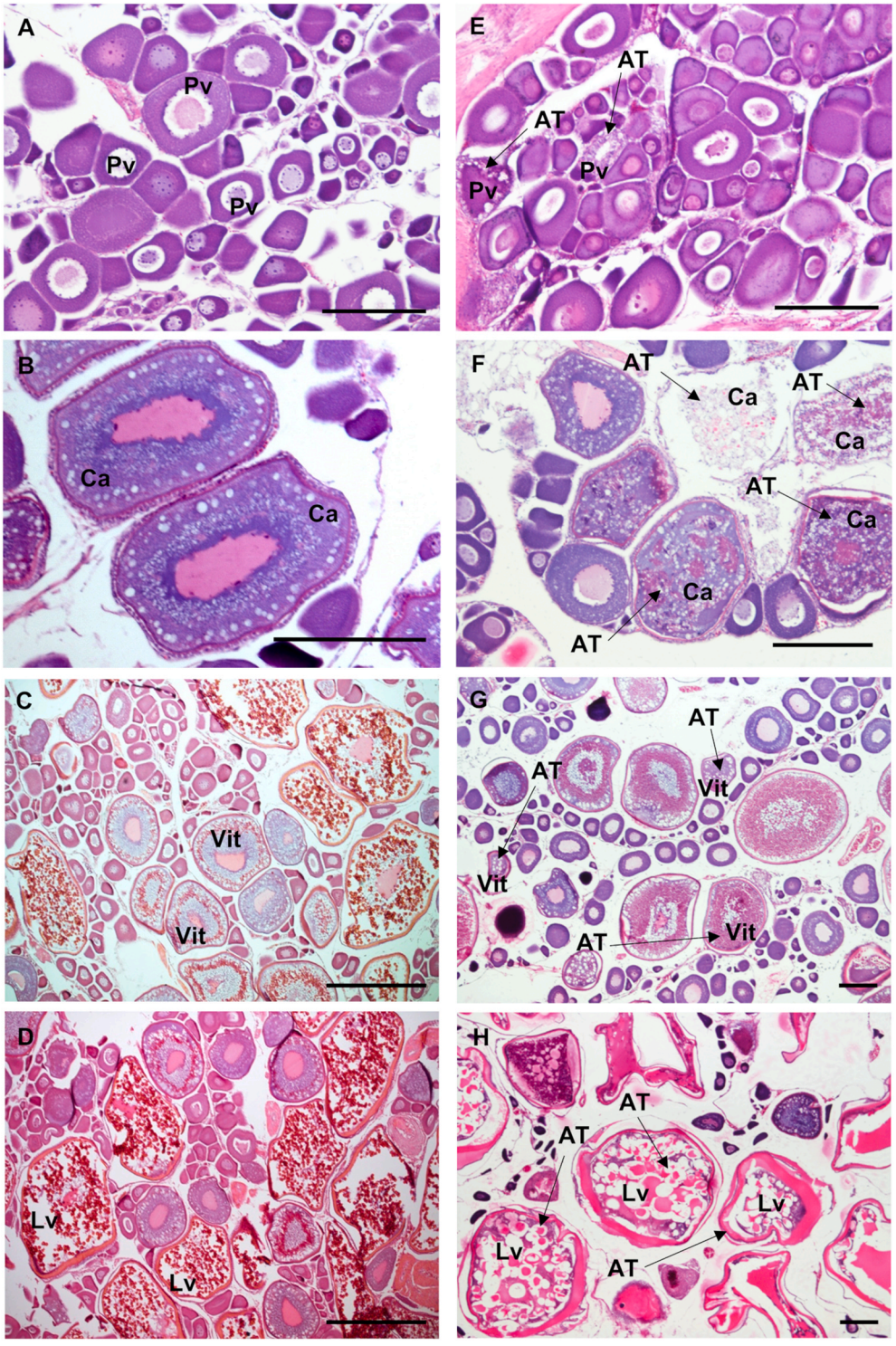


Fig. 1. Histological analysis and ovary development staging in European hake. Asynchronous developing ovaries in European hake with oocytes at different developmental stages: (A to D) ovaries with non-atretic oocytes at different stages of development (Pv = Previtellogenic oocytes; Ca = Cortical alveoli; Vit = Vitellogenic oocytes; Lv = Late vitellogenic oocytes). (E–H) Ovaries with atretic oocytes in all stages of development (AT = Atretic). Bars = 100 μm in all micrographs except C and D where bars = 500 μm.

develop tools that could be utilized to identify atresia and not a fisheries study.

