CLONING AND SEQUENCE ANALYSIS OF A VASA HOMOLOG IN THE
EUROPEAN SEA BASS (*Dicentrarchus labrax*): TISSUE DISTRIBUTION AND mRNA
EXPRESSION LEVELS DURING EARLY DEVELOPMENT AND SEX
DIFFERENTIATION

Mercedes Blázquez¹,², Alicia González¹,³, Constantinos C. Mylonas⁴ and Francesc Piferrer¹*

¹Institut de Ciències del Mar, Consejo Superior de Investigaciones Científicas (CSIC),
Passeig Marítim, 37-49, 08003 Barcelona, Spain.
²Current address: Instituto de Acuicultura Torre la Sal, Consejo Superior de Investigaciones
Científicas (CSIC), Torre de la Sal s/n, 12595 Ribera de Cabanes, Castellón, Spain.
³Current address: Departamento de Fisiología y Farmacología, Facultad de Medicina,
Universidad de Cantabria, Avda. Herrera Oria s/n, 39011 Santander, Spain.
²Hellenic Centre for Marine Research, Institute of Aquaculture, P.O. Box 2214, Heraklion,
Crete 71003, Greece.

* Corresponding author: Francesc Piferrer. Institut de Ciències del Mar, Consejo Superior de
Investigaciones Científicas (CSIC), Passeig Marítim, 37-49, 08003 Barcelona, Spain.
e-mail: piferrer@icm.csic.es

Keywords: *vasa*, helicases, primordial germ cells, sex differentiation, gonadal development,
gametogenesis, European sea bass.

Short title: *vasa* in fish ontogenesis and sex differentiation
ABSTRACT

Vasa is a protein expressed mainly in germ cells and conserved across taxa. However, sex-related differences and environmental influences on vasa expression have not been documented. This study characterized the cDNA of a vasa homolog in the European sea bass, *Dicentrarchus labrax*, (sb-vasa), a gonochoristic fish with temperature influences on gonadogenesis. The 1911 bp open reading frame predicted a 637-amino acid protein with the eight conserved domains typical of Vasa proteins. Comparisons of the deduced amino acid sequence with those of other vertebrates and invertebrates revealed the highest homology (68–85%) with those of other teleosts. An updated tree with the full-length sequences for Vasa proteins in 66 species belonging to six different Phyla was constructed, establishing the evolutionary relationships of Vasa amino acid sequences. European sea bass vasa was highly expressed in gonads with little or no expression in other tissues. Real time RT-PCR quantification of the temporal expression of sb-vasa from early development throughout sex differentiation showed that mRNA levels were high in unfertilized eggs, decreased during larval development and increased again during the period of germ cell proliferation. Rearing of fish at high temperature resulted in further increased sb-vasa levels, most likely reflecting temperature effects on both somatic and gonadal growth. Differences in expression were also found well before sex differentiation and persisted until the end of the first year, with higher levels present in females. These differences in expression demonstrate the implication of vasa during the initial stages fish sex differentiation and gametogenesis and suggest that, through its helicase activity, it might be implicated in the translational regulation of mRNAs involved in the specification and differentiation of gonadal-specific cell types.
1. Introduction

Germ cells, also known as pole cells, constitute a highly specialized cell type committed to produce the gametes that will give rise to future offspring (Wylie, 2000). Development of the germline depends on asymmetric distribution of the germ plasm or pole plasm, a specialized cytoplasm containing electron-dense structures, originally present in the oocytes, which contains maternal RNAs and proteins (Houston and King, 2000; Saffman and Lasko, 1999). The germ plasm is further segregated to the primordial germ cells (PGCs), which become differentiated from somatic cells during early embryonic stages. Traditionally, morphological criteria using light and electron microscopy have been applied to discriminate PGCs from somatic cells (Braat et al., 1999b; Eddy, 1975; Hamaguchi, 1982). More recently, the discovery of several molecular markers has aided in the identification of PGCs. In this regard, the first molecular marker for PGCs in teleost fish was the vasa gene (Olsen et al., 1997; Yoon et al., 1997).

The vasa gene encodes a putative ATP-dependent RNA helicase of the DEAD-box family and was identified originally in pole cells of Drosophila, i.e. the true progenitors of the germ line (Hay et al., 1988; Lasko and Ashburner, 1988). DEAD box proteins share eight characteristic sequence motifs and are involved in several important cell processes such as RNA splicing, editing and processing, initiation of mRNA translation, nuclear export and degradation (Luking et al., 1998). Drosophila vasa homologues with germ-line specific expression have been reported from invertebrates to higher vertebrates (Raz, 2000; Saffman and Lasko, 1999), indicating that vasa has been conserved during evolution. In this regard, Braat et al. (1999b) demonstrated that vasa RNA expressing cells in zebrafish, Danio rerio, were indeed PGCs, and concluded that vasa could be used as a molecular marker of PGCs. In the same species it was shown that vasa RNA segregates asymmetrically in cleavage embryos, distinguishing germ cell precursors from somatic cells. Furthermore, at the late blastula stage, vasa mRNA segregation
changed from asymmetric to symmetric, a maternally programmed process that precedes PGC proliferation and perinuclear localization of Vasa protein (Knaut et al., 2000).

Studies of *vasa* mRNA expression and protein localization in fish during embryonic development include those in zebrafish (Yoon et al., 1997), goldfish, *Carassius auratus* (Otani et al., 2002), ukigori, *Gymnogobius spp.* (Saito et al., 2004), shiro-uo, *Leucopsarion petersii* (Miyake et al., 2006) and medaka, *Oryzias latipes* (Herpin et al., 2007). In medaka, *vasa* transcripts were detected exclusively in the cytoplasm of germ cells of both sexes but not in gonadal somatic cells (Shinomiya et al., 2000). *Vasa* has been reported to be differentially expressed during sex differentiation in tilapia, *Oreochromis niloticus* (Kobayashi et al., 2002), and zebrafish (Krøvel and Olsen, 2004; Wang et al., 2007). In addition, sex-related differences during gametogenesis have been shown in several fish species, including tilapia (Kobayashi et al., 2000) and gibel carp, *Carassius auratus gibelio* (Xu et al., 2005). Moreover, the hormonal regulation of *vasa* mRNA expression during oogenesis in gilthead sea bream, *Sparus aurata*, has also been studied (Cardinali et al., 2004). These authors found that growth hormone (GH), estradiol-17β (E₂) and the combination of gonadotropin-releasing hormone (GnRH) with GH were able to induce an increase in *vasa* mRNA expression. However, GnRH or GH alone decreased *vasa* mRNA, indicating the existence of an interplay between these hormones and Vasa during oogenesis.

