Mechanisms of oocyte development in European sea bass (*Dicentrarchus labrax*, L.): Investigations via application of unilateral ovariectomy

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Abstract

Unilateral ovariectomy (ULO) was performed in European sea bass (*Dicentrarchus labrax*, L.) during late pre-vitellogenesis/early vitellogenenesis. Plasma steroid levels and the expression of a suite of potential oogenesis-relevant genes in the ovary, brain and pituitary were evaluated with the aim of understanding their involvement in the compensatory oocyte development occurring within the remaining ovarian lobe. Sixty-nine days after surgery, the remaining ovarian lobe in ULO fish was gravimetrically equivalent to an intact paired ovary of sham operated, control fish. This compensatory ovarian growth was based on an increased number of early perinucleolar oocytes and mid-late stage vitellogenic follicles without an apparent recruitment of primary oocytes into the secondary growth phase. Plasma steroid levels were similar in ULO and control females at all time points analysed, suggesting an increased steroid production of the remaining ovarian lobe in hemi-castrated females. Results of the gene expression survey conducted indicate that the signalling pathways mediated by follicle stimulating hormone and gonadotropin-releasing hormone 1 constitute the central axes orchestrating the observed ovarian compensatory growth. In addition, steroid receptors, steroidogenic acute regulatory protein, insulin-like growth factors, and members of the transforming growth factor, beta superfamily including anti-Mullerian hormone and bone morphogenetic protein 4 were identified as potentially relevant players within this process, although their specific actions and interactions remain to be established. Our results demonstrate that ULO provides an excellent *in vivo* model for elucidating the interconnected endocrine and molecular mechanisms controlling oocyte development in European sea bass.

Introduction

Oogenesis in teleost fish is a highly complex process including several successive steps, *i.e.* (1) mitotic proliferation of oogonia, (2) transformation of oogonia into primary oocytes with the onset of meiosis (primary growth phase), (3) secondary growth phase characterized by a significant oocyte enlargement due to the accumulation of lipids and yolk proteins (vitellogenesis), (4) maturation characterized by the resumption of meiosis, germinal vesicle breakdown, lipid and yolk coalescence, and hydration, and (5) ovulation (Patiño & Sullivan 2002). The process of oogenesis is controlled by an intricate regulatory network both outside and within the follicle complex, the latter consisting of the oocyte, an inner surrounding granulosa cells, and two outer theca layers (Grier 2000). The main players within this regulatory network include gonadotropin releasing hormones (GnRH) in the brain, the pituitary gonadotropins, *i.e.* follicle-stimulating hormone (FSH) and luteinizing hormone (LH), as well as sex steroids and peptide growth factors acting locally in the ovary in an autocrine or paracrine fashion or, in case of steroids, on extra-ovarian sites as endocrine hormones (*e.g.*, regulating the hepatic synthesis of vitellogenin or exerting feedback actions on GnRH and gonadotropin production and release at the brain and pituitary) (Ge 2005, Lubzens *et al.* 2010). Despite our knowledge on the specific roles of the above-mentioned factors, oogenesis is a dynamic and complex process and, thus, many aspects of its regulation remain to be elucidated.

In teleost fish, as for other vertebrate taxa, the surgical removal of one of the ovarian lobes, referred to as unilateral ovariectomy (ULO) or hemi-castration, induces a compensatory growth in the remaining ovarian lobe ultimately generating a single lobe equivalent in mass to the intact paired ovary of control individuals (Goswami & Sundaraj 1968a, Dadzie & Hyder 1976, Tyler *et al.* 1994, 1996, 1997, Luckenbach *et al.*

2008). The mechanisms involved in such compensatory growth, which may vary across species and with the reproductive status of the animal, include increased mitotic activity of oogonia, new recruitment of primary follicles into the secondary growth phase, increased size of pre-existing follicles, and/or reduced follicular atresia (see references above). In teleost fish, the endocrine processes driving these compensatory mechanisms are not fully understood, although the results of ULO studies in rainbow trout (*Oncorhynchus mykiss*) point towards FSH as one of the main regulators (Tyler *et al.* 1997). Based on the dramatic physiological changes that ULO provokes, this procedure constitutes a powerful *in vivo* technique for studying multiple aspects of the integral regulatory mechanisms driving oogenesis, especially the early stages of oocyte development, which are largely unexplored in teleost fish.

The European sea bass (*Dicentrarchus labrax*) is one of the most important commercial teleost fish cultured in the Mediterranean area. Ovarian development of this species is defined as group-synchronous, *i.e.* follicles at different developmental stages are simultaneously present in the ovary and are spawned progressively in 3-4 batches during a *ca*. 2 months reproductive period in mid winter (Mayer *et al.* 1990, Asturiano *et al.* 2000, 2002). Diverse descriptive and experimental studies have provided some information concerning the physiological control of European sea bass reproductive processes, including oocyte development (Zanuy *et al.* 2001, Taranger *et al.* 2010), but there are still significant knowledge gaps on the controlling mechanisms. To start to address some of these knowledge gaps, ULO was applied as an experimental tool to study the integrated regulatory mechanisms spanning the ovary, pituitary and brain, responsible for mediating oocyte development in European sea bass.