3.2. In-situ TUNEL assay and apoptosis

In-situ TUNEL analysis of histological sections of European hake ovarian tissue revealed TUNEL-positive cells with a distinct staining and displaying stained nuclei in contrast to the TUNEL negative controls devoid of any staining (Fig. 2B).

Positively stained nuclei were identified in atretic previtellogenic oocytes although labelling was also conspicuous in nuclei of hypertrophied granulosa cells (Figs. 2B and 3B-D) whereas oocytes in normal non-atretic follicles were TUNEL-negative (Figs. 2B and 3B). Atresia in follicles at more advanced developmental stages resulted in TUNEL-positive staining of theca and follicular cells but it was not evident in oocytes themselves (Fig. 3D–F).

3.3. 5S/18S rRNA ratio calculation

The 5S/18S rRNA ratio was useful to discreetly identify the level of development of the ovaries in European hake. Ovaries were classified as containing mainly previtellogenic oocytes when they displayed the highest 5S/18S rRNA ratio values (Rojo-Bartolomé et al., 2016, 2017). Values decreased as the ovaries contained more oocytes that had entered the vitellogenesis stage that is characterised by a high production of 18S and 28S rRNA's. The lowest values were observed when ovaries contained many oocytes reaching final maturation (Fig. 4). Hake ovary develops asynchronously (Figs. 1 and 3), and our histological ranking of development took into consideration the presence of at least one oocyte in the most advanced developmental stage without distinguishing which is the most dominant oocyte developmental stage in each moment. With the 5S/18S rRNA ratio, a fine-grain continuous numerical ranking of ovarian development was made possible, thus eliminating the possibility of bias with histological staging. Within the developmental continuum from early development to full maturity this ranking system matched well with the histological staging that was previously done. In all cases, there were individuals showing ratio values that would rank them outside their histologically assigned developmental stage.

3.4. Gene transcription analyses

Relative transcription levels in each individual were plotted against their individual 5S/18S rRNA ratio comparing transcription levels between ovaries identified histologically as atretic or non-atretic. The ratio classified the asyncrhonous ovaries in European hake in terms of their stage of development and was used to plot the qPCR values along ovarian development. Lower 5S/18S rRNA ratio values identified ovaries with oocytes in vitellogenesis and entering final maturation, while higher values identified ovaries with oocytes in previtellogenesis. The histological classification ranks in the same group an ovary with only one vitellogenic oocyte and another one with most of its oocytes in vitellogenesis (vitellogenic); whereas the ratio reflects the gonad development stage along a numerical continuum. The trends in relative transcription levels of *p53* and *caspase-3* (*casp3*) (Fig. 5) and *cathepsin D* (*ctsd*) and *death-associated protein kinase 1* (*dapk1*) (Fig. 6) were identical. Significant differences in *p53* transcription levels were observed between vitellogenic atretic follicles and non-atretic follicles with 5S/18S index values ranging from 3.01 to 10.65, with *p53* transcripts being upregulated in atretic oocytes. However, *p53* showed no transcription level differences in atretic vs. non-atretic follicles during very early previtellogenesis and late vitellogenesis. The same expression patterns were observed for *casp3, ctsd* and *dapk1* transcripts but this time in between 5S/18S rRNA ratio values of 2.95–10.01, 3.24–10.25 and 3.47–10.21 respectively. In the case of *beclin-1* and *ptenb*, upregulation was observed in atretic follicles along their whole developmental spectrum, including very early previtellogenesis and late vitellogenesis (Fig. 6). For *mdm2, rpl11, rpl5, bcl2, fshr* and *lhr,* no significant transcription level differences were observed between atretic and non-atretic follicles.

4. Discussion

The focus of the present study was to understand the molecular mechanisms governing follicular atresia in fish ovaries to develop early warning biomarkers of atresia and interruption of the normal oogenic cycle that could eventually be applied to better monitor fish stock fecundity. The transcriptional profile of apoptosis and autophagy-related genes (*p53, mdm2, rpl11, rpl5, caspase-3, bcl2, ptenb, fshr, lhr, beclin-1, ctsd, dapk1*) were analysed in the ovaries of the European hake (*Merluccius merluccius*), a species whose ovaries develop asynchronously and that shows episodes of enhanced oocyte atresia. Ovarian developmental stages were ranked histologically and inferred molecularly by applying the 5S/18S rRNA ratio (Rojo-Bartolomé et al., 2016) and TUNEL assay was used to histologically identify apoptosis.