In mammals, the role of the germ line in sex differentiation is far from clear (Brennan and Capel, 2004). Germ cells are not essential for testicular differentiation although they participate in several aspects of ovarian differentiation, including folliculogenesis (Choi and Rajkovic, 2006). Similarly, in zebrafish the germ line is essential for ovarian differentiation (Siegfried and Nüsslein-Volhard, 2008), since morpholino-silenced expression of *dnd*, a gene essential for germ line survival (Weidinger et al., 2003), resulted in fish lacking *vasa* expression and
exhibiting testicular development and typical male mating behavior. Immunocytochemical studies with zebrafish showed that Vasa protein was present at high levels in undifferentiated spermatogonia decreasing as spermatogenesis progressed until they became absent in spermatids and spermatozoa (Leal et al., 2009).

The European sea bass, *Dicentrarchus labrax*, is an economically important gonochoristic marine teleost and the subject of both applied and basic research in reproduction. In this species, germ cells are readily visible after 25 days post fertilization (dpf). The gonadal ridges form around 35 dpf and are subsequently colonized by germ cells, which increase rapidly in number between 50-100 dpf (Roblin and Bruslé, 1983). The first sex-related differences in aromatase (*cyp19a1a*) expression, a molecular marker of ovarian differentiation, take place around 120 dpf (Blázquez et al., 2009), and sex differentiation can be detected histologically at around 150 dpf, at a size of 79-95 mm standard length (Saillant et al., 2003). This process occurs earlier in females than in males (Piferrer et al., 2005) and is more dependent on length than on age (Blázquez et al., 1999).

In the studies cited above, Vasa protein was identified by immunohistochemistry, whereas *vasa* mRNA was detected by in situ hybridization or conventional PCR. However, to the best of our knowledge, accurate quantification of *vasa* mRNA levels during sex differentiation has never been carried out in fish. The objective of the present study was to quantify the expression of *vasa* during early development in the European sea bass subjected to different temperatures during early life and in relation to sex differentiation. To this end, the European sea bass *vasa* homolog (*sb-vasa*) was cloned and characterized. Further, an updated phylogenetic tree including is presented including the complete Vasa protein sequences from species belonging to all taxa were the presence of this protein has been described. Tissue distribution of *sb-vasa*
mRNA and expression patterns during larval development and sex differentiation were also studied and quantified by real time RT-PCR.

2. Materials and methods

2.1. Fish and rearing conditions

Adult European sea bass used for cloning and tissue distribution studies were obtained from the facilities of the Barcelona Aquarium during the spawning season (December-March). Fish were anesthetized with MS-222 (Sigma, St. Louis, MO, USA) and sacrificed after decapitation. Tissues were removed rapidly under sterile conditions, snap frozen in liquid nitrogen and kept at -80°C until further analysis.

Experiment 1 was designed to study sb-vasa mRNA expression during early development (results reported in Fig. 4). Fertilized European sea bass eggs obtained from a commercial hatchery (Base Viva; St. Pere Pescador, Girona Spain) were transported immediately and reared at the Experimental Aquarium and Chamber Facility (ZAЕ) at the Institute of Marine Sciences (Barcelona, Spain). Fish were reared following standard procedures (Moretti et al., 1999) up to 120 days post fertilization (dpf) at 21 ± 1°C, the temperature used routinely for European sea bass culture (Navarro-Martín et al., 2009; Piferrer et al., 2005). Experiment 2 was designed to assess the effects of the rearing temperature on sb-vasa mRNA expression from 30 to 150 dpf, i.e., the period encompassing gonadal ridge formation up to the beginning of sex differentiation (results reported in Fig. 5). For this purpose, another group of fish from the same genetic origin as those of experiment 1 was reared in parallel at 15 ± 1°C from 0-120, dpf, a temperature that does not induce distorting effects on European sea bass sex ratios (Navarro-Martín et al., 2009; Piferrer et al., 2005). After 120 dpf, temperature was progressively increased at about 0.5°C /day until it reached 21°C. Finally, experiment 3 was aimed at the study of sb-vasa mRNA expression during sex differentiation (results reported in Fig. 6). Monosex populations created after
crossing sex-reversed individuals are not feasible in the European sea bass due to its polygenic system of sex determination. Therefore, male- and female-dominant groups were obtained by repeated size-grading based on the association between somatic growth and phenotypic sex, with the larger fish being usually females and the smaller being usually males (Blázquez et al., 1999; Vandeputte et al., 2007). For this experiment, the fish used were those described in detail in Papadaki et al. (2005) and Blázquez et al. (2008). These fish were reared in the facilities of the Hellenic Center for Marine Research, Heraklion, Crete, Greece. Briefly, successive gradings were performed when fish were about 2, 4, 5, and 7 months of age to finally obtain a female-dominant group (97% females) and a male-dominant group (70% males), as assessed histologically (Papadaki et al., 2005). Samplings of these fish were carried out between 50 and 300 dpf (see below for details). In all instances, fish were treated in agreement with the European regulations of animal welfare (European convention for the protection of vertebrate animals used for experimental and other scientific purposes; ETS Nº 123, 01/01/91).