Results

Morphological and morphometrical evaluation

At the time of ULO operations, fish in both experimental groups showed no significant differences in total body weight (mean: 1.77±0.09 kg), ovarian lobe weight (Fig. 1A), gonadosomatic index (GSI) (Fig. 1B), total number of oocytes, or number of oocytes at different stages of follicular development (Fig. 1D). Ovaries at this time were mainly comprised of oocytes at early and late perinucleolar and lipid vesicles stages together with very low numbers of early vitellogenic follicles (Fig. 1D and F).

Sixty nine days after surgical procedures, the ovarian lobe weights of ULO females were 2.3-fold higher ($p<0.05$) than those in the sham operated control (SHAM) individuals (Fig. 1A and C), while total body weights (mean: 1.88±0.08 kg) and GSI values (calculated taking into account both ovarian lobes in SHAM females but only the remaining ovarian lobe in ULO fish) were similar in both groups (Fig. 1B). Evaluation of oocyte stages present in the ovary showed that ULO females (in comparison to SHAM fish) had on average a 1.6-fold fewer total number of oocytes ($p<0.05$) in concordance with 1.9-fold fewer late perinucleolar oocytes ($p<0.05$), 1.5-fold fewer lipid vesicles stage 1 oocytes ($p<0.05$), 1.6-fold fewer lipid vesicles stage 2 oocytes ($p<0.05$), and 2.2-fold lower number of early vitellogenic follicles ($p<0.05$) (Fig. 1E, G and H). In contrast, ovaries of ULO females had similar number of early perinucleolar oocytes and mid and late stage vitellogenic follicles compared to those of SHAM individuals (Fig. 1E, G and H). Incidence of oocyte atresia was low and similar in both experimental groups (not shown).

Total number of oocytes contained in the paired ovaries of control fish showed an average 33% decrease from day 0 (INITIAL group) to day 69 (SHAM group) (Fig. 1D and E). This decrease was associated with a weight increase of the fish (oocyte numbers

are corrected for individual body weight) together with a decline of the estimated number of early perinucleolar oocytes. Due to the small size of this stage of development and their particular arrangement within sections from vitellogenic ovaries (they are scarce and scattered among much bigger follicles), we found that our counting strategy may have underestimated their numbers in vitellogenic ovaries.

Linear regression analyses (Table 1) showed positive correlations between the GSI and the number of lipid vesicles stage 2 oocytes at the time of ULO, between the GSI 69 days after ULO and both the GSI and the number of lipid vesicles stage 2 oocytes at the time of ULO, between the total number of oocytes 69 days after ULO and both the total number of oocytes and the number of late perinucleolar oocytes at the time of ULO, and between the GSI and the number of mid and late vitellogenic oocytes 69 days after ULO. Correlations between the GSI, the total number of oocytes, and the number of oocytes at any developmental stage other than that mentioned above were not significant.

Plasma steroid levels

The temporal profiles for circulating levels of 17β-estradiol (E_2) and testosterone (T) were similar in both INITIAL-SHAM and ULO females (Fig. 2); low steroid levels were measured at days -25, 0, and 21 after operations, with increasing concentrations of steroids at days 47 and 69 after ULO. There were no significant differences in steroid concentrations between the two groups at any sampling point, although there appeared to be a possible tendency towards relatively lower levels in operated females 47 days after ULO.

Gene expression analysis in the ovary, pituitary and brain

The expression levels of all the genes analyzed in the ovary were similar in both groups at the time of ULO operations (Fig. 3). At 69 days after surgery, ovarian expression levels of *follicle stimulating hormone receptor* (*fshr*), *steroidogenic acute regulatory protein* (*star*), *androgen receptor a* (*ara*), *bone morphogenetic protein 4* (*bmp4*), and *lipoprotein lipase* (*lpl*) were significantly higher in ULO females compared with SHAM individuals. In contrast, ovarian transcripts amounts for *insulin-like growth factor 1* (*igf1*), *anti-Mullerian hormone* (*amh*), and *vitellogenin receptor* (*vtgr*) were all significantly down-regulated. There were no significant changes for all other transcripts analysed in the ovary, including *luteinizing hormone/choriogonadotropin receptor* (*lhcgr*), *thyroid stimulating hormone receptor* (*tshr*), *cytochrome P450, family 19, subfamily A, polypeptide 1a* (*cyp19a1a*), *estrogen receptors 1* (*esr1*), *2a* (*esr2a*), and *2b* (*esr2b*), *androgen receptor b* (*arb*), *insulin-like growth factor 2* (*igf2*), *transforming growth factor, beta 1a* (*tgfb1a*), and *SRY-box containing gene 17* (*sox17*).