Histological analysis revealed the presence of atretic follicles in the ovaries of a high percentage of the hakes analysed, the atresia becoming evident in follicles at any stage of development. It must be noted that individuals were captured from March to July after the peak winter spawning period and this could help explain the high prevalence of atresia identified in the study population. In European hake, oocyte recruitment is a continuous process, with oocytes of different sizes and stages coexisting in an ovary during maturity (Korta et al., 2010). Ranking the developmental stage of an ovary as vitellogenic due to the presence of a single oocyte in vitellogenesis and thus disregarding the predominance of cortical alveoli and previtellogenesis stage oocytes (West, 1990) may not reflect the relative maturity stage of the individual ovary. Instead of taking a stereological approach to quantify the extent of coverture of each type of oocyte in each ovary, we aimed to rank the developmental stage of the study ovaries more granularly using the 5S/18S rRNA ratio.

The 5S/18S rRNA ratio has been proposed as a good quantitative and unbiased proxy to establish the ovarian developmental stage of any

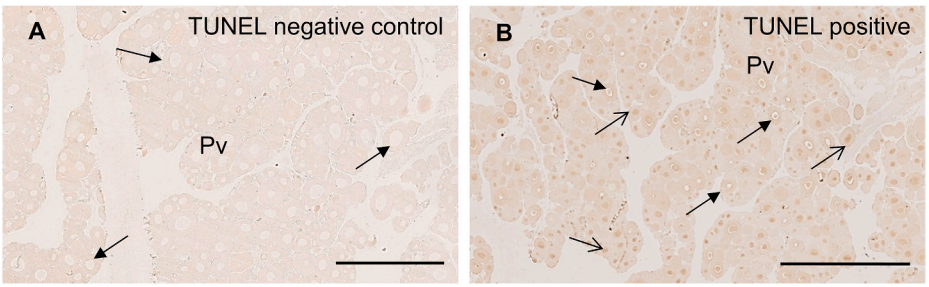


Fig. 2. *In-situ* TUNEL staining in ovaries of European hake.
(A) TUNEL assay negative control. Bar = 500 μm; (B) TUNEL-positive staining in cells of ovarian follicles. Bar = 1 mm. Open arrows show positively labelled previtellogenic oocyte (Pv) nuclei and full arrows indicate unstained oocyte nuclei in this early developmental stage ovary.

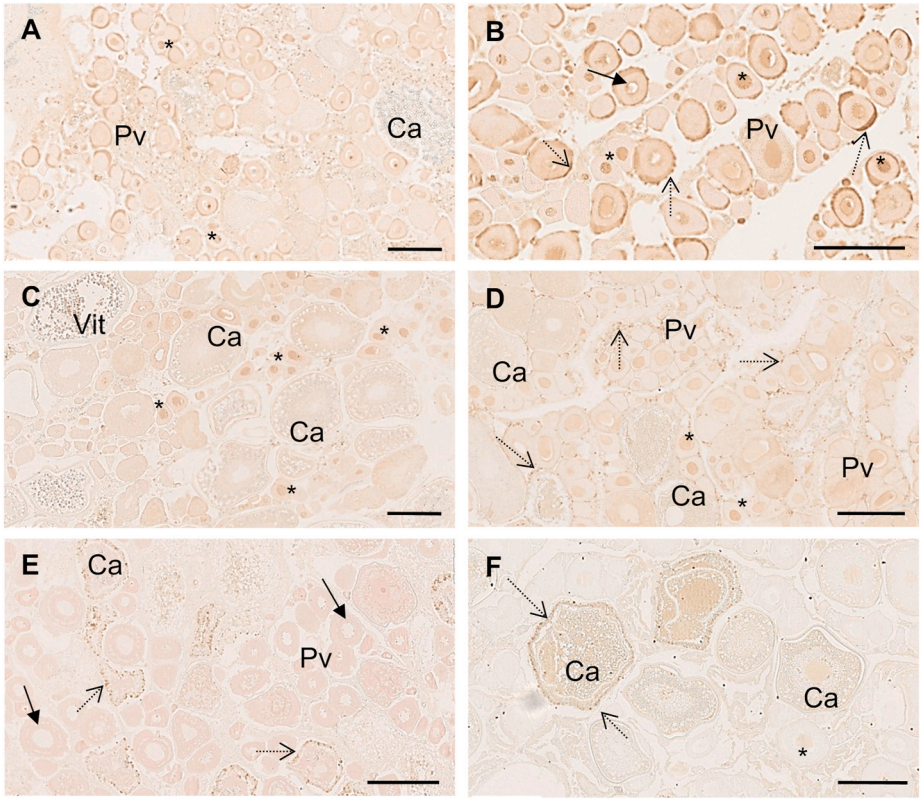


Fig. 3. *In-situ* TUNEL staining in ovaries of the asynchronous developing European hake.
Apoptotic oocytes with TUNEL-positive staining can be observed. Asterisks show positively labelled oocyte nuclei all of them at previtellogenic stage (Pv). Broken arrows label positively labelled follicular cells mainly around cortical alveoli (Ca) and vitellogenic (Vit) stage oocytes. Full arrows mark non-apoptotic and unlabelled oocyte nuclei. Bars = 250 μm.