2.2. RNA isolation and cDNA cloning

Gonads from adult European sea bass were used for total RNA isolation using Trizol reagent (Invitrogen Life Technologies, Paisley, Scotland, UK) following the manufacturer’s instructions. Briefly, tissues were homogenized in Trizol, isopropanol precipitated and washed in 75% ethanol. The quality and concentration of the RNA were assessed by spectrophotometry ($A_{260nm}/A_{280nm}$ ratios > 1.8) and checked on a 1% agarose/formaldehyde gel. Transcription efficiency and absence of genomic DNA contamination were checked in a PCR for 18S rRNA ($r18S$) using both the resulting cDNAs and 0.5 μg of non-reverse transcribed RNAs as templates, respectively.
Five micrograms of total RNA isolated from an adult European sea bass ovary were used for cDNA synthesis by RT-PCR using superscript-II (200 units; Invitrogen) and oligo dT-(18) primer following the manufacturer’s instructions. The resulting cDNA was used as a template to amplify a core partial clone of *sb-vasa* (382 bp) using a degenerate primer pair located within highly conserved regions of previously reported *vasa* sequences from rainbow trout, *Oncorhynchus mykiss*, tilapia, medaka and zebrafish available in the GenBank (nucleotide accession numbers AB032566, AB032467, AB063484, and AB005147, respectively). The forward primer included part of the sequence corresponding to the ATPase-A motif (*vasa*-Fwd1; 5’-ATG GCC TG(T/C) GC(T/C) CAG AC(T/C) G-3’, whereas the reverse primer was located just after the ATPase-B motif (*vasa*-Rev1; 5’-(A/G)AA (C/G)CC CAT (G/A)TC CA(A/G) CAT-3’. The PCR reaction was performed in a final volume of 50 μl containing 5 μl of 10x reaction buffer, 2 mM MgCl2, 1 ml of a 10 mM solution mix containing each deoxy (d)ATP, dCTP, dGTP, dTTP, 50 pmol of each primer and 2.5 units of Taq polymerase (Promega, Madison, Wi, USA). After an initial 5 min denaturing step at 95°C, 5 cycles of amplification were performed using a cycle profile of 95°C for 30 s, 40°C for 1 min and 72°C for 1 min. Thirty-eight cycles of amplification were subsequently performed using a profile of 95°C for 30 s, 58°C for 1 min and 72°C for 1 min. A final last cycle of elongation was extended to 10 min at 72°C. The resulting PCR product of the expected size was gel excised and isolated using the QIAquick® gel extraction kit (Qiagen, Hilden, Germany), cloned in a bacterial vector using the pGEM®-T Easy kit (Promega) and transformed in *E.coli* competent cells following the manufacturer’s instructions. White colonies were selected from X-Gal/IPTG ampicillin LB agar plates and grown in LB/ampicillin liquid media and plasmids were further purified using the QIAprep spin miniprep kit (Qiagen). The cloned fragment was sequenced using an ABI prism 377 automatic sequencer (PE Applied Biosystems; Warrington, UK) and submitted to FASTA for comparison to known sequences accessible in GenBank/EMBL. Sequence alignments revealed that the cloned fragment shared an 81% homology with *vasa* from rainbow trout,
*Oncorhynchus mykiss*. A new specific primer pair was designed based on the sequence obtained above (sense: *vasa*-Fw2; 5’- GTA AAA CGG CTG CTT TCC TG -3’; antisense: *vasa*-Rv2; 5’- TAC CGC ACC TTA CTC AAC CC -3’). When tested in a PCR reaction, the primer combination resulted in a 316 bp fragment that was sequenced to verify its authenticity and used to screen a European sea bass ovarian cDNA library.

2.3. Probe labelling and cDNA library screening

The European sea bass *vasa* cDNA clone (316 bp) isolated previously was randomly labelled with [α-32P]dCTP using Ready-to-Go™ labelling beads (-dCTP) following the manufacturer’s instructions and used to screen a European sea bass ovarian cDNA library, constructed using the ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA, USA) kindly donated by Prof. S. Zanuy (Institute of Aquaculture of Torre de la Sal, Spain). Membrane lifts were taken in duplicate to discard false positives. The membranes were denatured for 2 min in 1.5M NaCl/0.5M NaOH, neutralized for 5 min in 0.5 M Tris-HCl (pH 8.0) and finally rinsed for 1 min in 2 x SSC. Membranes were air-dried, cross-linked at 80ºC for 2 h and pre-hybridized in Rapid-Hyb buffer (Amersham, Little Chalfont, UK) at 65ºC for 30 min. The labelled probe was denatured for 5 min at 95ºC, added to the tubes containing the membranes with the Rapid-Hyb buffer and hybridized for 4 h at 65ºC. After hybridization, membranes were sequentially washed at 65ºC from 2% SSC/0.1% SDS. Specific signals were visualized on X-ray film after 2-day exposure at -80ºC with an intensifying screen. Two independent positive clones were isolated after three sequential rounds of screening, *in vivo* excised from the ZAPII vector into pBluescript SK(-) and sequenced on both strands using an ABI prism 377 automatic sequencer (Applied Biosystems).

2.4. Phylogenetic analysis

The *sb-vasa* sequence was used to search in the GenBank/EMBL for sequences that encode *vasa* orthologues in fish and other taxa. The obtained sequences were used for amino acid
comparisons and phylogenetic analyses. Amino acid sequences were aligned using the ClustalW algorithm, version 1.7 (Thompson et al., 1994) using default settings. Evolutionary distances were estimated using the Poisson correction method (Zuckerkandl and Pauling, 1965) and the final consensus phylogenetic tree was constructed with the neighbor-joining method (Saitou and Nei, 1987). The bootstrapping method (Felsenstein, 1985) was used to check for the statistical validity of the nodes generated in the tree with a total of 1000 nonparametric bootstrap replicates performed for the analysis. A member of the DEAD box protein family, the germ-line helicase (gl1) amino acid sequence of Caenorhabditis elegans was used as outgroup to root the tree. All phylogenetic analyses were carried out in MEGA version 4 (Tamura et al., 2007).

2.5. Tissue-specific expression of sb-vasa

Total RNA was isolated from testis, ovary, brain, head kidney, liver, gut, spleen, gill, heart, muscle, and visceral fat of adult European sea bass as described above. Five micrograms of total RNA were used for cDNA synthesis using Superscript II (200 units; Invitrogen) and 250 ng of random hexamers (pdN6) following the manufacturer’s instructions. Transcription efficiency and absence of DNA contamination was assessed as described above. RT-PCR was performed using specific primers for sb-vasa (sense: vasa-Fw2, antisense: vasa-Rv2). PCR conditions were as follows: 95°C for 5 min followed by 35 cycles at 95°C for 1 min, 58°C for 30 sec and 72°C for 30 sec and finally, a single elongation step at 72°C for 5 min.