In the pituitary of ULO females (Fig. 4), transcripts levels of *glycoprotein hormones, alpha polypeptide* (*cga*), *follicle stimulating hormone, beta polypeptide* (*fshb*), and *gonadotropin-releasing hormone receptor 2a* (*gnrhr2a*) were significantly higher compared with SHAM control fish, while there were no significant changes in the expression levels of *luteinizing hormone, beta polypeptide* (*lhb*), *esr1*, *esr2a*, *esr2b*, *ara*, and *arb*.

In the brain of ULO females, mRNA expression of *gonadotropin-releasing hormone 1* (*gnrh1*) was significantly up-regulated while *esr1* and *esr2a* transcripts amounts were significantly down-regulated compared to SHAM control fish. Transcript levels for *gonadotropin-releasing hormone 3* (*gnrh3*), *cytochrome P450, family 19, subfamily A, polypeptide 1b* (*cyp19a1b*), and *esr2b* were similar in both groups (Fig. 5).

Discussion

Although research efforts have contributed significantly in widening-up our knowledge about the mechanisms regulating teleost fish oogenesis, we are still far from understanding the complete process. Here, we report the regulatory changes observed in European sea bass reproductive axis in response to ULO, a surgical procedure used for the first time in this species and with a strong potential as an *in vivo* model for investigating factors involved in the regulation of oocyte development in teleost fish.

Morphological responses to ULO

Macroscopically, the response of European sea bass to ULO was similar to those previously reported for other fish species (Goswami & Sundaraj 1968a, Dadzie & Hyder 1976, Tyler *et al.* 1994, 1996, 1997, Luckenbach *et al.* 2008), *i.e.* the ovarian lobe remaining after surgery underwent a compensatory growth ultimately reaching a total mass equivalent to that of intact paired gonads. The mechanism by which such compensatory growth is accomplished seems to vary across fish species and have been shown to depend on the ovarian stage at the time of surgery. In European sea bass, which were submitted to ULO at late pre-vitellogenesis/early vitellogenesis, we found that the number of early perinucleolar and mid-late vitellogenic oocytes was fully compensated 2 months after surgery, but oocyte stages from late perinucleolar to early vitellogenic (as well as total oocyte number) were not. This may indicate that even though new early perinucleolar oocytes were originated in response to ULO, as reported in stinging catfish (*Heteropneustes fossilis*) (Goswami & Sundaraj 1968a) and blue tilapia (*Oreochromis aureus*) (Dadzie & Hyder 1976), these new oocytes did not progress through the primary nor the secondary growth phases during the study period. Therefore, and unlike that reported for rainbow trout hemi-castrated at pre- or early

189 vitellogenesis (Tyler *et al.* 1994, 1996, 1997), the compensatory ovarian growth
190 observed in European sea bass after ULO was not associated with recruitment of a
191 complete new batch of primary oocytes into the secondary growth phase. Rather, such
192 compensation was based on an extra recruitment of follicles at mid and late
193 vitellogenesis, probably derived from the pool of early vitellogenic oocytes already
194 existing in the ovary.
195 European sea bass undertake several spawning events deriving the eggs from 3-4
196 different batches of post-vitellogenic oocytes. The first batch contains more oocytes
197 than the successive ones (up to 50% of total oocytes to be spawned), which mature and
198 are ovulated in unison (Mayer *et al.* 1990, Asturiano *et al.* 2002). Given these findings,
199 we speculate that the extra recruitment of mid-to-late vitellogenic follicles observed in
200 hemi-castrated European sea bass occurs to ensure a full complement of oocytes for
201 spawning in the first batch of eggs and thus, ensuring maintenance of fertility for the
202 first main ovulatory episode. Further studies would be required however covering the
203 full spawning period to assess for any overall effects on fertility collectively over the 3-
204 4 spawning events.
205 In rainbow trout subjected to ULO before mid vitellogenesis, the compensatory ovarian
206 growth was achieved by the recruitment of new primary oocytes (Tyler *et al.* 1994,
207 1996). In contrast, when surgery was performed at later stages, the compensation was
208 based on an over-enlargement of pre-existing follicles without any further recruitment
209 of primary oocytes into the maturing pool. Consequently, the term “critical window for
210 recruitment” was introduced and assigned to mid-vitellogenesis in this species (Tyler *et*
211 *al.* 1994, 1996). Whether such a critical window exists for European sea bass is less
212 clear, but if such a window does exist, it must occur before the appearance of lipid
213 vesicles stage 2/early vitellogenesis, *i.e.* before the developmental time point when

ULO was performed in this study. Our results support the idea that the presence of lipid vesicle stage 2 oocytes at ULO favoured the further development of the pre-existing follicles rather than the formation of new ones. This is based on the findings that (1) the number of lipid vesicle stage 2 oocytes at ULO was correlated with the GSI 69 days after, and (2) the weight of the ovary after ULO was determined by the number of mid and late vitellogenic oocytes and not by the total number of oocytes present. In contrast, the positive correlation between the number of late perinucleolar oocytes at the time of ULO and the total number of oocytes 69 days after may indicate that the presence of such staged oocytes favoured the formation of additional oocytes. In fact, when blue tilapia females were hemi-castrated at the protoplasmic stage (*i.e.*, ovaries containing oocytes with vacuole free cytoplasm [perinucleolar stage] as most advanced stage), new recruitment of both primary and secondary oocytes and accelerated vitellogenic development were evident, which ultimately led to complete compensatory ovarian growth (Dadzie & Hyder 1976). Further studies in European sea bass are needed to determine if the ovary has sufficient plasticity to recruit a new full complement of oocytes after ULO and to identify unequivocally the critical stage of ovarian development when this may occur.