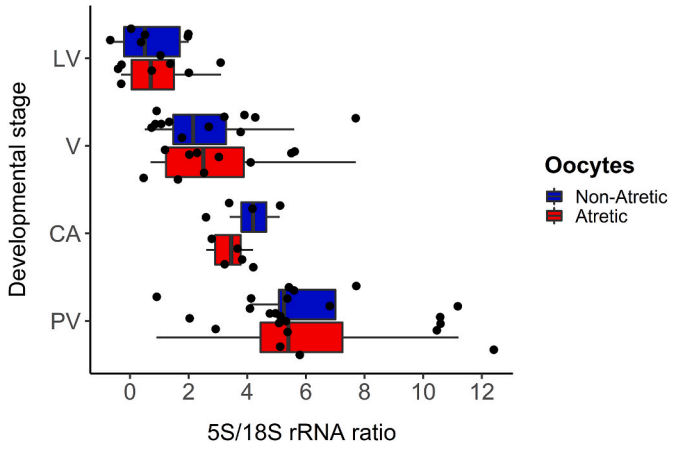


Fig. 4. 5S/18S rRNA ratio values during ovarian development in the European hake (atretic and non-atretic).
The developmental stages include previtellogenic (PV; atretic n = 14, non-atretic n = 7), cortical alveoli (CA; atretic n = 6, non-atretic n = 3), vitellogenic (V; atretic n = 19, non-atretic n = 5), late vitellogenic and maturing (LV; atretic n = 7, non-atretic n = 7) and were assigned according to the presence of the most advanced developmental stage present in the ovary. Boxplots represent the data within the 25th and 75th percentiles, with the median indicated by a line, and bottom and top whiskers representing minimum and maximum values, respectively. Each dot corresponds to an individual.

teleost species (Rojo-Bartolomé et al., 2016). This is so because 5S rRNA levels are very high in previtellogenic oocytes while 18S rRNA (and 28S rRNA) levels begin to increase after the cortical alveoli stage (Rojo-Bartolomé et al., 2016, 2017). Growing oocytes require rRNAs to synthesise new ribosomes for the proper development of the embryo after fertilisation and they initiate the process with the production of the less energy-demanding 5S rRNA molecule. Larger molecules begin to accumulate during vitellogenesis and secondary oocyte growth (Diaz De Cerio et al., 2012; Rojo-Bartolomé et al., 2016, 2017; Shen et al., 2016). Due to the dynamics of production of the ribosomal intermediates, the 5S/18S rRNA ratio ranks ovarian developmental status efficiently in synchronous species although it has been proved also to work in asynchronous species such as Atlantic, chub and horse mackerels, blue whiting, European anchovy and pilchard and bluegill and largemouth bass (Rojo-Bartolomé et al., 2016; Shen et al., 2016). Therefore, considering the dynamics of 5S and 18S rRNA production, the 5S/18S rRNA ratio proved to be a useful numerical and non-biased approach to rank ovaries, integrating the developmental stage of the pool of oocytes in each gonad. The results aligned quite well with the histological staging, but instead of creating four discrete groups (previtellogenic, cortical alveoli, vitellogenic, late-vitellogenic) identified each ovary along the continuum of the maturation process.

There is evidence that apoptosis plays a key role in gonad development and reproduction in birds and mammals, but also in fish (Habibi and Andreu-Vieyra, 2007; Janz and Van Der Kraak, 1997; Matsuda et al., 2012; Nozu et al., 2013; Tilly, 1996a; Tiwari et al., 2015; Wood and Van Der Kraak, 2001; Yamamoto et al., 2011; Yang et al., 2022). In birds and mammals, for example, the dominant follicle is normally recruited to the ovulatory pool, while the remaining follicles are eliminated through atresia, mediated by apoptosis initiated within granulosa cells (Andreu-Vieyra and Habibi, 2000; Bhardwaj and Sharma, 2012; Matsuda et al., 2012). In fish, widespread apoptosis-mediated atresia occurs when of the thousands of oocytes normally recruited, the vitellogenic oocytes fail to mature in a spawning season and regression occurs in preparation for a new reproductive cycle (Guraya, 1986; Miranda et al., 1999; Nozu et al., 2013; Rizzo and Bazzoli, 1995; Yamamoto et al., 2011). Results of the TUNEL assay demonstrate that apoptosis occurs in hake previtellogenic oocytes and their surrounding follicular cells. Apoptosis was additionally observed in cortical alveoli and vitellogenic follicles, but not within the oocytes. In such cases, apoptosis was limited to the nuclei of the surrounding follicular cells. Similar responses in advanced-stage follicles were observed in other asynchronous

