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

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

22

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

24

25 **ABSTRACT**

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

47

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

73 changed from asymmetric to symmetric, a maternally programmed process that precedes PGC
74 proliferation and perinuclear localization of Vasa protein (Knaut et al., 2000).
75
76 Studies of *vasa* mRNA expression and protein localization in fish during embryonic
77 development include those in zebrafish (Yoon et al., 1997), goldfish, *Carassius auratus* (Otani
78 et al., 2002), ukigori, *Gymnogobius spp*. (Saito et al., 2004), shiro-uo, *Leucopsarion petersii*
79 (Miyake et al., 2006) and medaka, *Oryzias latipes* (Herpin et al., 2007). In medaka, *vasa*
80 transcripts were detected exclusively in the cytoplasm of germ cells of both sexes but not in
81 gonadal somatic cells (Shinomiya et al., 2000). *Vasa* has been reported to be differentially
82 expressed during sex differentiation in tilapia, *Oreochromis niloticus* (Kobayashi et al., 2002),
83 and zebrafish (Krøvel and Olsen, 2004; Wang et al., 2007). In addition, sex-related differences
84 during gametogenesis have been shown in several fish species, including tilapia (Kobayashi et
85 al., 2000) and gibel carp, *Carassius auratus gibelio* (Xu et al., 2005). Moreover, the hormonal
86 regulation of *vasa* mRNA expression during oogenesis in gilthead sea bream, *Sparus aurata*,
87 has also been studied (Cardinali et al., 2004). These authors found that growth hormone (GH),
88 estradiol-17β (E_2) and the combination of gonadotropin-releasing hormone (GnRH) with GH
89 were able to induce an increase in *vasa* mRNA expression. However, GnRH or GH alone
90 decreased *vasa* mRNA, indicating the existence of an interplay between these hormones and
91 Vasa during oogenesis.
92
93 In mammals, the role of the germ line in sex differentiation is far from clear (Brennan and
94 Capel, 2004). Germ cells are not essential for testicular differentiation although they participate
95 in several aspects of ovarian differentiation, including folliculogenesis (Choi and Rajkovic,
96 2006). Similarly, in zebrafish the germ line is essential for ovarian differentiation (Siegfried and
97 Nüsslein-Volhard, 2008), since morpholino-silenced expression of *dnd*, a gene essential for
98 germ line survival (Weidinger et al., 2003), resulted in fish lacking *vasa* expression and

99 exhibiting testicular development and typical male mating behavior. Immunocytochemical
100 studies with zebrafish showed that Vasa protein was present at high levels in undifferentiated
101 spermatogonia decreasing as spermatogenesis progressed until they became absent in spermatids
102 and spermatozoa (Leal et al., 2009).

103

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

114

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

124 mRNA and expression patterns during larval development and sex differentiation were also
125 studied and quantified by real time RT-PCR.
126

127 **2. Materials and methods**

128 ***2.1. Fish and rearing conditions***

129 Adult European sea bass used for cloning and tissue distribution studies were obtained from the
130 facilities of the Barcelona Aquarium during the spawning season (December-March). Fish were
131 anesthetized with MS-222 (Sigma, St. Louis, MO, USA) and sacrificed after decapitation.
132 Tissues were removed rapidly under sterile conditions, snap frozen in liquid nitrogen and kept at
133 -80ºC until further analysis.
134

135 Experiment 1 was designed to study *sb-vasa* mRNA expression during early development
136 (results reported in Fig. 4). Fertilized European sea bass eggs obtained from a commercial
137 hatchery (Base Viva; St. Pere Pescador, Girona Spain) were transported immediately and reared
138 at the Experimental Aquarium and Chamber Facility (ZAE) at the Institute of Marine Sciences
139 (Barcelona, Spain). Fish were reared following standard procedures (Moretti et al., 1999) up to
140 120 days post fertilization (dpf) at 21 ± 1ºC, the temperature used routinely for European sea
141 bass culture (Navarro-Martín et al., 2009; Piferrer et al., 2005). Experiment 2 was designed to
142 assess the effects of the rearing temperature on *sb-vasa* mRNA expression from 30 to 150 dpf,
143 i.e., the period encompassing gonadal ridge formation up to the beginning of sex differentiation
144 (results reported in Fig. 5). For this purpose, another group of fish from the same genetic origin
145 as those of experiment 1 was reared in parallel at 15 ± 1ºC from 0-120, dpf, a temperature that
146 does not induce distorting effects on European sea bass sex ratios (Navarro-Martín et al., 2009;
147 Piferrer et al., 2005). After 120 dpf, temperature was progressively increased at about 0.5ºC /day
148 until it reached 21ºC. Finally, experiment 3 was aimed at the study of *sb-vasa* mRNA expression
149 during sex differentiation (results reported in Fig. 6). Monosex populations created after

150 crossing sex-reversed individuals are not feasible in the European sea bass due to its polygenic
151 system of sex determination. Therefore, male- and female-dominant groups were obtained by
152 repeated size-grading based on the association between somatic growth and phenotypic sex, with
153 the larger fish being usually females and the smaller being usually males (Blázquez et al., 1999;
154 Vandeputte et al., 2007). For this experiment, the fish used were those described in detail in
155 Papadaki et al. (2005) and Blázquez et al. (2008). These fish were reared in the facilities of the
156 Hellenic Center for Marine Research, Heraklion, Crete, Greece. Briefly, successive gradings
157 were performed when fish were about 2, 4, 5, and 7 months of age to finally obtain a female-
158 dominant group (97% females) and a male-dominant group (70% males), as assessed
159 histologically (Papadaki et al., 2005). Samplings of these fish were carried out between 50 and
160 300 dpf (see below for details). In all instances, fish were treated in agreement with the
161 European regulations of animal welfare (European convention for the protection of vertebrate
162 animals used for experimental and other scientific purposes; ETS Nº 123, 01/01/91).
163
164
165 ***2.2. RNA isolation and cDNA cloning***
166 Gonads from adult European sea bass were used for total RNA isolation using Trizol reagent
167 (Invitrogen Life Technologies, Paisley, Scotland, UK) following the manufacturer's
168 instructions. Briefly, tissues were homogenized in Trizol, isopropanol precipitated and washed
169 in 75% ethanol. The quality and concentration of the RNA were assessed by spectrophotometry
170 (A_{260nm}/A_{280nm} ratios > 1.8) and checked on a 1% agarose/formaldehyde gel. Transcription
171 efficiency and absence of genomic DNA contamination were checked in a PCR for *18S* rRNA
172 (*r18S*) using both the resulting cDNAs and 0.5 μg of non-reverse transcribed RNAs as
173 templates, respectively.
174