Endocrine responses to ULO

Pituitary gonadotropins, FSH and LH, acting through their respective receptors Fshr and Lhcgr, are the main direct regulators of teleost fish gonadal functions (Ge 2005, Lubzens *et al.* 2010). In this regard, the patterns of gene expression of gonadotropin subunits in the pituitary and gonadotropin receptors in the ovary of ULO females suggest that the FSH/Fshr-mediated signalling may constitute the central gonadotropic axis orchestrating the ovarian compensatory growth observed in response to ULO in

European sea bass. Nevertheless, the involvement of LH-dependent signalling in the process cannot be ruled out taking into account the higher *cga* levels measured in ULO females, which together with the relatively higher levels of expression of *lhb* seen in the pituitary could lead to an over-production of LH. In support of this hypothesis previous reports studying the effects of ULO in teleost fish have demonstrated the involvement of gonadotropins, particularly FSH, in the gonadal compensation observed in response to hemi-castration (Goswami & Sundaraj 1968b, Dadzie & Hyder 1976, Tyler *et al.* 1997).

Assessment of temporal profiles of plasma concentrations of E_2 and T showed no significant differences between operated and control fish, in spite of the removal of half the mass of the gonadal tissue resulting from hemi-castration. This suggests that, as pointed out previously for rainbow trout (Tyler *et al.* 1997), the dynamics of steroid synthesis and release by ovarian tissue of ULO females differed from that of the intact control fish; the production of sex steroids by the remaining ovary in the ULO fish must have far exceeded that in the intact fish. Star serves to translocate cholesterol into the mitochondria, a process referred to as the rate-limiting step in steroidogenesis (Stocco *et al.* 2005). Thus, the almost 2-fold higher amount of *star* transcripts in the ovary of ULO fish suggest involvement of Star in effecting the compensatory responses of circulating steroid concentrations in the hemi-castrated European sea bass. Previous studies have shown that FSH-dependent signalling is involved in the up-regulation of *star* transcription during teleost fish follicular development (Lubzens *et al.* 2010; Luckenbach *et al.* 2011), findings supporting a putative influence of the FSH/Fshr axis on Star actions in our study. In European sea bass females, expression of *star* is approximately 8-times higher in mid and late vitellogenic follicles compared to earlier stages (García-López *et al.*, unpublished) and thus, the elevated *star* mRNA levels

observed in ULO females could also constitute a reflection of the proportionally higher number of these type of follicles.

The up-regulation in the transcription of *gnrh1* in the brain and of *gnrhr2a* in the pituitary suggests the involvement of the GnRH system in the ovarian compensatory response in hemi-castrated European sea bass. Interestingly, Gnrh1 has been identified as the most relevant GnRH form controlling oocyte development and reproductive activity in several fish species (Holland *et al.* 1998, Andersson *et al.* 2001, Zmora *et al.* 2002, Okuzawa *et al.* 2003, Molés *et al.* 2007). Previous studies have reported overlapping expression of *gnrhr2a* with both *fshb* and *lhb* in pituitary cells of European sea bass (González-Martínez *et al.* 2004), findings supporting a putative influence of GnRH-derived signalling on gonadotropin subunits transcription and gonadotropin release. However, such an effect has only been verified experimentally in European sea bass for *cga* and *lhb* mRNAs and for LH secretion, while *fshb* transcription was shown to be unaffected by GnRH treatment (Forniés *et al.* 2002, Mateos *et al.* 2002) and no information for FSH release is presently available. These findings agree with data obtained for zebrafish (Lin & Ge 2009), although other studies have reported significant effects of GnRH treatments on pituitary *fshb* mRNA levels and FSH release in female teleost fish (Levavi-Sivan *et al.* 2010). Collectively such results suggest, on the one hand, the involvement of the Gnrh1/Gnrhr2a signalling cascade in the stimulation of *cga* transcription observed in the pituitary of hemi-castrated European sea bass while the involvement of such pathway in the up-regulation of *fshb* transcription also observed in our study remains to be proven experimentally.