2.6. Real time RT-PCR

Gene-specific primers based on the sequence reported in this study (nucleotide accession number GU987023) were designed with Primer Express (Applied Biosystems). According to zebrafish gene sequence (AJ311625), sb-vasa forward primer (vasaRT-Fw: CAG AAG CAT GGC ATT CCA ATC) was located in putative exon 19 whereas forward primer was located in putative exon 20 (vasaRT-Rv: TGC AGA ATA GGG AGC AGG AAA). The PCR reaction
yielded a product 102 bp long. Primers for 18S ribosomal RNA (r18S), used as the reference
gene, were based on a partial European sea bass sequence (AY831388). European sea bass r18S
forward primer (r18SRT-Fw: CCG CTT TGG TGA CTC TAG ATA ACC) and r18S reverse
primer (r18SRT-Rv: CAG AAA GTA CCA TCG AAA GTT GAT AGG) have been previously
used, yielding a 110 bp fragment (Blázquez et al., 2008). Assays were optimized and validated
for real-time PCR using SYBR Green as previously described (Blázquez et al., 2008). Detection
ranges covered at least three orders of magnitude. A melting curve analysis (95°C for 15 s, 60°C
for 15 s and 95°C for 15s) was performed at the end of the amplification phase to check for
primers specificity. The amplification efficiency (E) of each primer pair was calculated based on
the slope of a linear regression from a dilution series (mean threshold cycle (Ct) values plotted
against log amount input cDNA), and normalized to r18S as a reference where $E = 10^{(-1/slope)}$.
For the standard curves of both the target (sb-vasa) and the reference (r18S) genes, Ct values vs
log cDNA dilution, resulted in slopes of -3.33, E of 2.0 and linear correlations ($R^2$) between the
mean Ct and the cDNA dilution higher than 0.99. The identity of the PCR products was further
confirmed by sequencing.

Real-time PCR was performed on an ABI Prism® 7900HT sequence detection system (Applied
Biosystems) using SYBR Green I. Samples were run in triplicate in optically clear 384-well
plates in a final volume of 20 μl containing 1 μl of diluted cDNA (1:5), 10 pmol of each primer,
and 10 μl of Power SYBR® Green PCR master mix (Applied Biosystems). Cycling parameters
were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1
min. Data were collected and compiled using SDS 2.3 software (Applied Biosystems) and RQ
Manager 1.2 (Applied Biosystems) used to calculate gene expression levels. For vasa and r18S a
non-template control was included to confirm the absence of DNA contamination. In addition,
the same control sample was used in all runs to calculate the intra- and inter-assay variations. Ct
values were adjusted for differences in $E$ of each primer set using Q-gene (Müller et al., 2002).
Values were normalized (normalized expression; NE) to the reference gene following the equation \( \text{NE} = \frac{(E_{\text{ref}})^{C_{\text{ref}}}}{(E_{\text{target}})^{C_{\text{target}}}} \). Replicates were averaged and shown as mean normalized expression (MNE) ± SEM.

2.7. Ontogenic expression of sb-vasa mRNA during embryonic and larval development

Expression profiles of sb-vasa were assessed in eggs and larvae reared at 21ºC, the temperature routinely used in European sea bass cultures (Piferrer et al., 2005), during the first 120 dpf by real time PCR (experiment 1). An extra sample consisting of unfertilized eggs taken from the ovary of a spawning female was also included in the analysis. Samples (n = 3) were collected from 0 dpf (unfertilized eggs) to 14 dpf at 2-day intervals, and from 20 dpf to 30 dpf at 5-day intervals. Each sample consisted of a pool of 20 eggs/embryos (0-2 dpf), a pool of 20 larvae (4-25 dpf), and a pool of ten larvae (30 dpf).

2.8. Effects of temperature on sb-vasa mRNA during early development

To check for possible temperature-induced differences in sb-vasa mRNA levels (experiment 2), an extra group of eggs was reared at 15ºC up to 120 dpf, in parallel to that used to assess sb-vasa ontogenic expression (experiment 1) reared at 21ºC during the same period. Both groups originated from the same natural spawning and the experimental fish were therefore siblings. For this purpose, samples were taken at different developmental times that have already been described in this species (Roblin and Bruslé, 1983). These times were: 1) 30 and 44 dpf, before the proliferation of germ cells; each sample consisting on ten larvae, 2) 60 and 72 dpf, coinciding with the initiation of germ cell proliferation; each sample consisting on five larvae, and finally 3) 120 and 150 dpf, coinciding with the rapid colonization of the gonadal ridges by germ cells and the formation of the gonads; each sample consisting on one body trunk. The decrease in the number of larvae used in the different sampling points in experiments 1 and 2 was in accordance with the increase in the amount of tissue during development.
2.9. Expression of \textit{sb-vasa} during the first year of age in male-and female dominant populations

Profiles of \textit{sb-vasa} mRNA expression levels during ontogenesis were studied in gonads from male- and female-dominant populations by real time-PCR as described above (experiment 3). Tissue samples (n = 6 per group and per sampling time) were collected at 50, 100, 150, 200, 250, and 300 days post hatching (dph) for total RNA extraction (note that hatching took place at 4 dpf). In samplings ranging from 150 dph to 300 dph, each sample consisted of both gonads from each individual. Due to the difficulty to dissect gonads from fish at 50 and 100 dph, three body trunks per sample were pooled at this sampling point. Using a dissecting microscope, special care was taken to discard parts of the trunks, i.e., skin, a high amount of muscle, bones and part of the viscera located far from the putative gonads before RNA extraction. In this regard, only minimal amounts of muscle and other extragonadal tissues were included for RNA extractions; therefore the vast majority of the resulting RNA corresponded to the developing gonads. Moreover, previously we showed that the chance of underestimating gene expression when using bodies or body trunks was negligible (Blázquez et al., 2008). Samples were frozen in liquid nitrogen and kept at -80ºC until further analysis. At each sampling time, the gonads of 15-20 fish were histologically examined in order to identify phenotypic sex. Final sex ratios (n = 100 fish per group) were determined at 300 dph (Papadaki et al., 2005). It is very important to note that from 200 dph onwards, all fish used for the analysis of \textit{sb-vasa} mRNA expression from the male-dominant populations were males, and all fish from the female-dominant population were females, as determined histologically.

2.10. Statistical analysis of data

Statistical analyses of data were performed using the SPSS 15.0 package. Differences in \textit{sb-vasa} during early development (experiment 1) were analyzed by a one way analysis of variance
(ANOVA I) followed by a Tukey’s multiple range test. Differences in gene expression between fish reared at two different temperatures (experiment 2) and between male- and female-dominant groups (experiment 3) within and across ages were analyzed by a two-way analysis of variance, followed by a Tukey’s multiple range test for pairwise multiple comparisons (Sokal and Rohlf, 1997). In addition, a Student’s t-test was also used to detect differences in $sb$-vasa between the different temperatures within a specific sampling date. Prior to the analyses data were tested for normality and gene expression levels log-transformed to ensure homocedasticity of variances. In all tests, differences were accepted as statistically different when $P < 0.05$.