175 Five micrograms of total RNA isolated from an adult European sea bass ovary were used for
176 cDNA synthesis by RT-PCR using superscript-II (200 units; Invitrogen) and oligo dT-(18)
177 primer following the manufacturer´s instructions. The resulting cDNA was used as a template to
178 amplify a core partial clone of *sb-vasa* (382 bp) using a degenerate primer pair located within
179 highly conserved regions of previously reported *vasa* sequences from rainbow trout,
180 *Oncorhynchus mykiss*, tilapia, medaka and zebrafish available in the GenBank (nucleotide
181 accession numbers AB032566, AB032467, AB063484, and AB005147, respectively). The
182 forward primer included part of the sequence corresponding to the ATPase-A motif (*vasa*-Fwd1;
183 5'-ATG GCC TG(T/C) GC(T/C) CAG AC(T/C) G-3', whereas the reverse primer was located
184 just after the ATPase-B motif (*vasa*-Rev1; 5'-(A/G)AA (C/G)CC CAT (G/A)TC CA(A/G)
185 CAT-3'. The PCR reaction was performed in a final volume of 50 μl containing 5 μl of 10x
186 reaction buffer, 2 mM MgCl2, 1 ml of a 10 mM solution mix containing each deoxy (d)ATP,
187 dCTP, dGTP, dTTP, 50 pmol of each primer and 2.5 units of Taq polymerase (Promega,
188 Madison, Wi, USA). After an initial 5 min denaturing step at 95ºC, 5 cycles of amplification
189 were performed using a cycle profile of 95ºC for 30 s, 40ºC for 1 min and 72ºC for 1 min.
190 Thirty-eight cycles of amplification were subsequently performed using a profile of 95ºC for 30
191 s, 58ºC for 1 min and 72ºC for 1 min. A final last cycle of elongation was extended to 10 min at
192 72ºC. The resulting PCR product of the expected size was gel excised and isolated using the
193 QIAquick® gel extraction kit (Qiagen, Hilden, Germany), cloned in a bacterial vector using the
194 pGEM®-T Easy kit (Promega) and transformed in *E.coli* competent cells following the
195 manufacturer's instructions. White colonies were selected from X-Gal/IPTG ampicillin LB agar
196 plates and grown in LB/ampicillin liquid media and plasmids were further purified using the
197 QIAprep spin miniprep kit (Qiagen). The cloned fragment was sequenced using an ABI prism
198 377 automatic sequencer (PE Applied Biosystems; Warrington, UK) and submitted to FASTA
199 for comparison to known sequences accessible in GenBank/EMBL. Sequence alignments
200 revealed that the cloned fragment shared an 81% homology with *vasa* from rainbow trout,

201 *Oncorhynchus mykiss*. A new specific primer pair was designed based on the sequence obtained
202 above (sense: *vasa*-Fw2; 5'- GTA AAA CGG CTG CTT TCC TG -3'; antisense: *vasa*-Rv2; 5'-
203 TAC CGC ACC TTA CTC AAC CC -3'). When tested in a PCR reaction, the primer
204 combination resulted in a 316 bp fragment that was sequenced to verify its authenticity and used
205 to screen a European sea bass ovarian cDNA library.
206

207 ***2.3. Probe labelling and cDNA library screening***

208 The European sea bass *vasa* cDNA clone (316 bp) isolated previously was randomly labelled
209 with [α^{-32}P]dCTP using Ready-to-Go^{TM} labelling beads (-dCTP) following the manufacturer's
210 instructions and used to screen a European sea bass ovarian cDNA library, constructed using the
211 ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA, USA) kindly donated by Prof. S. Zanuy
212 (Institute of Aquaculture of Torre de la Sal, Spain). Membrane lifts were taken in duplicate to
213 discard false positives. The membranes were denatured for 2 min in 1.5M NaCl/0.5M NaOH,
214 neutralized for 5 min in 0.5 M Tris-HCl (pH 8.0) and finally rinsed for 1 min in 2 x SSC.
215 Membranes were air-dried, cross-linked at 80ºC for 2 h and pre-hybridized in Rapid-Hyb buffer
216 (Amersham, Little Chalfont, UK) at 65ºC for 30 min. The labelled probe was denatured for 5
217 min at 95ºC, added to the tubes containing the membranes with the Rapid-Hyb buffer and
218 hybridized for 4 h at 65ºC. After hybridization, membranes were sequentially washed at 65ºC
219 from 2% SSC/0.1% SDS. Specific signals were visualized on X-ray film after 2-day exposure at
220 -80ºC with an intensifying screen. Two independent positive clones were isolated after three
221 sequential rounds of screening, *in vivo* excised from the ZAPII vector into pBluescript SK(-) and
222 sequenced on both strands using an ABI prism 377 automatic sequencer (Applied Biosystems).
223

224 ***2.4. Phylogenetic analysis***

225 The *sb-vasa* sequence was used to search in the GenBank/EMBL for sequences that encode *vasa*
226 orthologues in fish and other taxa. The obtained sequences were used for amino acid

227 comparisons and phylogenetic analyses. Amino acid sequences were aligned using the ClustalW
228 algorithm, version 1.7 (Thompson et al., 1994) using default settings. Evolutionary distances
229 were estimated using the Poisson correction method (Zuckerkandl and Pauling, 1965) and the
230 final consensus phylogenetic tree was constructed with the neighbor-joining method (Saitou and
231 Nei, 1987). The bootstrapping method (Felsenstein, 1985) was used to check for the statistical
232 validity of the nodes generated in the tree with a total of 1000 nonparametric bootstrap replicates
233 performed for the analysis. A member of the DEAD box protein family, the germ-line helicase
234 (gl1) amino acid sequence of *Caenorhabditis elegans* was used as outgroup to root the tree. All
235 phylogenetic analyses were carried out in MEGA version 4 (Tamura et al., 2007).
236
237 ***2.5. Tissue-specific expression of sb-vasa***
238 Total RNA was isolated from testis, ovary, brain, head kidney, liver, gut, spleen, gill, heart,
239 muscle, and visceral fat of adult European sea bass as described above. Five micrograms of total
240 RNA were used for cDNA synthesis using Superscript II (200 units; Invitrogen) and 250 ng of
241 random hexamers (pdN6) following the manufacturer's instructions. Transcription efficiency
242 and absence of DNA contamination was assessed as described above. RT-PCR was performed
243 using specific primers for *sb-vasa* (sense: *vasa*-Fw2, antisense: *vasa*-Rv2). PCR conditions were
244 as follows: 95ºC for 5 min followed by 35 cycles at 95ºC for 1 min, 58ºC for 30 sec and 72ºC
245 for 30 sec and finally, a single elongation step at 72ºC for 5 min.
246
247 ***2.6. Real time RT-PCR***
248 Gene-specific primers based on the sequence reported in this study (nucleotide accession
249 number **GU987023**) were designed with Primer Express (Applied Biosystems). According to
250 zebrafish gene sequence (AJ311625), *sb-vasa* forward primer (*vasa*RT-Fw: CAG AAG CAT
251 GGC ATT CCA ATC) was located in putative exon 19 whereas forward primer was located in
252 putative exon 20 (*vasa*RT-Rv: TGC AGA ATA GGG AGC AGG AAA). The PCR reaction

253 yielded a product 102 bp long. Primers for 18S ribosomal RNA (*r18S*), used as the reference
254 gene, were based on a partial European sea bass sequence (AY831388). European sea bass *r18S*
255 forward primer (*r18S*RT-Fw: CCG CTT TGG TGA CTC TAG ATA ACC) and *r18S* reverse
256 primer (*r18S*RT-Rv: CAG AAA GTA CCA TCG AAA GTT GAT AGG) have been previously
257 used, yielding a 110 bp fragment (Blázquez et al., 2008). Assays were optimized and validated
258 for real-time PCR using SYBR Green as previously described (Blázquez et al., 2008). Detection
259 ranges covered at least three orders of magnitude. A melting curve analysis (95ºC for 15s, 60ºC
260 for 15 s and 95ºC for 15s) was performed at the end of the amplification phase to check for
261 primers specificity. The amplification efficiency (*E*) of each primer pair was calculated based on
262 the slope of a linear regression from a dilution series (mean threshold cycle (Ct) values plotted
263 against log amount input cDNA), and normalized to *r18S* as a reference where $E = 10^{(-1/\text{slope})}$.
264 For the standard curves of both the target (*sb-vasa*) and the reference (*r18S*) genes, Ct values *vs*
265 log cDNA dilution, resulted in slopes of -3.33, *E* of 2.0 and linear correlations (R^2) between the
266 mean Ct and the cDNA dilution higher than 0.99. The identity of the PCR products was further
267 confirmed by sequencing.