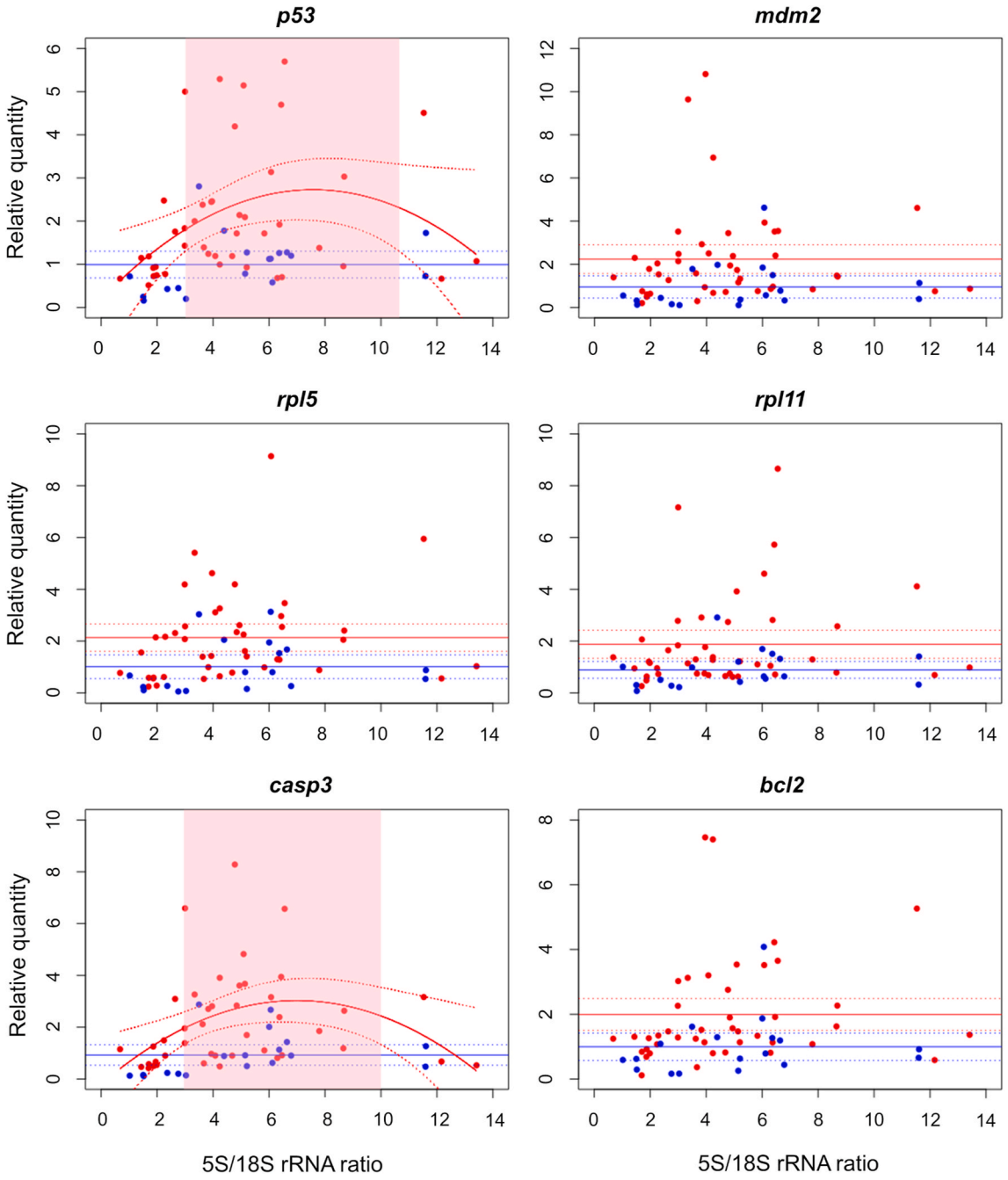
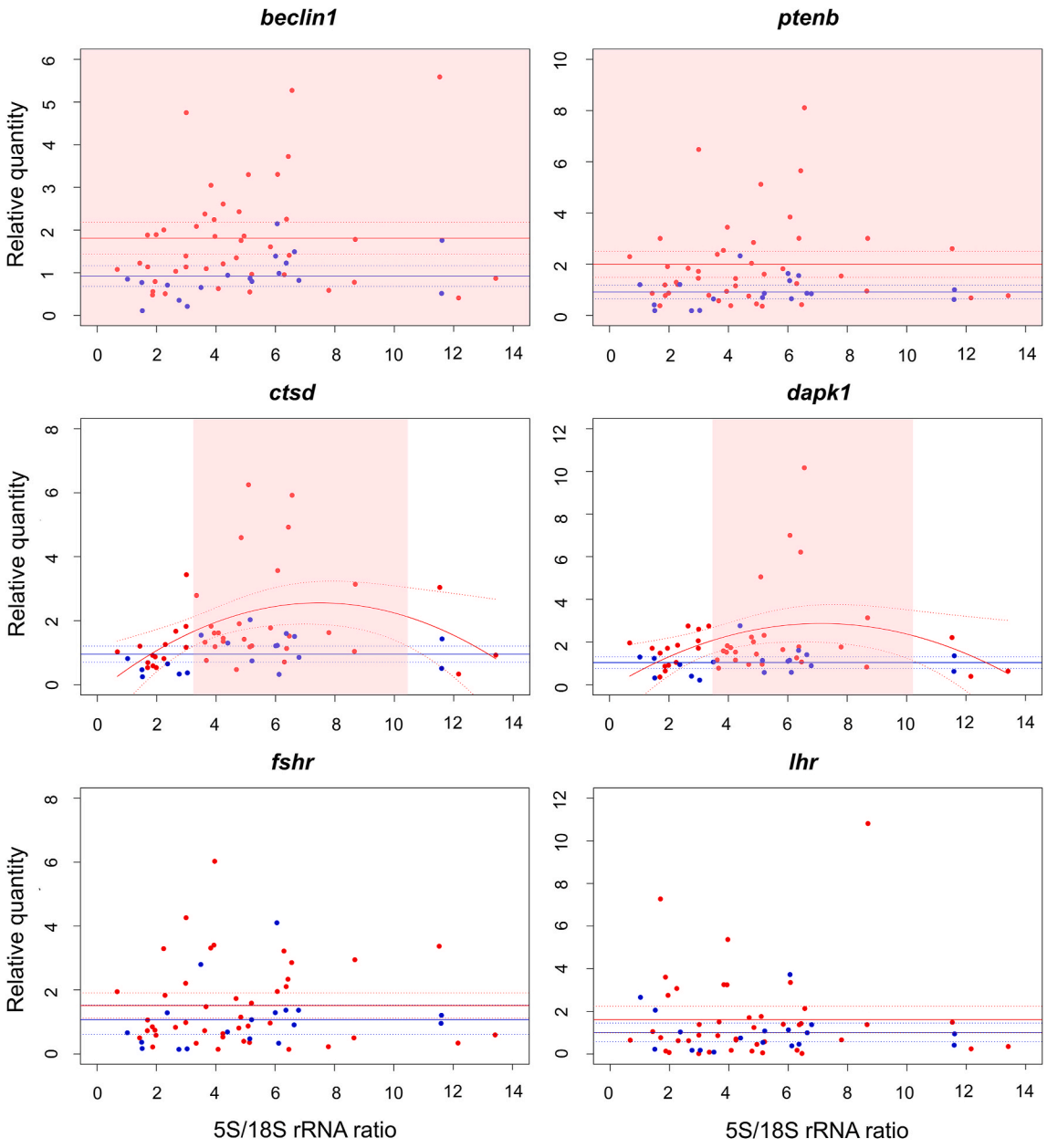