3. Results

3.1. Isolation and characterization of $sb$-vasa cDNA sequence

The screening of an ovarian European sea bass library resulted in the isolation of two cDNA clones of approximately 2.3 kb that proved to be identical when sequenced, containing an ORF of 1911 nucleotides flanked by noncoding regions at both ends. The 5’ untranslated terminal region (UTR) was 42 bp long and the 3’UTR was 325 bp long and contained a polyadenilation signal and the polyA tail (Fig. 1). The deduced amino acid sequence of the ORF encodes a protein 637 amino acids long with a theoretical pI of 5.16 and a calculated molecular weight of 69.121 kDa. The amino acid sequence contained eight consensus sequences for the dead protein family (Fujiwara et al., 1994; Linder et al., 1989), including the ATPase-A motif (AQTGSGKT), the ATPase-B motif (DEAD), the RNA unwinding motif (SAT) and the RNA binding motif (HRIGRTGR) (Pause and Sonenberg, 1992). About 30% of the amino acids contained between the N-terminus and position 140 were glycines. Moreover, this region also comprised seven arginine-glycine (RG) repeats and seven arginine-glycine-glycine triad repeats (RGG). In addition, the well conserved triptophan (W), glutamic acid (E) and aspartic acid (D) residues near the start and stop codons, characteristic of Vasa proteins, were also present. Moreover, the sequence EARKF at residues 296-300 in the European sea bass is found
specifically in the Vasa subfamily (Shibata et al., 1999). Comparisons of the deduced amino acid sequence with those of other full-length Vasa cloned in fish revealed that the highest homology was shared with giant gouramy, Osphronemus goramy, Pacific bluefin tuna, Thunnus orientalis, and red sea bream, Pagrus major, (86-85% similarity). Lowest similarity values were shared with grass carp, Ctenopharyngodon idella, zebrafish, Danio rerio, common carp, Cyprinus carpio, and southern catfish, Silurus meridionalis (67-68% similarity).

To support the homology of the deduced amino acid sequence, a phylogenetic analysis was performed considering other characterized Vasa proteins. A search in the Genbank resulted in the identification of full-length sequences for Vasa proteins in 66 species belonging to 6 phyla, including Nematoda, Cnidaria, Arthropoda, Echinodermata, Mollusca and Chordata, with this last phylum including 32 species from the subphylum Vertebrata and 5 species from the subphylum Tunicata. Vasa amino acid sequences were used to construct the phylogenetic tree using two different statistical packages, Phylip and Mega 4. Phylogenetic analysis based on protein distances (Fitch, Kitsch and Neighbor-Joning) resulted in trees with similar topologies. A consensus tree resulting with the Neighbor-Joining method and performed with the Mega4 program was finally adopted (Fig. 2). The tree showed eight main branches, four of them corresponding to individual phyla including Nematoda, Cnidaria, Echinodermata and Mollusca. Two branches belonged to the phylum Arthropoda, one grouping the insects and the other one the crustaceans. The remaining two branches corresponded to the phylum Chordata, represented by the tunicates, all from the class Asciidiacea, and by the vertebrates, among which teleost fish, amphibians, birds and mammals are included. These results are consistent with the idea of the universal occurrence of Vasa. The phylogenetic analysis clustered (100% bootstrap value) all Vasa protein sequences from teleost fish in a single group (Fig. 2).
3.2. Tissue-specific expression of sb-vasa

Tissue-specific expression of sb-vasa in adult European sea bass was studied by RT-PCR. sb-vasa mRNA expression was virtually restricted to the gonads with a very strong signal in ovaries and testis (Fig. 3). Expression in the nine other different tissues studied was almost non-detectable when compared to that found in gonads. The levels of 18S rRNA were used as an internal control and was found in all tissues studied at a similar intensity. In this regard, ovary and testis exhibited 1.80 and 1.41 relative expression values (sb-vasa/18S) whereas in the other tissues these values ranged between 0.16 and 0.26 (Fig. 3C).

3.3. sb-vasa levels during early development, sex differentiation and effects of temperature

The results from embryonic and larval development (experiment 1) are shown in Fig. 4. The highest levels were found in in eggs obtained by stripping from ovulated females, before insemination with sperm. Expression decreased abruptly, reaching values 75-fold lower as early as 6 dpf, and the lowest values were detected at 25-30 dpf (269-fold lower). From that stage, sb-vasa expression increased steadily (Fig. 5) from 45-72 dpf up to 150 dpf, by the time histological sex differentiation could be first detected (Papadaki et al., 2005). Briefly, the onset of sex differentiation was signaled by the appearance of the ovarian cavity and the first oocytes in females, whereas presumptive testes were still not differentiated. However, the rest of the fish could be identified as males because the morphological arrangement of the gonad was typical of that of a testis (Fig. 6). In addition, temperature had a significant effect on the expression levels of sb-vasa with high temperature resulting in higher levels than those in the group reared at low temperature (Fig. 5). This statistically significant ($P < 0.05$) up-regulation due to high temperature was first detected at 60 dpf (2-fold higher at 21°C), continued at 72 dpf (3.7-fold higher at 21°C), and persisted at 120 dpf (10.3-fold higher at 21°C), reaching the highest differences at 150 dpf (44-fold higher at 21°C), 30 days after the end of the thermal treatment. In
a closer study of the expression of *sb-vasa* during sexual differentiation (experiment 3), we took advantage of the development of two European sea bass populations based on their size, since in this species females grow larger than males (Blázquez et al., 1999; Vandeputte et al., 2007). The results showed a parallel increase of *sb-vasa* in the gonads in both the female- and the male-dominant groups during development (Fig. 7). Differential *sb-vasa* mRNA expression between groups was analyzed by a two-way analysis of variance (ANOVA-II). Significant differences between groups (*P* < 0.05) were detected already at 100 dph with values in the female-dominant group about 13-fold higher than those in the male-dominant group. The differences persisted during the period comprising the first year of age and including sex differentiation (5.1-fold, 2.7-fold 3-fold and 4.7-fold higher in females than in males at 150, 200, 250, and 300 dph, respectively).