268

269 Real-time PCR was performed on an ABI Prism® 7900HT sequence detection system (Applied
270 Biosystems) using SYBR Green I. Samples were run in triplicate in optically clear 384-well
271 plates in a final volume of 20 µl containing 1 µl of diluted cDNA (1:5), 10 pmol of each primer,
272 and 10 µl of Power SYBR® Green PCR master mix (Applied Biosystems). Cycling parameters
273 were 50ºC for 2 min, 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 s and 60ºC for 1
274 min. Data were collected and compiled using SDS 2.3 software (Applied Biosystems) and RQ
275 Manager 1.2 (Applied Biosystems) used to calculate gene expression levels. For *vasa* and *r18S* a
276 non-template control was included to confirm the absence of DNA contamination. In addition,
277 the same control sample was used in all runs to calculate the intra- and inter-assay variations. Ct
278 values were adjusted for differences in *E* of each primer set using Q-gene (Müller et al., 2002).

279 Values were normalized (normalized expression; NE) to the reference gene following the
280 equation NE = $(E_{\mathrm{ref}})^{\mathrm{Ctref}}/(E_{\mathrm{target}})^{\mathrm{Cttarget}}$. Replicates were averaged and shown as mean normalized
281 expression (MNE) ± SEM.
282

283 ***2.7. Ontogenic expression of sb-vasa mRNA during embryonic and larval development***

284 Expression profiles of *sb-vasa* were assessed in eggs and larvae reared at 21ºC, the temperature
285 routinely used in European sea bass cultures (Piferrer et al., 2005), during the first 120 dpf by
286 real time PCR (experiment 1). An extra sample consisting of unfertilized eggs taken from the
287 ovary of a spawning female was also included in the analysis. Samples (n =3) were collected
288 from 0 dpf (unfertilized eggs) to 14 dpf at 2-day intervals, and from 20 dpf to 30 dpf at 5-day
289 intervals. Each sample consisted of a pool of 20 eggs/embryos (0-2 dpf), a pool of 20 larvae (4-
290 25 dpf), and a pool of ten larvae (30 dpf).
291

292 ***2.8. Effects of temperature on sb-vasa mRNA during early development***

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

305

306 ***2.9. Expression of sb-vasa during the first year of age in male-and female dominant***
307 ***populations***

308 Profiles of *sb-vasa* mRNA expression levels during ontogenesis were studied in gonads from
309 male- and female-dominant populations by real time-PCR as described above (experiment 3).
310 Tissue samples (n = 6 per group and per sampling time) were collected at 50, 100, 150, 200,
311 250, and 300 days post hatching (dph) for total RNA extraction (note that hatching took place at
312 4 dpf). In samplings ranging from 150 dph to 300 dph, each sample consisted of both gonads
313 from each individual. Due to the difficulty to dissect gonads from fish at 50 and 100 dph, three
314 body trunks per sample were pooled at this sampling point. Using a dissecting microscope,
315 special care was taken to discard parts of the trunks, i.e., skin, a high amount of muscle, bones
316 and part of the viscera located far from the putative gonads before RNA extraction. In this
317 regard, only minimal amounts of muscle and other extragonadal tissues were included for RNA
318 extractions; therefore the vast majority of the resulting RNA corresponded to the developing
319 gonads. Moreover, previously we showed that the chance of underestimating gene expression
320 when using bodies or body trunks was negligible (Blázquez et al., 2008). Samples were frozen in
321 liquid nitrogen and kept at -80ºC until further analysis. At each sampling time, the gonads of 15-
322 20 fish were histologically examined in order to identify phenotypic sex. Final sex ratios (n =
323 100 fish per group) were determined at 300 dph (Papadaki et al., 2005). It is very important to
324 note that from 200 dph onwards, all fish used for the analysis of *sb-vasa* mRNA expression
325 from the male-dominant populations were males, and all fish from the female-dominant
326 population were females, as determined histologically.

327

328 ***2. 10. Statistical analysis of data***

329 Statistical analyses of data were performed using the SPSS 15.0 package. Differences in *sb-vasa*
330 during early development (experiment 1) were analyzed by a one way analysis of variance

331 (ANOVA I) followed by a Tukey's multiple range test. Differences in gene expression between
332 fish reared at two different temperatures (experiment 2) and between male- and female-
333 dominant groups (experiment 3) within and across ages were analyzed by a two-way analysis of
334 variance, followed by a Tukey's multiple range test for pairwise multiple comparisons (Sokal
335 and Rohlf, 1997). In addition, a Student's *t*-test was also used to detect differences in *sb-vasa*
336 between the different temperatures within a specific sampling date. Prior to the analyses data
337 were tested for normality and gene expression levels log-transformed to ensure homocedasticity
338 of variances. In all tests, differences were accepted as statistically different when $P < 0.05$.
339

340 **3. Results**

341 ***3.1. Isolation and characterization of sb- vasa cDNA sequence***

342 The screening of an ovarian European sea bass library resulted in the isolation of two cDNA
343 clones of approximately 2.3 kb that proved to be identical when sequenced, containing an ORF
344 of 1911 nucleotides flanked by noncoding regions at both ends. The 5' untranslated terminal
345 region (UTR) was 42 bp long and the 3'UTR was 325 bp long and contained a polyadenilation
346 signal and the polyA tail (Fig. 1). The deduced amino acid sequence of the ORF encodes a
347 protein 637 amino acids long with a theoretical pI of 5.16 and a calculated molecular weight of
348 69.121 kDa. The amino acid sequence contained eight consensus sequences for the dead protein
349 family (Fujiwara et al., 1994; Linder et al., 1989), including the ATPase-A motif
350 (AQTGSGKT), the ATPase-B motif (DEAD), the RNA unwinding motif (SAT) and the RNA
351 binding motif (HRIGRTGR) (Pause and Sonenberg, 1992). About 30% of the amino acids
352 contained between the N-terminus and position 140 were glycines. Moreover, this region also
353 comprised seven arginine-glycine (RG) repeats and seven arginine-glycine-glycine triad repeats
354 (RGG). In addition, the well conserved triptophan (W), glutamic acid (E) and aspartic acid (D)
355 residues near the start and stop codons, characteristic of Vasa proteins, were also present.
356 Moreover, the sequence EARKF at residues 296-300 in the European sea bass is found

357 specifically in the Vasa subfamily (Shibata et al., 1999). Comparisons of the deduced amino
358 acid sequence with those of other full-length Vasa cloned in fish revealed that the highest
359 homology was shared with giant gouramy, *Osphronemus goramy*, Pacific bluefin tuna, *Thunnus*
360 *orientalis*, and red sea bream, *Pagrus major*, (86-85% similarity). Lowest similarity values were
361 shared with grass carp, *Ctenopharyngodon idella*, zebrafish, *Danio rerio*, common carp,
362 *Cyprinus carpio,* and southern catfish, *Silurus meridionalis* (67-68% similarity).
363

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

381

382 ***3.2. Tissue-specific expression of sb-vasa***

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

390

391 ***3.3. sb-vasa levels during early development, sex differentiation and effects of temperature***

392 The results from embryonic and larval development (experiment 1) are shown in Fig. 4. The
393 highest levels were found in in eggs obtained by stripping from ovulated females, before
394 insemination with sperm. Expression decreased abruptly, reaching values 75-fold lower as early
395 as 6 dpf, and the lowest values were detected at 25-30 dpf (269-fold lower). From that stage, *sb-*
396 *vasa* expression increased steadily (Fig. 5) from 45-72 dpf up to 150 dpf, by the time
397 histological sex differentiation could be first detected (Papadaki et al., 2005). Briefly, the onset
398 of sex differentiation was signaled by the appearance of the ovarian cavity and the first oocytes
399 in females, whereas presumptive testes were still not differentiated. However, the rest of the fish
400 could be identified as males because the morphological arrangement of the gonad was typical of
401 that of a testis (Fig. 6). In addition, temperature had a significant effect on the expression levels
402 of *sb-vasa* with high temperature resulting in higher levels than those in the group reared at low
403 temperature (Fig. 5). This statistically significant ($P < 0.05$) up-regulation due to high
404 temperature was first detected at 60 dpf (2-fold higher at 21ºC), continued at 72 dpf (3.7-fold
405 higher at 21ºC), and persisted at 120 dpf (10.3-fold higher at 21ºC), reaching the highest
406 differences at 150 dpf (44-fold higher at 21ºC), 30 days after the end of the thermal treatment. In