Fig. 5. Transcription levels of genes related to apoptosis and autophagy in ovaries of the European hake at different developmental stages.
5S/18S rRNA ratio indicates the level of maturity of the ovaries analysed ranging from ovaries with oocytes in vitellogenesis (values tending to 0) to pre-vitellogenesis (values tending to 14). Each dot represents one individual. Red dots represent ovaries with atretic follicles and blue dots represent ovaries with non-atretic follicles. Red and blue lines represent the regression curves (continuous lines) and 95% confidence intervals (dashed lines) of ovaries with atretic and non-atretic follicles respectively. Pink shading covers the rank of individuals along the developmental continuum as staged by the 5S/18S rRNA ratio that shows significant differences between atretic and non-atretic individuals ($p < 0.05$).

developing species, the goldfish (*Carassius auratus*) (Wood and Van Der Kraak, 2001) and farmed Nile tilapia (*Oreochromis niloticus*) (Qiang et al., 2021). This points to an atresia process that is directed from outside to inside in the follicular structure, as has been suggested for other fish species (Qiang et al., 2022; Wood et al., 2005). Previous studies of atresia in ovaries of non-mammalian vertebrates showed that the degeneration of follicular cells takes place only after the resorption of vitellogenic materials from vitellogenic oocytes is completed (Saidapur, 1978); thus, facilitating the efficient recycling of oocyte contents (Wood and Van Der Kraak, 2001).