Changes in the transcription levels of estrogen and androgen receptors in the brain and ovary of ULO females observed in our study suggest that modulation of cellular responsiveness to steroid stimulation was one of the mechanisms involved in the

289 regulatory response leading to the ovarian compensatory growth in European sea bass.
290 At the level of the ovary, up-regulation of *ara* transcription observed in ULO females
291 suggest some, as yet uncharacterized, role(s) of androgen-dependent signalling in
292 gonadal compensation. Unpublished own experiments showed that the number of
293 androgen and estrogen receptor transcripts varies differentially according to the follicle
294 stage in European sea bass, although the significance of such changes for teleost fish
295 follicular development remains to be established. In addition, estrogens and androgens
296 are able to exert negative and positive feedback effects on the activity of multiple
297 reproduction-relevant neural systems, including the GnRH producing neurons,
298 depending on the physiological-reproductive status of the animal (Klenke 2006, Zohar
299 *et al.* 2010). In the case of our study, it is tempting to hypothesize that the down-
300 regulation of *esr1* and *esr2a* in the brain of hemi-castrated European sea bass was part
301 of a steroidal feedback loop ultimately leading to the stimulation of *gnrh1* transcription.
302 Previous studies in stinging catfish submitted to ULO showed that administration of
303 estradiol benzoate (an estrogenic compound) effectively blocked the compensatory
304 growth of the remaining ovary (Goswami & Sundaraj 1968b). Collectively, these and
305 our data support the concept that correctly balanced steroid signalling is essential for the
306 proper compensatory development of oocytes after hemi-castration in teleost fish.
307 Expression of both *igf1* and *igf2* normally show progressive decreasing levels during
308 European sea bass oocyte development (García-López *et al.*, unpublished) but,
309 interestingly, here they showed opposing modulatory responses after ULO. This
310 indicates that their respective encoded proteins may serve disparate regulatory functions
311 within the compensatory ovarian growth in European sea bass. Although the specific
312 biological actions of Igf related peptides have not been clearly established in any fish
313 species, such functions could include the stimulation of primary oocyte growth, the

modulation of pituitary *fshb* expression, and/or the regulation of follicular steroidogenesis (Lin & Ge 2009, Lankford & Weber 2010, Reinecke 2010).

AMH has been shown to decrease follicular sensitivity to FSH as well as the expression of *Lhcgr* and *Cyp19a1a* in mouse ovary (Di Clemente *et al.* 1994, Durlinger *et al.* 2001). Accordingly, the three-fold down-regulation of *amh* transcripts observed in hemi-castrated European sea bass suggests that Amh plays a role within the local interplay ultimately leading to increased *fshr* mRNA levels observed after ULO and consequently to an increased sensitivity of follicles to FSH. Recombinant BMP4 has been found to modulate FSH signalling in a way that promotes E_2 production in cultured rat granulosa cells (Shimasaki *et al.* 2004). Hence, elevated *bmp4* transcript levels in hemi-castrated European sea bass suggest Bmp4 to be important in compensating steroid levels after ULO.

Finally, in the regulatory network of responses to ULO, ovarian transcription of *vtgr*, which mediates the endocytic uptake of vitellogenin through the oocyte membrane (Perazzolo *et al.* 1999; Hiramatsu *et al.* 2004), was down-regulated, while *lpl*, which is involved in supplying the oocyte with fatty acids (Ibáñez *et al.* 2008), was up-regulated. As *vtgr* transcripts decreases as translation of the receptor and its translocation to the oocyte membrane take place, *i.e.* as oocyte development progresses (Perazzolo *et al.* 1999; Hiramatsu *et al.* 2004; García-López *et al.*, unpublished), the responses observed for both *vtgr* and *lpl* could be associated with the fact that there were proportionally more follicles actively incorporating both vitellogenin and fatty acids in the ovary of hemi-castrated European sea bass (*i.e.*, mid and late vitellogenic follicles). Although the respective circulating levels of vitellogenin and fatty acids were not measured in our study, previous reports suggest that they may not be modified as circulating vitellogenin amounts do not change in response to ULO in rainbow trout (Tyler *et al.* 1996) and

plasma total lipid levels remain stable during vitellogenesis in European sea bass (Cerdà *et al.* 1995).

Conclusions

ULO in European sea bass at the late pre-vitellogenesis/early vitellogenesis stage resulted in a compensatory ovarian growth and this was the result of increased numbers of early perinucleolar oocytes and mid-late vitellogenic follicles, without an apparent recruitment of primary oocytes into the secondary growth phase. Targeted gene expression analyses across the brain-pituitary-ovary indicated that FSH/Fshr and the Gnrh1/Gnrhr2a signalling pathways acted as the main axes orchestrating the ovarian compensatory growth. Steroid receptors, steroidogenesis-related proteins, including Star, Igf related peptides, and members of the transforming growth factor, beta superfamily, such as Amh and Bmp4, were also identified as potentially relevant players in the compensatory response process, although any direct connections between them remain to be established. We have shown that the ULO technique provides a highly tractable approach for elucidating the interconnected endocrine and molecular mechanisms controlling oocyte development in teleost fish.