407 a closer study of the expression of *sb-vasa* during sexual differentiation (experiment 3), we took
408 advantage of the development of two European sea bass populations based on their size, since in
409 this species females grow larger than males (Blázquez et al., 1999; Vandeputte et al., 2007). The
410 results showed a parallel increase of *sb-vasa* in the gonads in both the female- and the male-
411 dominant groups during development (Fig. 7). Differential *sb-vasa* mRNA expression between
412 groups was analyzed by a two-way analysis of variance (ANOVA-II). Significant differences
413 between groups ($P < 0.05$) were detected already at 100 dph with values in the female-dominant
414 group about 13-fold higher than those in the male-dominant group. The differences persisted
415 during the period comprising the first year of age and including sex differentiation (5.1-fold,
416 2.7-fold 3-fold and 4.7-fold higher in females than in males at 150, 200, 250, and 300 dph,
417 respectively).
418

419 **4. Discussion**

420 The present study reports the isolation, cloning and sequencing of a cDNA clone corresponding
421 to European sea bass *vasa*. The predicted amino acid sequence contained the eight consensus
422 motifs present in the DEAD box protein family (Fujiwara et al., 1994; Linder et al., 1989; Pause
423 and Sonenberg, 1992) and other signatures found specifically in the Vasa subfamily, including
424 an N-terminus rich in glycine and multiple RGG repeats, and the EARKF sequence (Shibata et
425 al., 1999). The European sea bass Vasa protein shared the highest similarity with Vasa proteins
426 from other fish (67-85%). However, it only shared 44% similarity with zebrafish pl10, 38% with
427 a germ-line helicase (gl1) of *Caenorhabditis elegans* and 27% with human p68 homologue (data
428 not shown), all of them members of the DEAD box protein family (Olsen et al., 1997).
429 Together, these results further indicate that the cDNA cloned in the European sea bass is indeed
430 a member of the *vasa* family. Moreover, the phylogenetic tree revealed that European sea bass
431 Vasa protein clusters (100% bootstrap replicates) with Vasa proteins from other teleosts. The
432 topology of the tree is in agreement with the universal occurrence of Vasa, showing its presence

433 in six different phyla, including Nematoda, Cnidaria, Arthropoda, Echinodermata, Mollusca and
434 Chordata, all of them clustering independently and supported by bootstrap values higher than
435 80%. The tree showed that vertebrates are divided into three main branches including fish
436 (teleosts), amphibians and mammals. The only bird sequence that was included in the analysis
437 clustered with mammalian sequences, a situation that most likely will change when additional
438 bird sequences become available for inclusion in the dataset. On the other hand, teleost fish
439 clustered in two main branches and shared a common ancestor, in agreement with currently
440 accepted evolutionary relationships (Nelson, 1994). One branch grouped salmonids and
441 cyprinids, whereas the other clustered all the remaining species, including the European sea
442 bass.

443

444 Germ-cell specific expression of *vasa*, either the mRNA and/or the protein, has been reported in
445 several species including lower metazoans such as hydra and planaria (Mochizuki et al., 2001;
446 Shibata et al., 1999), *Caenorhabditis* (Gruidl et al., 1996), *Drosophila* (Hay et al., 1988; Lasko
447 and Ashburner, 1988), zebrafish (Olsen et al., 1997; Yoon et al., 1997), rainbow trout
448 (Yoshizaki et al., 2000), tilapia (Kobayashi et al., 2000), medaka (Shinomiya et al., 2000),
449 Xenopus, (Ikenishi et al., 1996; Komiya et al., 1994), chicken (Tsunekawa et al., 2000), rat
450 (Komiya and Tanigawa, 1995), and mouse (Fujiwara et al., 1994). The present results show that
451 *sb-vasa* is highly expressed in ovaries and testis, whereas in other tissues its expression was very
452 weak. This is in agreement with the prominent role of this gene in germline development (Braat
453 et al., 1999a). Nevertheless, *vasa* mRNA expression in extra-gonadal tissues, although at very
454 low levels, has also been reported, e.g., in the adrenal and mesonephric tissues of the mouse
455 (Zamboni and Upadhyay, 1983), in somatic cells of Xenopus*s* (Ikenishi and Tanaka, 2000;
456 Ikenishi et al., 1996), in heart, kidney, muscle and brain in adult frog, *Rana nigromaculata* (Jia
457 et al., 2009), in the mandibular segment (Dearden, 2006) and in the fat body (Tanaka and
458 Hartfelder, 2009) of the honeybee, and in the heart and brain of the rainbow trout (Yoshizaki et

459 al., 2000). This has been related with the possibility that the Vasa protein, through its helicase
460 activity, might be implicated in the translational regulation of mRNAs involved in the
461 specification and differentiation of other tissue-specific cell types (Ikenishi and Tanaka, 2000).
462 Notwithstanding, more in-depth studies are needed to determine the functional significance of
463 extragonadal *vasa* expression.

464

465 Traditionally, PGCs have been identified by morphological criteria using light and electron
466 microscopy (Hamaguchi, 1982). The development of techniques such as *in situ* hybridization
467 and PCR, have aided the study of *vasa* mRNA expression during different developmental
468 stages, including early development and gametogenesis. Several studies in vertebrates and
469 invertebrates have shown that *vasa* mRNA is a maternally supplied transcript (Braat et al.,
470 1999a; Saffman and Lasko, 1999). Thus, for example, in tilapia *vasa* mRNA and protein were
471 present in full-grown oocytes, ovulated eggs and embryos at the 1- and 4-cell stage (Kobayashi
472 et al., 2000; Kobayashi et al., 2002). This maternal transfer has also been reported in zebrafish,
473 both in oocytes (Braat et al., 1999b) and in freshly fertilized eggs (Braat et al., 1999b; Krøvel
474 and Olsen, 2004; Yoon et al., 1997), and also in medaka (Shinomiya et al., 2000). The present
475 study shows that *sb-vasa* is present in unfertilized eggs and in the developing embryo at 2 and 4
476 dpf, just before hatching, strongly suggesting its maternal transfer. The observation that during
477 embryo and larval development *sb-vasa* mRNA levels were drastically reduced was probably
478 because the amount of the maternally inherited *vasa* transcript became diluted within the total
479 RNA content of a growing individual. A similar explanation has been offered for the decrease of
480 *vasa* mRNA during vitellogenesis in tilapia (Kobayashi et al., 2000). In our study, *sb-vasa* levels
481 increased again between 45-72 dpf, concomitant with the start of PGC divisions and
482 proliferation reported previously in this species (Roblin and Bruslé, 1983). A similar pattern has
483 been reported in zebrafish where high *vasa* levels during embryogenesis decreased abruptly

484 during larval development and increased during gametogenesis, suggesting a switch from
485 maternal to *de novo vasa* expression (Krøvel and Olsen, 2004).