4. Discussion

The present study reports the isolation, cloning and sequencing of a cDNA clone corresponding to European sea bass *vasa*. The predicted amino acid sequence contained the eight consensus motifs present in the DEAD box protein family (Fujiwara et al., 1994; Linder et al., 1989; Pause and Sonenberg, 1992) and other signatures found specifically in the Vasa subfamily, including an N-terminus rich in glycine and multiple RGG repeats, and the EARKF sequence (Shibata et al., 1999). The European sea bass Vasa protein shared the highest similarity with Vasa proteins from other fish (67-85%). However, it only shared 44% similarity with zebrafish pl10, 38% with a germ-line helicase (gl1) of *Caenorhabditis elegans* and 27% with human p68 homologue (data not shown), all of them members of the DEAD box protein family (Olsen et al., 1997). Together, these results further indicate that the cDNA cloned in the European sea bass is indeed a member of the *vasa* family. Moreover, the phylogenetic tree revealed that European sea bass Vasa protein clusters (100% bootstrap replicates) with Vasa proteins from other teleosts. The topology of the tree is in agreement with the universal occurrence of Vasa, showing its presence
in six different phyla, including Nematoda, Cnidaria, Arthropoda, Echinodermata, Mollusca and Chordata, all of them clustering independently and supported by bootstrap values higher than 80%. The tree showed that vertebrates are divided into three main branches including fish (teleosts), amphibians and mammals. The only bird sequence that was included in the analysis clustered with mammalian sequences, a situation that most likely will change when additional bird sequences become available for inclusion in the dataset. On the other hand, teleost fish clustered in two main branches and shared a common ancestor, in agreement with currently accepted evolutionary relationships (Nelson, 1994). One branch grouped salmonids and cyprinids, whereas the other clustered all the remaining species, including the European sea bass.

Germ-cell specific expression of *vasa*, either the mRNA and/or the protein, has been reported in several species including lower metazoans such as hydra and planaria (Mochizuki et al., 2001; Shibata et al., 1999), *Caenorhabditis* (Gruidl et al., 1996), *Drosophila* (Hay et al., 1988; Lasko and Ashburner, 1988), zebrafish (Olsen et al., 1997; Yoon et al., 1997), rainbow trout (Yoshizaki et al., 2000), tilapia (Kobayashi et al., 2000), medaka (Shinomiya et al., 2000), *Xenopus*, (Ikenishi et al., 1996; Komiya et al., 1994), chicken (Tsunekawa et al., 2000), rat (Komiya and Tanigawa, 1995), and mouse (Fujiwara et al., 1994). The present results show that *sb-vasa* is highly expressed in ovaries and testis, whereas in other tissues its expression was very weak. This is in agreement with the prominent role of this gene in germline development (Braat et al., 1999a). Nevertheless, *vasa* mRNA expression in extra-gonadal tissues, although at very low levels, has also been reported, e.g., in the adrenal and mesonephric tissues of the mouse (Zamboni and Upadhyay, 1983), in somatic cells of *Xenopus* (Ikenishi and Tanaka, 2000; Ikenishi et al., 1996), in heart, kidney, muscle and brain in adult frog, *Rana nigromaculata* (Jia et al., 2009), in the mandibular segment (Dearden, 2006) and in the fat body (Tanaka and Hartfelder, 2009) of the honeybee, and in the heart and brain of the rainbow trout (Yoshizaki et
This has been related with the possibility that the Vasa protein, through its helicase activity, might be implicated in the translational regulation of mRNAs involved in the specification and differentiation of other tissue-specific cell types (Ikenishi and Tanaka, 2000). Notwithstanding, more in-depth studies are needed to determine the functional significance of extragonadal vasa expression.

Traditionally, PGCs have been identified by morphological criteria using light and electron microscopy (Hamaguchi, 1982). The development of techniques such as in situ hybridization and PCR, have aided the study of vasa mRNA expression during different developmental stages, including early development and gametogenesis. Several studies in vertebrates and invertebrates have shown that vasa mRNA is a maternally supplied transcript (Braat et al., 1999a; Saffman and Lasko, 1999). Thus, for example, in tilapia vasa mRNA and protein were present in full-grown oocytes, ovulated eggs and embryos at the 1- and 4-cell stage (Kobayashi et al., 2000; Kobayashi et al., 2002). This maternal transfer has also been reported in zebrafish, both in oocytes (Braat et al., 1999b) and in freshly fertilized eggs (Braat et al., 1999b; Krøvel and Olsen, 2004; Yoon et al., 1997), and also in medaka (Shinomiya et al., 2000). The present study shows that sb-vasa is present in unfertilized eggs and in the developing embryo at 2 and 4 dpf, just before hatching, strongly suggesting its maternal transfer. The observation that during embryo and larval development sb-vasa mRNA levels were drastically reduced was probably because the amount of the maternally inherited vasa transcript became diluted within the total RNA content of a growing individual. A similar explanation has been offered for the decrease of vasa mRNA during vitellogenesis in tilapia (Kobayashi et al., 2000). In our study, sb-vasa levels increased again between 45-72 dpf, concomitant with the start of PGC divisions and proliferation reported previously in this species (Roblin and Bruslé, 1983). A similar pattern has been reported in zebrafish where high vasa levels during embryogenesis decreased abruptly.
during larval development and increased during gametogenesis, suggesting a switch from maternal to *de novo* *vasa* expression (Krøvel and Olsen, 2004).

High rearing temperatures during larval and post-larval stages accelerate somatic and gonadal growth in European sea bass (Ayala et al., 2001; Blázquez et al., 1998; Navarro-Martín et al., 2009). However, past a certain threshold, high temperatures can become germotoxic. Thus, in medaka high rearing temperatures inhibited the proliferation and development of germ cells in genetic females but had no effect in genetic males (Selim et al., 2009). The higher sensitivity of female gonads has also been reported in pejerrey, *Odontesthes bonariensis* (Ito et al., 2003). Our results show significantly higher *sb-vasa* values in fish reared at high temperature with respect to those of fish reared at low (i.e., “natural”) temperature. Species-specific differences aside, the differences observed in the different studies may be due to the fact that while the high temperature used in the European sea bass was still within its normal thermal range, in medaka and pejerrey they may have been close to the thermal maximum of these species. In addition, the lack of increase in *vasa* expression observed in the European sea bass reared at low temperature from 0-120 dpf was also probably influenced by the marked effect that this prolonged exposure has on gonadal growth and maturation (Blázquez et al., 1998), something that was not reported in medaka (Selim et al., 2009). Nevertheless, growth acceleration in the group reared at high temperature was already found at the start of PGC proliferation in this species by 60 dpf, persisted at 150 dpf with the onset of histological sex differentiation, and was still present at the end of the first year. High temperature also influenced gonadal growth and maturation, as confirmed by lower gonadosomatic index values in fish reared under similar conditions (Navarro-Martín et al., 2009). The high temperature-induced increase in *sb-vasa* expression could be due to (1) a direct effect at the transcriptional level, (2) an indirect effect through increased developmental rates, accelerating gonadal growth with a concomitant increase in the
number of PGCs that specifically express \textit{sb-vasa}, or (3) a combination of both. Thus, the effects of the temperature on \textit{sb-vasa} levels merits further investigation.