The molecular mechanisms that control follicular atresia in teleost fishes may open avenues for early assessment of the onset of the process under environmental stress and in this respect, a set of core genes involved in apoptosis and autophagocytosis have been highlighted as potential markers across teleost species (González-Kother et al., 2020), although some others may exist. Reproductive hormones in teleosts closely integrate physiological status and environmental cues to direct gonad development (Habibi and Andreu-Vieyra, 2007; Juntti and Fernald, 2016; Qiang et al., 2022). In this way, increased levels of follicle-stimulating (FSH) and luteinizing (LH) hormone are known to



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promote oocyte development and maturation (Qiang et al., 2022), with FSH controlling apoptosis-mediated atresia in ovaries (Lubzens et al., 2010; Shen et al., 2016). In this study, the transcript levels of genes encoding the receptors of both gonadotropins (*fshr* and *lhr*) did not change in ovaries with atretic follicles. Nonetheless, the role of FSH as an anti-apoptotic hormone has been clearly demonstrated in the Coho salmon (*Oncorhynchus kisutch*) and the Nile tilapia, and upregulation of *fshr* and *lhr* has been reported in connection with reduced apoptosis-mediated follicular atresia (Guzmán et al., 2014).

p53 is the guardian of the cell cycle both in its functions as a tumour suppressor and regulator of apoptosis and its transcriptional regulation has often been studied as a marker of stress (Rau Embry et al., 2006) although not in relation to follicular atresia. Its role in triggering apoptosis in fish oocytes is proved by its role in sex differentiation in *Danio rerio*. All newly hatched zebrafish develop a juvenile ovary with previtellogenic oocytes and this ovary regresses in the case of males for the development of testis. This sex reversal process is triggered by p53 and executed by caspase-3, causing apoptosis of oocytes (Rodríguez-Marí et al., 2010). In hake, we observed an upregulation of the apoptosis-related gene *p53* in ovaries with atretic vitellogenic (5S/18S index 3.01 to 10.65) follicles and no difference in transcription levels between ovaries with atretic and non-atretic follicles when these were early previtellogenic or late vitellogenic. In these two cases, differences in p53 activities can not be ruled out since p53 is largely regulated post-transcriptionally through ubiquitination and proteolytic cleavage via the E3 ubiquitin ligase Mdm2 (Boehme and Blattner, 2009;

Deisenroth and Zhang, 2010; Rau Embry et al., 2006; Shi and Gu, 2012). Another gene corroborates the preponderance of apoptotic response in atresia affecting vitellogenic stage follicles in opposition to those in previtellogenesis or late vitellogenesis. Transcript levels of *caspase-3* were also significantly higher in ovaries with vitellogenic (5S/18S rRNA ratio 3 to 10) atretic follicles, without differences atretic vs non-atresia in ovaries histologically ranked as previtellogenic or late vitellogenic. Caspase-3 largely mediate the apoptotic response triggered by p53 and its proteolytic activity, also in fish oocytes (Rodríguez-Marí et al., 2010). p53 controls the proteolytic cascade by regulating the transcription of anti-apoptotic Bcl2 whose function is to stabilise the mitochondrial membrane. When this membrane is destabilised, cytochrome *c* is released to the cytosol and caspase-3 is activated (Donati et al., 2013; Maiuri et al., 2007). However, even if *bcl2* transcriptional regulation has been reported in some teleost species suffering atresia (González-Kother et al., 2020), transcription levels were similar in hake ovaries with and without atretic follicles.

As already said Mdm2 is the major regulator of p53 through ubiquitination but no differences in *mdm2* transcription levels were observed between ovaries with atretic and non-atretic follicles at any stage. A strict relationship could exist between p53, Mdm2 and 5S rRNA, considering the enormous amount of 5S rRNA in fish oocytes that could account for a major mechanism deciding the fate of oocytes during development in fish ovaries. In conjunction with ribosomal proteins L5 (Rpl5) and L11 (Rpl11), 5S rRNA has been demonstrated to bind Mdm2 in mammalian cells suffering ribosomal stress. Such binding rescues p53 from ubiquitination and degradation and results in its activation in such circumstances (Deisenroth and Zhang, 2010; Donati et al., 2013; Sloan et al., 2013). Chakraborty et al. (2009) observed that in *Danio rerio* embryos, the loss of Rpl11 results in a decreased production of mature 18S rRNA molecules, activation of p53 and impaired ribosome biogenesis. In this study, however, the transcription levels of *rpl5* and *rpl11* followed a pattern similar to that of *mdm2* without significant transcriptional regulation with regard to atresia. Obviously, the mechanism does not need to be regulated at the transcriptional level.