486

487 High rearing temperatures during larval and post-larval stages accelerate somatic and gonadal
488 growth in European sea bass (Ayala et al., 2001; Blázquez et al., 1998; Navarro-Martín et al.,
489 2009). However, past a certain threshold, high temperatures can become germotoxic. Thus, in
490 medaka high rearing temperatures inhibited the proliferation and development of germ cells in
491 genetic females but had no effect in genetic males (Selim et al., 2009). The higher sensitivity of
492 female gonads has also been reported in pejerrey, *Odontesthes bonariensis* (Ito et al., 2003). Our
493 results show significantly higher *sb-vasa* values in fish reared at high temperature with respect
494 to those of fish reared at low (i.e., “natural”) temperature. Species-specific differences aside, the
495 differences observed in the different studies may be due to the fact that while the high
496 temperature used in the European sea bass was still within its normal thermal range, in medaka
497 and pejerrey they may have been close to the thermal maximum of these species. In addition, the
498 lack of increase in *vasa* expression observed in the European sea bass reared at low temperature
499 from 0-120 dpf was also probably influenced by the marked effect that this prolonged exposure
500 has on gonadal growth and maturation (Blázquez et al., 1998), something that was not reported
501 in medaka (Selim et al., 2009). Nevertheless, growth acceleration in the group reared at high
502 temperature was already found at the start of PGC proliferation in this species by 60 dpf,
503 persisted at 150 dpf with the onset of histological sex differentiation, and was still present at the
504 end of the first year. High temperature also influenced gonadal growth and maturation, as
505 confirmed by lower gonadosomatic index values in fish reared under similar conditions
506 (Navarro-Martín et al., 2009). The high temperature-induced increase in *sb-vasa* expression
507 could be due to (1) a direct effect at the transcriptional level, (2) an indirect effect through
508 increased developmental rates, accelerating gonadal growth with a concomitant increase in the

509 number of PGCs that specifically express *sb-vasa*, or (3) a combination of both. Thus, the
510 effects of the temperature on *sb-vasa* levels merits further investigation.
511
512 The study of *vasa* mRNA expression levels during early ontogenesis, sex change and gonadal
513 maturation, including oogenesis and spermatogenesis, has been reported in several fish species
514 including zebrafish (Krøvel and Olsen, 2004), medaka (Shinomiya et al., 2000), gilthead sea
515 bream (Cardinali et al., 2004), gibel carp (Xu et al., 2005), tilapia (Kobayashi et al., 2000;
516 Kobayashi et al., 2002), and rice field eel, *Monopterus albus* (Ye et al., 2007). Two *vasa* splice
517 variants with different expression patterns at the time of sex differentiation have been found in
518 tilapia (Kobayashi et al., 2002) and zebrafish (Krøvel and Olsen, 2004). The long isoform
519 showed a pattern similar to that observed in the present study , with females exhibiting higher
520 levels than males, but an accurate quantification of *vasa* expression was not reported (Krøvel
521 and Olsen, 2004). Thus, to the best of our knowledge, a complete study of *vasa* expression
522 patterns encompassing early development and sex differentiation had not been carried out to
523 date in any fish. Furthermore, in all studies cited above Vasa protein and mRNA were localized
524 by immunohistochemistry and *in situ* hybridization or semiquantitative PCR, but expression
525 levels were not quantified. In fact, PCR techniques were used only as a tool to assess the
526 presence of *vasa* mRNA expression. In the present study, using real time RT-PCR, it was shown
527 that *sb-vasa* levels in the female-dominant group were always higher than those found in the
528 male-dominant group, with statistical differences starting as early as 100 dph, i.e., prior to the
529 first observable signs of female sex differentiation (which occur at 150 dpf). These differences
530 could not be attributed solely to sex, but also to differential growth since at this developmental
531 stage, the female-dominant group exhibited 37.8% higher growth (length) than that of the male-
532 dominant group (Papadaki et al., 2005). Interestingly, at 150 and 200 dph, coinciding with the
533 onset of histological sex differentiation, *sb-vasa* levels were 5.1- and 2.7-fold higher in females
534 than in males, respectively, reaching values up to 4.7-fold-higher at 300 dph. It should be noted

535 that siblings of the fish used in this experiment had higher levels of plasma levels of E_2 at 150
536 and 200 dph (Papadaki et al., 2004). This would support the observations made in gilthead sea
537 bream, where a stimulatory effect of E_2 was observed (Cardinali et al., 2004). These differences
538 in *sb-vasa* expression levels can be influenced by sex-related differences in size in favor of
539 females (11.7% at 150 dph and 9.6% at 200 dph), in agreement with the reported progressive
540 reduction in sexual growth dimorphism in European sea bass as sex differentiation proceeds
541 (Saillant et al., 2001). However, it seems very unlikely that the small magnitude in the growth
542 differences reported, particularly during sex differentiation, could account for these much higher
543 levels in females. Thus, other factors should be considered. In this regard, there are two
544 observations that may support a true influence of sex on *vasa* expression. Firstly, from 200 dph
545 onwards only females from the female-dominant group and only males from the male-dominant
546 group were used for the study. Thus, at least in the period 200-300 dph comparisons are between
547 fish of verified phenotype (see Fig. 6).Secondly, a significant number of males started
548 maturation before the end of the first year (precocious maturation), compared to the absence of
549 any mature females at the same time. Taken together, these observations suggest that —body
550 size, and maturation differences aside— *sb-vasa* levels are higher in females than in males.
551
552 During natural sex reversal in the rice field eel, *vasa* was shown to be expressed in oocytes at all
553 stages of development, including degenerating oocytes in the ovotestis, and also in
554 spermatogonia and spermatocytes (Ye et al., 2007). The signal in primary spermatocytes was
555 nevertheless weaker than in spermatogonia, similarly to what has been reported in tilapia
556 (Kobayashi et al., 2000), gibel carp (Xu et al., 2005) and zebrafish (Leal et al., 2009). In the
557 present study, localization of the cellular origin of *vasa* expression by *in situ* hybridization was
558 not attempted. However, in a recent study of our group carried out using laser capture
559 microdissection combined with real time RT-PCR using the primers for *vasa* developed in the
560 present study, it was found that, at least in adult European sea bass male gonads, *vasa* was

561 expressed in germ cells in all stages of spermatogenesis, with the highest levels detected in
562 spermatogonia, followed by spermatocytes, spermatids and maintained at low levels in
563 spermatozoa (Viñas and Piferrer, 2008).

564

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

577

578 In conclusion, the present study reports the full-length sequence of a *vasa* homolog in the
579 European sea bass (*sb-vasa*), a species with temperature influences on sex differentiation.
580 European sea bass *vasa* is mainly expressed in gonads although some expression in extra-
581 gonadal tissues cannot be neglected. This suggests the possibility of other functions related to its
582 helicase activity such as the translational regulation of mRNAs in other tissue types. In this
583 study the evolutionary relationships of Vasa amino acid sequences belonging to six different
584 phyla have been also established. Further, the obtained results add evidence to the importance of
585 *vasa* in gonadal development in fish and suggest a role of this gene in the process of sexual
586 differentiation and maturation. In this regard, significant differences in *sb-vasa* expression were

587 detected not only prior to histological sex differentiation, with higher levels in females than in
588 males, but particularly during sex differentiation and testicular maturation. Whether this reflects
589 possible constitutive differences in *vasa* expression between female and male germ cells
590 deserves further study.

591

592 **Acknowledgments**

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

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762

763 **Figure captions**
764
765 **Fig. 1.** Nucleotide and deduced amino acid sequence of *sb-vasa* in the European sea bass
766 (*Dicentrarchus labrax*). Shaded boxes correspond to the eight conserved regions of the DEAD-
767 box protein family. Arginine-glycine (RG) repeats and arginine-glycine-glycine (RGG) repeats
768 in the N-terminal region are underlined and double underlined, respectively. Acidic amino acid
769 residues (aspartic acid; D and glutamic acid; E) and tryptophan (W) in the N-terminal and C-
770 terminal regions are shown inside circles. Numbers indicate the nucleotide position starting at
771 the A of the initial ATG codon (top line) and the amino acid positions starting at the initial
772 methionine (M, bottom line). An asterisk indicates the stop codon (TAG). The polyadenylation
773 signal and the poly-A tail are marked in boldface. The present *sb-vasa* nucleotide sequence has
774 been deposited in the GenBank under accession number **GU987023**
775
776 **Fig. 2.** Phylogenetic tree of Vasa proteins. Scientific name of the species used to generate the
777 tree are given at the right of the branches and GenBank protein ID numbers appear between
778 parentheses. The tree was constructed using the Neighbor-Joining method (Saitou and Nei,
779 1987). The bootstrap consensus tree inferred from 1000 replicates represents the evolutionary
780 history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 80%
781 bootstrap replicates are collapsed. Bootstrap values (percent) are shown next to the branches.
782 The evolutionary distances were computed using the Poisson correction method and are in the
783 units of the number of amino acid substitutions per site. All positions containing gaps and
784 missing data were eliminated only in pairwise sequence comparisons with a total of 1208
785 positions in the final dataset. The tree was rooted with the germ-line helicase gl1 from
786 *Caenorhabditis elegans*. Phylogenetic analyses were conducted in MEGA version 4.
787
788 **Fig. 3.** Tissue-specific expression of *sb-vasa* in adult European sea bass. Tissues include testis
789 (T), ovary (O), male brain (B_m), female brain (B_f), head kidney (K), liver (L), gut (G), spleen