Material and Methods

Fish and general rearing and handling conditions

Pit-tagged adult female and male European sea bass (3-4 years old) were maintained in 5 m^3 tanks under natural photoperiod (Puerto Real, Cádiz, Spain) and constant temperature (18-20 °C). Fish were fed *ad libitum* on commercial dry pellets (Vitalis Repro, Skretting; Burgos, Spain) three days a week. Prior to sampling, fish were sedated with 2-phenoxyethanol at a 300 ppm dose in order to reduce handling stress. For

364 euthanasia, a lethal anaesthetic dose (600 ppm) was applied followed by decapitation of
365 the fish. All experimental procedures involving care and use of live animals were
366 carried out according to Spanish national and European bioethical regulations and were
367 approved by the CSIC Bioethical Committee.
368
369 ***Surgical procedures***
370 On 6th October 2009 (day 0), *i.e.* one month before the expected onset of vitellogenesis
371 (Prat *et al.* 1999), randomly selected females (mean total body weight: 1.77±0.09 kg)
372 were either submitted to ULO operation (n=8; ULO group), sham operated (n=4;
373 SHAM group), or euthanized (n=5; INITIAL control group). The starting point was
374 chosen in accordance to results in rainbow trout showing that the mid vitellogenesis was
375 the critical developmental stage for inducing a new recruitment of primary oocytes in
376 the remaining ovarian lobe after ULO (Tyler *et al.* 1994, 1996). One ULO female died
377 within 24 hours following the operation, but no further mortalities occurred during the
378 time-course of the experiment.
379 Ovarian lobes, whole brain, and pituitary were excised from fish in the INITIAL group.
380 Extraction of the right ovary in females of the ULO group was accomplished by two 3-4
381 cm longitudinal incisions made into the abdominal cavity. A cephalic incision was made
382 in order to ligate and cut the vessel connecting the liver and the ovary and to detach the
383 anterior part of the gonad from the body wall. Then, the whole right ovarian lobe was
384 eased outwards through the caudal incision, the joint with the left lobe was clamped
385 with haemostatic forceps, and finally the ovary was excised fully. Following this
386 procedure, blood loss was minimal and thus, no cauterization was required. Fish in the
387 SHAM group received only one incision into the abdominal cavity. All incisions were
388 closed with non-absorbable silk sutures, which were removed 21 days later as healing of

389 the incisions proceeded correctly and no herniation was evident.

390 Sixty nine days after operations, on 14th December 2009, the experiment was finished;
391 females in the ULO and SHAM groups were euthanized and ovarian lobes, whole brain
392 and pituitary dissected out. The end point of the experiment was chosen in accordance
393 to the vitellogenic growth period in European sea bass (Prat *et al.* 1999); *i.e.* and at the
394 time when most of the oocytes of the first main egg clutch are expected to have reached
395 the mid-late vitellogenesis stage (500-800 µm of diameter; Mayer *et al.* 1990; Asturiano
396 *et al.* 2000).

397 Ovarian lobes collected at days 0 and 69 days after surgery were weighed individually,
398 their volume determined by water displacement, and divided into several subsamples
399 for histological and molecular analyses. Since no differences in weight or volume
400 between both ovarian lobes were found in females of the INITIAL and SHAM groups
401 (results not shown), total ovarian weight and volume of ULO fish at the time of
402 operations was calculated by doubling the value obtained from the single ovarian lobe
403 dissected. At sixty nine days, the weight of the single ovarian lobe was considered as
404 total gonad weight in ULO females. Twenty five days before surgery and thereafter at
405 21-26 days intervals until the termination of the experiment (see dates in Fig. 2), fish
406 were weighed and blood sampled. The GSI was calculated as 100 × total gonad weight
407 × total body weight^{-1}.

408

409 ***Histological and stereological procedures***

410 Ovarian fragments were processed for plastic embedding and histological examination
411 as described in García-López *et al.* (2007), except that the tissue was fixed in 4%
412 glutaraldehyde in phosphate buffer saline pH 7.4 at 4ºC for 24 hours.

413 To estimate the total number of oocytes at each developmental stage, the stereological

414 method of Weibel (1979) as described in Medina *et al.* (2002) and García-López *et al.*
415 (2007) was applied. Oocyte classification was based on Mayer *et al.* (1988) and
416 included the following stages: early perinucleolar (20-50 µm of diameter), late
417 perinucleolar (50-110 µm of diameter), lipid vesicles stage 1 (100-160 µm of diameter),
418 lipid vesicles stage 2 (140-250 µm of diameter), early vitellogenic (250-400 µm of
419 diameter), mid vitellogenic (400-600 µm of diameter), and late vitellogenic (600-800
420 µm of diameter). Ovarian fragments analyzed were taken from the central part of each
421 lobe and regarded as being representative for the whole ovarian lobe (no longitudinal
422 differences in oocyte composition are found in European sea bass ovary; Navas 2003).
423 No significant differences in oocyte composition between the left and right ovarian
424 lobes were found in the fish of the INITIAL and SHAM groups (not shown) and thus,
425 the total oocyte number of the ULO operated fish at 0 days was calculated by doubling
426 the values obtained for the excised ovary. For ULO females at 69 days after surgery, the
427 oocyte number in the left ovary was considered as the total number of oocytes. Data are
428 reported as total numbers of oocytes at different stages of follicular development
429 corrected for the total body weight of the fish ("relative fecundity"). Thus, total
430 numbers of oocytes at 69 days after surgery were calculated taking into account both
431 ovarian lobes in SHAM females but only the remaining ovarian lobe in ULO fish.
432
433 ***Steroid immunoassays***
434 Plasma was obtained by centrifugation (3000 ×g; 4ºC; 10 min) of blood samples and
435 stored at -80ºC. Levels of E_2 and T in plasma were then measured by commercially
436 available enzyme immunoassays (Cayman Chemical Company, Ann Arbor, MI, USA)
437 validated for their use in European sea bass in our laboratory (results not shown). The
438 sensitivity of the assays, defined as 90% binding, were 98 pg ml^{-1} for the E_2 assay and