The study of \textit{vasa} mRNA expression levels during early ontogenesis, sex change and gonadal maturation, including oogenesis and spermatogenesis, has been reported in several fish species including zebrafish (Krøvel and Olsen, 2004), medaka (Shinomiya et al., 2000), gilthead seabream (Cardinali et al., 2004), gibel carp (Xu et al., 2005), tilapia (Kobayashi et al., 2000; Kobayashi et al., 2002), and rice field eel, \textit{Monopterus albus} (Ye et al., 2007). Two \textit{vasa} splice variants with different expression patterns at the time of sex differentiation have been found in tilapia (Kobayashi et al., 2002) and zebrafish (Krøvel and Olsen, 2004). The long isoform showed a pattern similar to that observed in the present study, with females exhibiting higher levels than males, but an accurate quantification of \textit{vasa} expression was not reported (Krøvel and Olsen, 2004). Thus, to the best of our knowledge, a complete study of \textit{vasa} expression patterns encompassing early development and sex differentiation had not been carried out to date in any fish. Furthermore, in all studies cited above Vasa protein and mRNA were localized by immunohistochemistry and \textit{in situ} hybridization or semiquantitative PCR, but expression levels were not quantified. In fact, PCR techniques were used only as a tool to assess the presence of \textit{vasa} mRNA expression. In the present study, using real time RT-PCR, it was shown that \textit{sb-vasa} levels in the female-dominant group were always higher than those found in the male-dominant group, with statistical differences starting as early as 100 dph, i.e., prior to the first observable signs of female sex differentiation (which occur at 150 dpf). These differences could not be attributed solely to sex, but also to differential growth since at this developmental stage, the female-dominant group exhibited 37.8\% higher growth (length) than that of the male-dominant group (Papadaki et al., 2005). Interestingly, at 150 and 200 dph, coinciding with the onset of histological sex differentiation, \textit{sb-vasa} levels were 5.1- and 2.7-fold higher in females than in males, respectively, reaching values up to 4.7-fold-higher at 300 dph. It should be noted
that siblings of the fish used in this experiment had higher levels of plasma levels of E$_2$ at 150 and 200 dph (Papadaki et al., 2004). This would support the observations made in gilthead sea bream, where a stimulatory effect of E$_2$ was observed (Cardinali et al., 2004). These differences in sb-vasa expression levels can be influenced by sex-related differences in size in favor of females (11.7% at 150 dph and 9.6% at 200 dph), in agreement with the reported progressive reduction in sexual growth dimorphism in European sea bass as sex differentiation proceeds (Saillant et al., 2001). However, it seems very unlikely that the small magnitude in the growth differences reported, particularly during sex differentiation, could account for these much higher levels in females. Thus, other factors should be considered. In this regard, there are two observations that may support a true influence of sex on vasa expression. Firstly, from 200 dph onwards only females from the female-dominant group and only males from the male-dominant group were used for the study. Thus, at least in the period 200-300 dph comparisons are between fish of verified phenotype (see Fig. 6). Secondly, a significant number of males started maturation before the end of the first year (precocious maturation), compared to the absence of any mature females at the same time. Taken together, these observations suggest that —body size, and maturation differences aside— sb-vasa levels are higher in females than in males.

During natural sex reversal in the rice field eel, vasa was shown to be expressed in oocytes at all stages of development, including degenerating oocytes in the ovotestis, and also in spermatogonia and spermatocytes (Ye et al., 2007). The signal in primary spermatocytes was nevertheless weaker than in spermatogonia, similarly to what has been reported in tilapia (Kobayashi et al., 2000), gibel carp (Xu et al., 2005) and zebrafish (Leal et al., 2009). In the present study, localization of the cellular origin of vasa expression by in situ hybridization was not attempted. However, in a recent study of our group carried out using laser capture microdissection combined with real time RT-PCR using the primers for vasa developed in the present study, it was found that, at least in adult European sea bass male gonads, vasa was
expressed in germ cells in all stages of spermatogenesis, with the highest levels detected in spermatogonia, followed by spermatocytes, spermatids and maintained at low levels in spermatozoa (Viñas and Piferrer, 2008).

In the present study, the first histological signs of ovarian differentiation were found at 150 dph, shown by the presence of clutches of oogonia, whereas spermatogonia could not be seen in presumptive males. At 200 dph, ovaries contained primary oocytes, but only some spermatogonia scattered among connective tissue could be seen in males. At 200 dph, testis completed differentiation showing the typical arrangement in lobules filled with spermatogonia (Fig. 6 C) and followed maturation with the presence of spermatocytes at 250 dph and spermatogonia, spermatocytes, spermatids and even sperm at 300 dph. Conversely, females did not mature and showed only primary spermatocytes during the first year of age. These observations are in agreement with the results showing that significantly higher sb-vasa levels were found consistently in females, and increased during development, whereas sb-vasa levels in males did not increase further during progression of spermatogenesis and testicular maturation.

In conclusion, the present study reports the full-length sequence of a vasa homolog in the European sea bass (sb-vasa), a species with temperature influences on sex differentiation. European sea bass vasa is mainly expressed in gonads although some expression in extra-gonadal tissues cannot be neglected. This suggests the possibility of other functions related to its helicase activity such as the translational regulation of mRNAs in other tissue types. In this study the evolutionary relationships of Vasa amino acid sequences belonging to six different phyla have been also established. Further, the obtained results add evidence to the importance of vasa in gonadal development in fish and suggest a role of this gene in the process of sexual differentiation and maturation. In this regard, significant differences in sb-vasa expression were
detected not only prior to histological sex differentiation, with higher levels in females than in males, but particularly during sex differentiation and testicular maturation. Whether this reflects possible constitutive differences in vasa expression between female and male germ cells deserves further study.

Acknowledgments

The authors wish to thank Sílvia Joly for technical assistance and the staff at the Experimental Aquarium and Chamber Facility (ZAE) for rearing European sea bass exposed to different temperatures. This work was supported by the EU grant PROBASS (Q5RS-2000-31365) to F.P and C.C.M. Research at the lab of F.P. is partially funded by project Consolider “Aquagenomics” CDS2007-0002. M. Blázquez was supported by a postdoctoral contract from the EU and a Ramón y Cajal contract from the Spanish Ministry of Science and Technology.