790 (S), gill (Gi), heart (H), muscle (M), and visceral fat (F). Five micrograms of total RNA from
791 each tissue were reverse transcribed to cDNA. After DNase treatment, samples were amplified
792 by PCR using a specific primer set for *sb-vasa* yielding s 316 bp fragment (panel A). A 485 bp
793 fragment of 18S ribosomal RNA (*r18S*) was used as an internal control to check for the integrity
794 of the cDNA template (panel B). A negative control (-) using sterile water as template was
795 included in the PCR analysis to check for possible contamination and thus discard false
796 positives. Panel C plots a graph with the relative values quantified by densitometry in the tissue-
797 specific expression study. Values of *sb-vasa* gene expression in each tissue were referenced to
798 values of 18S rRNA expression in the same tissue.

799

800 **Fig. 4**. European sea bass *vasa* gene expression during early developmental stages prior to the
801 formation of the gonadal ridges and the onset of sex differentiation. Samples were analyzed by
802 real-time fluorescence PCR (see materials and methods for further details). Data are presented
803 as the mean normalized gene expression (MNE) levels ± the standard error of the mean (SEM)
804 of three samples performed in triplicate. Each sample consisted of a pool of 20 eggs/embryos (0-
805 2 days post fertilization, dpf), a pool of 20 larvae (4-25 dpf), and a pool of ten larvae (30 dpf).
806 Values were normalized against the levels of 18S rRNA amplified from the same reverse
807 transcribed template and plotted in logarithmic scale. Different letters show statistical
808 differences ($P < 0.05$) between the different sampling dates after a Tukey's test.

809

810 **Fig. 5**. Effects of the rearing temperature on *sb-vasa* gene expression during early
811 developmental stages of European sea bass, prior to the onset of sex differentiation. Samples
812 were analyzed by real-time fluorescence PCR (see materials and methods for further details).
813 Data are presented as the mean normalized gene expression (MNE) levels ± the standard error of
814 the mean (SEM) of three samples performed in triplicate. Each sample consisted of a pool of ten
815 larvae (30-45 dpf), a pool of five body trunks (60-72 dpf) and one body trunk at 150 dpf. Values

816 were normalized against the levels of 18S rRNA amplified from the same reverse transcribed
817 template and plotted in logarithmic scale. Different letters show statistical differences ($P < 0.05$)
818 between the different sampling dates after a Tukey's test. Asterisks indicate statistical
819 differences between temperatures (* = $P < 0.05$; ** = $P < 0.001$) at each sampling time after a
820 Student's *t*-test.

821

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

832

833 **Fig. 7**. European sea bass *-vasa* gene expression during the first year of life. Samples were
834 analyzed by real-time fluorescence PCR. The study was performed in samples from male- and
835 female-dominant groups at different times during the first year of development (50-300 days
836 post hatching; dph). Values were plotted in logarithmic scale and expressed as the mean
837 normalized expression (MNE) of *sb-vasa* mRNA levels against the levels of 18S rRNA ± the
838 standard error of the mean (SEM) of six samples performed in triplicate. Different letters show
839 statistical differences between groups ($P < 0.05$) after a Tukey's test. At 200, 250, and 300 dph
840 (right to the dashed line), all fish from the male-dominant group used for the analysis were

841 males and all fish from the female-dominant group were females. Grey boxes at the bottom
842 represent different key developmental processes during European sea bass gonadogenesis.
843
844
845

Figure 1

Figure 1. Blázquez et al 2010

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                                        gacgaaccaagtaacgttacaagttcagtaaggtgaataaac      -1

ATGGATGAGTGGGAAGAAGAGGGAACTACTATTACTAGCACTACCACACTAACCACCACTAGTGAAGGCAGACAA      75
 M  D  E  W  E  E  E  G  T  T  I  T  S  T  T  T  L  T  T  T  S  E  G  R  Q       25

GAAGGGTCTTGGAATACTGCCAATGGTGAATCTGGAAGGGGTCGTGGTGGAAGGGGCAGAGGAGGAGGATTTAAA     150
 E  G  S  W  N  T  A  N  G  E  S  G  R  G  R  G  G  R  G  R  G  G  G  F  K       50

AGCTCATTCTCCTCAGATGGCGACAACTGGAACAGTACAGCTGGAGAAGGAGGTTTTAGAGGTAGAGGAGGCAGA     225
 S  S  F  S  S  D  G  D  N  W  N  S  T  A  G  E  G  G  F  R  G  R  G  G  R       75

GGGCGTGGCAGAGGATTCAGCCGAACGGATCGCAGTGAATTCAATGAGGATGACAATCAAGTGTGTGAAAATGGG     300
 G  R  G  R  G  F  S  R  T  D  R  S  E  F  N  E  D  D  N  Q  V  C  E  N  G      100

GGAGGCAGCCGAGGCGGCAGAGGAGGCAGAGGAGGTTTCAGATCAGGTGGTGACCAAGGTTGCAGAGGAGGATTT     375
 G  G  S  R  G  G  R  G  G  R  G  G  F  R  S  G  G  D  Q  G  C  R  G  G  F      125

GGAGGAGGCTACCGTGGAAAAGATGAGGAGGTCTTTTCTCAACGGGAAGATAAAGAAAATAAGGATGCAACCGAT     450
 G  G  G  Y  R  G  K  D  E  E  V  F  S  Q  R  E  D  K  E  N  K  D  A  T  D      150

GGTGAAAGACCAAGGGTCACATATATTCCCCCAACCCTCCCTGAAGATGAAGACTCCATTTTTGCCCACTATGAG     525
 G  E  R  P  R  V  T  Y  I  P  P  T  L  P  E  D  E  D  S  I  F  A  H  Y  E      175

ACGGGCATCAACTTTGACAAGTATGATGACATCCTGGTGGATGTCAGTGGAGCCAATCCACCACAGGCTATAATG     600
 T  G  I  N  F  D  K  Y  D  D  I  L  V  D  V  S  G  A  N  P  P  Q  A  I  M      200

ACTTTTGATGAGGCAGGATTGTGTGAGTCGCTGAGAAAAACAGTCAGCAAGTCTGGTTATGTGAAGCCGACCCCT     675
 T  F  D  E  A  G  L  C  E  S  L  R  K  T  V  S  K  S  G  Y  V  K  P  T  P      225

GTGCAGAAGCATGGCATTCCAATCATCTCTGCTGGTAGAGATCTTATGGCCTGTGCCCAGACTGGATCTGGTAAA     750
 V  Q  K  H  G  I  P  I  I  S  A  G  R  D  L  M  A  C  A  Q  T  G  S  G  K      250

ACGGCTGCTTTCCTGCTCCCTATTCTGCAGCAGCTGATGGCAGACGGTGTGGCAGCCAGTTCCTTCAGTGAGCTG     825
 T  A  A  F  L  L  P  I  L  Q  Q  L  M  A  D  G  V  A  A  S  S  F  S  E  L      275