439 55 pg ml^{-1} for the T assay. Respective inter-assay coefficients of variation (calculated at
440 50% binding) were 10.4% and 4.2%.
441
442 ***Gene expression analyses***
443 Brain, pituitary, and ovary samples were snap frozen in liquid nitrogen and stored at -
444 80ºC. Total RNA was extracted using the NucleoSpin® RNA II and RNA XS kits
445 (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. RNA
446 integrity was checked using the Agilent RNA 6000 Nano kit in a Agilent 2100
447 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), while RNA quantity and
448 purity were analyzed spectrophotometrically.
449 For each sample, 500 ng of total RNA was transcribed into cDNA using the qScript™
450 cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, USA). Reverse
451 transcription products were diluted to 1/40, and 8 µl were used for each real-time PCR
452 reaction, which were performed in duplicate measurements on an ep realplex^2
453 Mastercycler (Eppendorf, Hamburg, Germany) using primer pairs specifically designed
454 for each target gene (100-400 nM; Table 2) and PerfeCTa™ SYBR® Green FastMix™
455 reaction mix (Quanta Biosciences). PCR conditions were: initial denaturation at 95ºC
456 for 5 min, 40 amplification cycles with one step at 95ºC for 15 s followed by a second
457 step at 60ºC for 30 s. To correct for variability in amplification efficiency among
458 samples, standard curves were prepared for each tissue analyzed by serially diluting
459 cDNA samples obtained from retro-transcribed total RNA pools of all experimental
460 samples. The relative amount of each transcript for each sample was determined from
461 the corresponding standard curve.
462 For data normalization, expression levels of three previously used endogenous reference
463 genes in European sea bass tissues were measured: *18S ribosomal RNA* (*18s*),

elongation factor 1-alpha (*ef1a*), and *bactin* (*e.g.*, Blázquez *et al.* 2008, Mitter *et al.* 2009, Rocha *et al.* 2009). However, since none of them showed a constitutive expression among the experimental samples analyzed, we opted for reporting non-normalized relative amounts for every target gene studied. This strategy is supported by studies showing opposite trends between normalized and non-normalized data (Neuvians *et al.* 2005) in addition to contradictory results depending on the reference gene used for the same data set (Zhang *et al.* 2003).

Statistical Analyses

Significant differences between groups were identified by the Student´s unpaired t-test (comparison between two groups) or by two-way ANOVA followed by the Bonferroni post test (comparison among multiple groups along time). When necessary, data were log-transformed in order to comply with normality. Correlations between different parameters were evaluated by linear regression analysis. All statistical analyses were carried out adopting a significance level (P) of 0.05.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the Spanish Ministry of Education and Science (grant number AGL2007-61192/ACU). Á.G.-L. was supported by a contract from the CSIC-JAE program.

Acknowledgements

Authors would like to acknowledge S. Halm for her valuable comments on the manuscript as well as G. Martínez-Rodríguez, J.L. Coello, and R. Osta for their collaboration during fish maintenance and sample collection and analysis.

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661 **Figure legends**

662 **Figure 1** Morphological and morphometrical changes in European sea bass ovary in
663 response to unilateral ovariectomy (ULO). Bars in panels A, B, D, and E represent the
664 mean ± SEM. Panel C shows the macroscopic view of ovaries collected 69 days after
665 surgery. Panels D and E represent the total number of oocytes at different stages of
666 follicular development corrected for total body weight of the fish. For INITIAL (0 days)
667 and SHAM females (69 days after surgery), the total oocyte number was calculated by
668 summing up the oocyte number contained in both ovarian lobes. For ULO females, the
669 total oocyte number at 0 days was calculated by doubling the values obtained for the
670 excised ovary, while at 69 days after surgery, the oocyte number in the remaining left
671 ovary was considered as the total number of oocytes. White bars represent data from
672 INITIAL group at 0 days and from SHAM group at 69 days. For sake of clarity,
673 numbers of each follicular stage are shown using different potentials (indicated in the
674 top of the panels). GSI, gonadosomatic index; Epn, early perinucleolar; Lpn, late
675 perinucleolar; Lv1, lipid vesicles stage 1; Lv2, lipid vesicles stage 2; Evit, early
676 vitellogenic; Mvit, mid vitellogenic; Lvit, late vitellogenic. An asterisk denotes a
677 significant difference compared to the respective parameter in INITIAL-SHAM females
678 at each sampling date ($P<0.05$). Scale bars: 2 cm (C); 200 µm (F, G, and H).