REFERENCES


**Figure captions**

**Fig. 1.** Nucleotide and deduced amino acid sequence of *sb-vasa* in the European sea bass (*Dicentrarchus labrax*). Shaded boxes correspond to the eight conserved regions of the DEAD-box protein family. Arginine-glycine (RG) repeats and arginine-glycine-glycine (RGG) repeats in the N-terminal region are underlined and double underlined, respectively. Acidic amino acid residues (aspartic acid; D and glutamic acid; E) and tryptophan (W) in the N-terminal and C-terminal regions are shown inside circles. Numbers indicate the nucleotide position starting at the A of the initial ATG codon (top line) and the amino acid positions starting at the initial methionine (M, bottom line). An asterisk indicates the stop codon (TAG). The polyadenylation signal and the poly-A tail are marked in boldface. The present *sb-vasa* nucleotide sequence has been deposited in the GenBank under accession number **GU987023**.

**Fig. 2.** Phylogenetic tree of Vasa proteins. Scientific name of the species used to generate the tree are given at the right of the branches and GenBank protein ID numbers appear between parentheses. The tree was constructed using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates represents the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 80% bootstrap replicates are collapsed. Bootstrap values (percent) are shown next to the branches. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated only in pairwise sequence comparisons with a total of 1208 positions in the final dataset. The tree was rooted with the germ-line helicase gl1 from *Caenorhabditis elegans*. Phylogenetic analyses were conducted in MEGA version 4.

**Fig. 3.** Tissue-specific expression of *sb-vasa* in adult European sea bass. Tissues include testis (T), ovary (O), male brain (B_m), female brain (B_f), head kidney (K), liver (L), gut (G), spleen...
(S), gill (G), heart (H), muscle (M), and visceral fat (F). Five micrograms of total RNA from each tissue were reverse transcribed to cDNA. After DNase treatment, samples were amplified by PCR using a specific primer set for sb-vasa yielding a 316 bp fragment (panel A). A 485 bp fragment of 18S ribosomal RNA (r18S) was used as an internal control to check for the integrity of the cDNA template (panel B). A negative control (-) using sterile water as template was included in the PCR analysis to check for possible contamination and thus discard false positives. Panel C plots a graph with the relative values quantified by densitometry in the tissue-specific expression study. Values of sb-vasa gene expression in each tissue were referenced to values of 18S rRNA expression in the same tissue.

**Fig. 4.** European sea bass *vasa* gene expression during early developmental stages prior to the formation of the gonadal ridges and the onset of sex differentiation. Samples were analyzed by real-time fluorescence PCR (see materials and methods for further details). Data are presented as the mean normalized gene expression (MNE) levels ± the standard error of the mean (SEM) of three samples performed in triplicate. Each sample consisted of a pool of 20 eggs/embryos (0-2 days post fertilization, dpf), a pool of 20 larvae (4-25 dpf), and a pool of ten larvae (30 dpf). Values were normalized against the levels of 18S rRNA amplified from the same reverse transcribed template and plotted in logarithmic scale. Different letters show statistical differences (*P* < 0.05) between the different sampling dates after a Tukey’s test.

**Fig. 5.** Effects of the rearing temperature on *sb-vasa* gene expression during early developmental stages of European sea bass, prior to the onset of sex differentiation. Samples were analyzed by real-time fluorescence PCR (see materials and methods for further details). Data are presented as the mean normalized gene expression (MNE) levels ± the standard error of the mean (SEM) of three samples performed in triplicate. Each sample consisted of a pool of ten larvae (30-45 dpf), a pool of five body trunks (60-72 dpf) and one body trunk at 150 dpf. Values
were normalized against the levels of 18S rRNA amplified from the same reverse transcribed template and plotted in logarithmic scale. Different letters show statistical differences ($P < 0.05$) between the different sampling dates after a Tukey’s test. Asterisks indicate statistical differences between temperatures ($* = P < 0.05$; $** = P < 0.001$) at each sampling time after a Student’s $t$-test.

**Fig. 6.** Photomicrographs of European sea bass gonads during sex differentiation. (A) testis of an undifferentiated presumptive male at 150 dph. The gonadal artery (ar) and the gonadal vein (v) can be seen. (B) ovary of an early differentiating female at 150 dph. Arrowheads indicate the presence of oocytes and the asterisk the ovarian cavity. (C) testis of a differentiated immature male at 200 dph. The testis is arranged in testicular lobules containing spermatogonia (sg). (D) ovary of a differentiated immature female at 200 dph containing oocytes at the perinucleolar stage arranged within the ovarian lamellae. (E) testis of a mature spermiating male at 300 dph containing, spermatocytes (sc), spermatids (st) and spermatozoa (sz). (F) ovary of a differentiated immature female at 300 dph containing oocytes at the perinucleolar stage arranged within the ovarian lamellae. In all photomicrographs, the bar equals 10 μm.

**Fig. 7.** European sea bass -*vasa* gene expression during the first year of life. Samples were analyzed by real-time fluorescence PCR. The study was performed in samples from male- and female-dominant groups at different times during the first year of development (50-300 days post hatching; dph). Values were plotted in logarithmic scale and expressed as the mean normalized expression (MNE) of *sb-vasa* mRNA levels against the levels of 18S rRNA ± the standard error of the mean (SEM) of six samples performed in triplicate. Different letters show statistical differences between groups ($P < 0.05$) after a Tukey’s test. At 200, 250, and 300 dph (right to the dashed line), all fish from the male-dominant group used for the analysis were
males and all fish from the female-dominant group were females. Grey boxes at the bottom represent different key developmental processes during European sea bass gonadogenesis.
Figure 2. Blázquez et al. 2010.
Figure 3. Blázquez et al 2010
Figure 4. Blázquez et al 2010
Figure 5. Blázquez et al., 2010
Figure 6. Blázquez et al 2010
Figure 7. Blázquez et al 2010

Figure 7: Blázquez et al 2010

- **Female-dominant group**
- **Male-dominant group**

- Rapid proliferation of PGCs
- Female sex differentiation
- Male sex differentiation

Age (dph):

- 0
- 50
- 100
- 150
- 200
- 250
- 300

 logarithmic scale sh-vasa MNE