Autophagy is another mechanism that may lead to cell death in atretic follicles in fish (Maiuri et al., 2007; Thomé et al., 2009). Autophagy can be triggered as a stress response to degrade damaged cell proteins and organelles (Maiuri et al., 2007; Thomé et al., 2009). Beclin-1, an important autophagy marker, is responsible for forming the isolation membrane that engulfs cytoplasmic material to form the autophagosomes that initiate autophagy (Bhardwaj et al., 2022), and it has been implicated in the process of follicular atresia in different freshwater fish species (Morais et al., 2012). Our study observed that both *beclin-1* and *ptenb* were continuously upregulated in atretic follicles irrespective of their developmental stage. Ptenb protein, a transcriptional target of p53, is known to negatively regulate the PI3K/AKT/mTOR signalling pathway, which in turn affects autophagy as it has been reported in Japanese flounder suffering from a pathogen infection (Li et al., 2020).

In vitellogenic oocytes of the European hake, autophagy and also phagocytosis by follicular cells could help to digest oocyte yolk material during atresia. In this way, we have observed the upregulation of *ctsd* and *dapk1* in ovaries with vitellogenic (5S/18S rRNA ratio 3 to 10) atretic follicles. Cathepsin D (*ctsd*) is well known for its key role in oocyte development in fish, amphibians and birds, cleaving vitellogenin into smaller molecules for yolk formation and in turn, oocyte growth and maturation (Carnevali et al., 2006). Several studies have observed that the cleavage of vitellogenin occurs during the early stages of atresia, coinciding with the presence of cathepsin D and preceding the formation of autophagic vacuoles (Guzmán et al., 2014; Mayo and Donner, 2002). These studies reinforce the suggestion that *ctsd* is necessary for vitellogenin cleavage to occur before atresia can proceed (Chang et al., 2003; Janz and Van Der Kraak, 1997). At the same time, Dapk1 is known for its role in stress signalling, regulating cell death and autophagy processes through caspase activation (Gozuacik et al., 2008; Singh et al., 2016). Taking this into account, our study suggests that the overexpression of *beclin-1* and *ptenb* promotes autophagy and transcription of proteolytic enzymes to facilitate the removal of organelles and proteins, therefore recycling macromolecules during nutrient deprivation or energy deficiency. This would allow the recovery of the energy invested in oogenesis previous to follicle resorption through apoptosis-mediated atresia. Comparatively, apoptosis seems to take the leading role in regulating atresia in mammals while in fish as seen hereby, autophagy also plays an important role, especially in somatic cells surrounding the vitellogenic oocytes (Yang et al., 2022). Nonetheless, recent revision of follicular atresia in mammals points also to an important role of autophagy with special mention to molecular markers such as Beclin-1 as in the present study in hake.

5. Conclusions

It can be concluded that the 5S/18S rRNA ratio can be used as a useful non-biased method to rank the overall development stage in the asynchronous developing ovaries of the European hake. The novelty of the study stands in that we can relate the levels of transcription of atresia markers genes in relation to the exact developmental stage of the ovaries. In this way, we observed that apoptosis marker genes *p53* and *caspase-3* are upregulated in hake ovaries which are in vitellogenesis together with autophagocytosis marker genes *ctsd* and *dapk1*. The upregulation of *beclin-1* and *ptenb* transcripts in atretic follicles, occurred irrespective of their developmental stage, suggesting that autophagy is strongly involved in follicular atresia along the whole process of oogenesis. The upregulation of *p53*, *caspase-3, ctsd* and *dapk1* transcripts only during atresia affecting vitellogenic follicles, would suggest that vitellogenesis is the moment at which both apoptosis and autophagy are at their highest (Yang et al., 2022). Hereby a set of marker genes for the early estimation of atresia in female European hakes (that could be extended to other fish species) is provided. These marker genes could be studied for a better estimation of hake stock fecundity but further analysis at the protein level should be carried out to confirm our observations at the gene transcription level.

Author contributions

A.N. wrote the initial draft and performed the experimental procedures. A.A., M.A.H. and A.V. performed some of the experimental procedures. M.K. provided the samples of the commercial fish species and developed the methodology. O.D. and M.O. managed and coordinated the research activity, planning and execution. I.C. conceived, coordinated and supervised the research activity and acquired the financial support for the project leading to this publication. All authors revised the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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