CAGGAGCCTGAAGCCGTCATCGTGGCTCCAACTAGGGAGCTCATCAACCAGATTTACCTAGAGGCCAGGAAGTTC     900
 Q  E  P  E  A  V  I  V  A  P  T  R  E  L  I  N  Q  I  Y  L  E  A  R  K  F      300

GCCTTTGGGACATGTGTGCGTCCAGTAGTAGTCTATGGTGGAGTCAGCACTGGACATCAAATAAGAGAAATCTCA     975
 A  F  G  T  C  V  R  P  V  V  V  Y  G  G  V  S  T  G  H  Q  I  R  E  I  S      325

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 R  G  C  N  V  L  C  G  T  P  G  R  L  L  D  V  I  G  R  G  K  V  G  L  S      350

AAGGTGCGGTACTTTGTGCTGGATGAGGCTGACCGGATGTTGGATATGGGTTTTGAGCCTGACATGCGTCGCTTG    1125
 K  V  R  Y  F  V  L  D  E  A  D  R  M  L  D  M  G  F  E  P  D  M  R  R  L      375

GTGGGCTCCCCCGGAATGCCATCAAAAGAGAACCGGCAGACGCTGATGTTCAGTGCCACCTACCCTGAGGACATT    1200
 V  G  S  P  G  M  P  S  K  E  N  R  Q  T  L  M  F  S  A  T  Y  P  E  D  I      400

CAGAGGATGGCAGCAGACTTTCTCAAGACCGACTATTTGTTCTTGGCTGTGGGTGTGGTTGGCGGAGCCTGCAGT    1275
 Q  R  M  A  A  D  F  L  K  T  D  Y  L  F  L  A  V  G  V  V  G  G  A  C  S      425

GATGTGGAGCAGACATTTGTCCAGGTCACCAAGTTCTCCAAAAGGGAGCAACTCCTTGACCTCCTCAAGACAACT    1350
 D  V  E  Q  T  F  V  Q  V  T  K  F  S  K  R  E  Q  L  L  D  L  L  K  T  T      450

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 G  M  E  R  T  M  V  F  V  E  T  K  R  Q  A  D  F  I  A  T  Y  L  C  Q  E      475

AAGGTTCCTACCACCAGCATTCATGGTGACCGAGAGCAGCGGGAGCGAGAGCAGGCTCTGGCAGACTTCCGCTCT    1500
 K  V  P  T  T  S  I  H  G  D  R  E  Q  R  E  R  E  Q  A  L  A  D  F  R  S      500

GGCAAATGTCCAGTCCTAGTGGCAACCTCTGTGGCTGCCCGTGGTCTGGACATTCCAGATGTACAGCATGTGGTG    1575
 G  K  C  P  V  L  V  A  T  S  V  A  A  R  G  L  D  I  P  D  V  Q  H  V  V      525

AACTTTGACCTCCCCAACAACATTGACGAATATGTCCACCGTATTGGGAGAACTGGCCGCTGTGGTAACACTGGA    1650
 N  F  D  L  P  N  N  I  D  E  Y  V  H  R  I  G  R  T  G  R  C  G  N  T  G      550

AGGGCGGTGTCTTTCTTTGACCCAGATTCTGATGACCAATTGGCTCGCTCCCTAGTCACAATCCTGTCCAAGGCT    1725
 R  A  V  S  F  F  D  P  D  S  D  D  Q  L  A  R  S  L  V  T  I  L  S  K  A      575

CAGCAGGAAGTGCCTTCATGGTTGGAGGATTCTGCATTCAGTGGCCCTGGTAGCATGGGCGTCGCCCCCAGAAAG    1800
 Q  Q  E  V  P  S  W  L  E  D  S  A  F  S  G  P  G  S  M  G  V  A  P  R  K      600

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 T  F  A  S  S  D  S  R  K  G  P  Q  G  G  S  V  Q  D  D  S  V  Q  S  Q  S      625

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 A  A  Q  T  A  H  D  E  E  E  W  D  *                                          637

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Figure 2. Blázquez et al 2010.

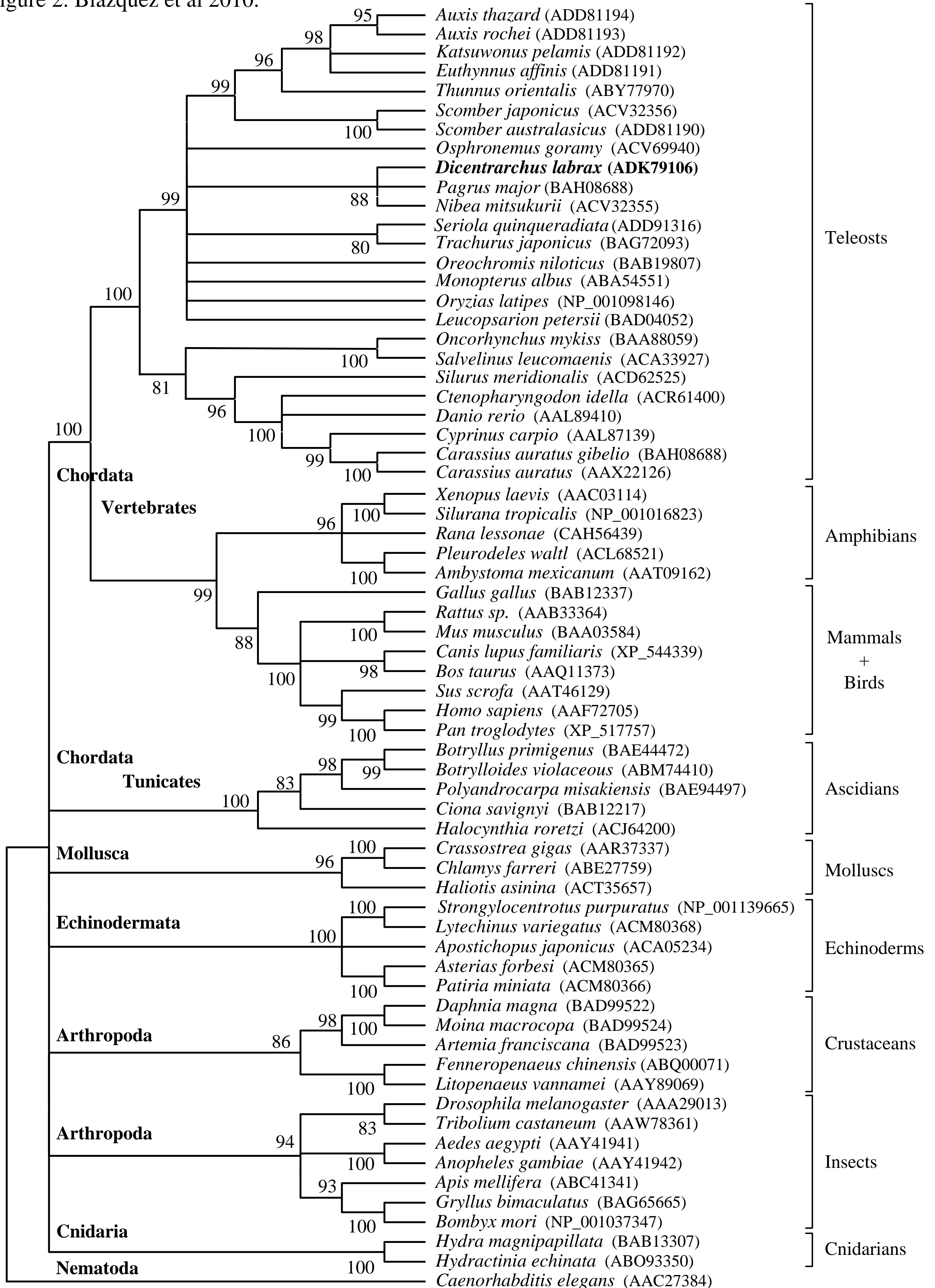

Figure 3

Figure 3. Blázquez et al 2010

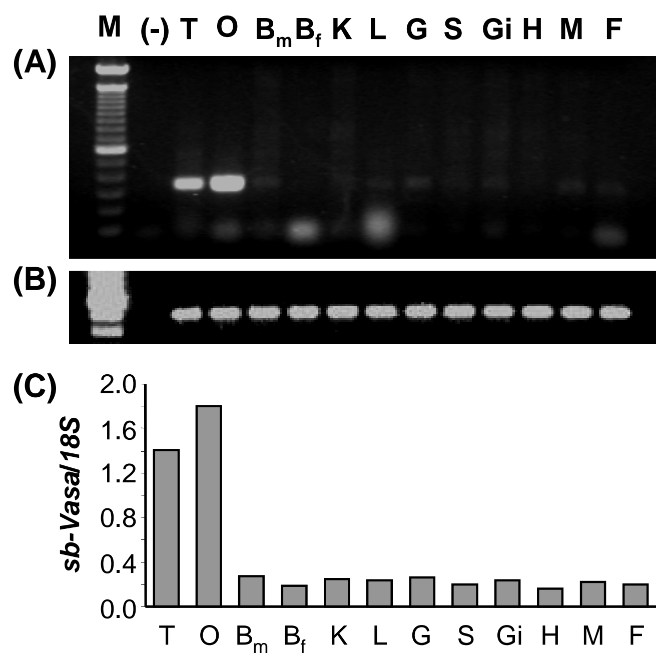

Figure 4

Figure 4. Blázquez et al 2010

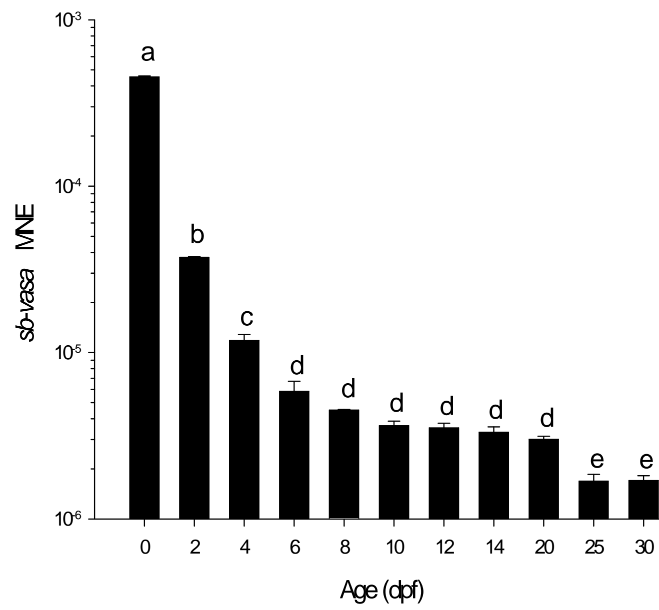

Figure 5

Figure 5. Blázquez et al., 2010

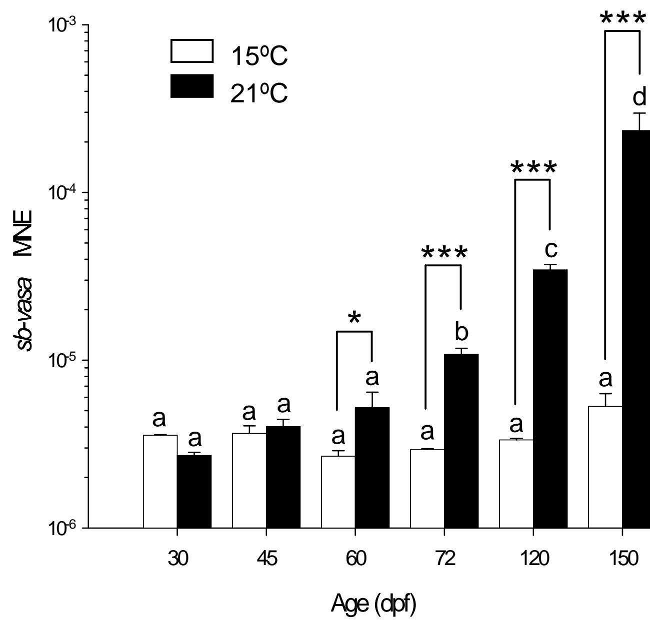

Figure 6

Figure 6. Blázquez et al 2010

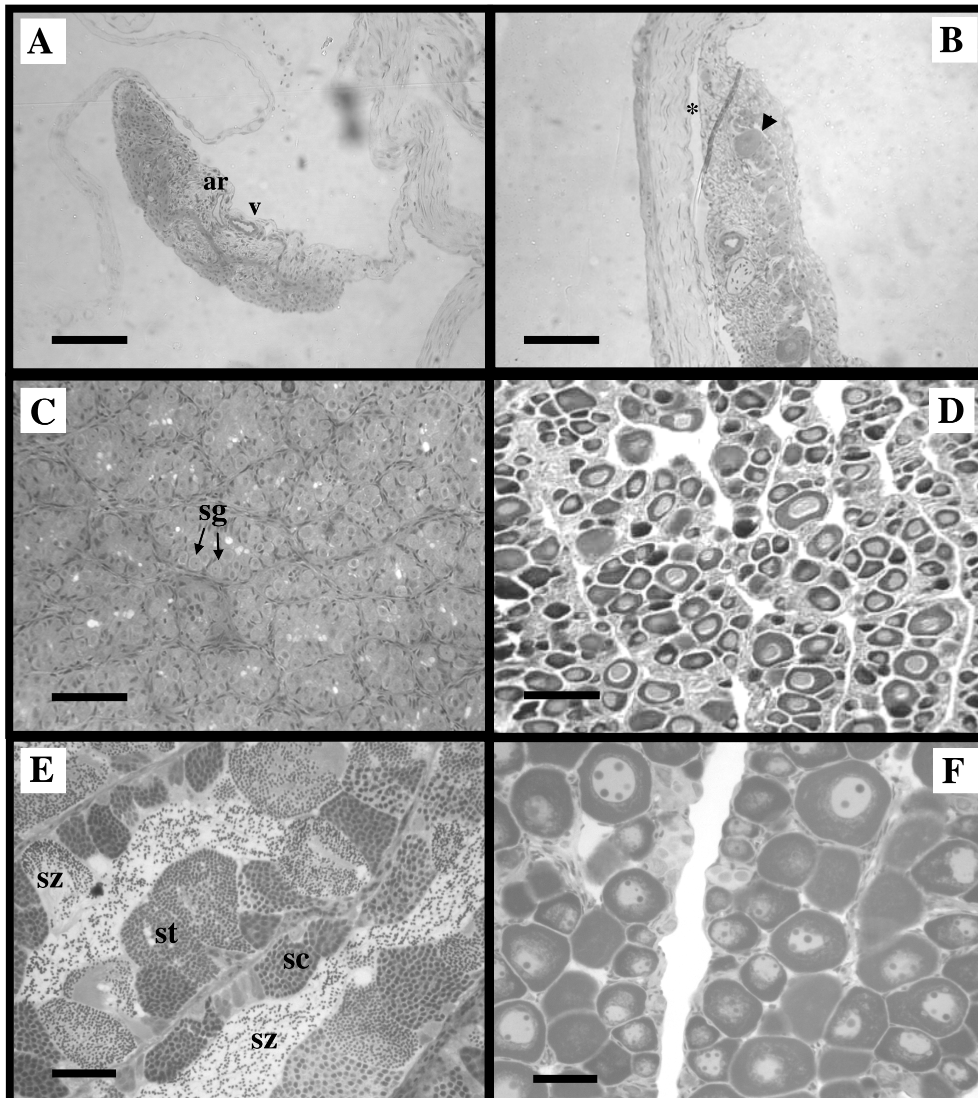

Figure 7

Figure 7. Blázquez et al 2010