679

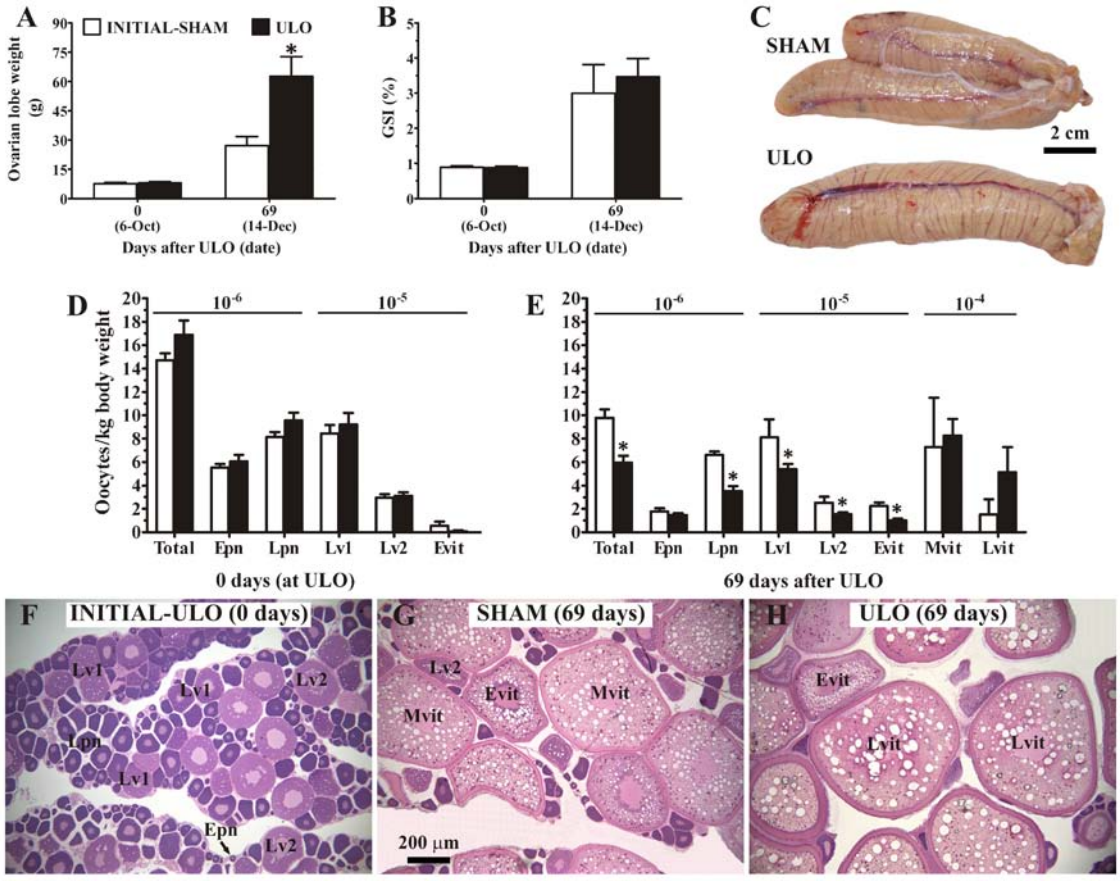
680 **Figure 2** Time course of plasma levels of 17β-estradiol (E_2) and testosterone (T) in
681 response to unilateral ovariectomy (ULO) in European sea bass. Values are expressed as
682 mean ± SEM. White bars represent combined data from INITIAL and SHAM groups at
683 -25 and 0 days and only from SHAM group at 21, 47 and 69 days. Different letters
684 denote significant differences during the experimental period within each group
685 ($P<0.05$). No significant differences between the two groups were found at any

sampling point ($P<0.05$).

Figure 3 Expression of target genes (shown in the individual panels) in the ovary of European sea bass in response to unilateral ovariectomy (ULO). For each gene, the expression levels at 0 and 69 days the after operations are indicated in arbitrary units (mean ± SEM). White bars represent data from INITIAL group at 0 days and from SHAM group at 69 days. An asterisk denotes a significant difference compared to the respective parameter in INITIAL-SHAM operated females at each sampling date ($P<0.05$).

Figure 4 Expression of target genes (shown in the individual panels) in the pituitary of European sea bass in response to unilateral ovariectomy (ULO). For each gene, the expression levels at 0 and 69 days after the operations are indicated in arbitrary units (mean ± SEM). White bars represent data from INITIAL group at 0 days and from SHAM group at 69 days. An asterisk denotes a significant difference compared to the respective parameter in SHAM operated females at 69 days after the operations ($P<0.05$).

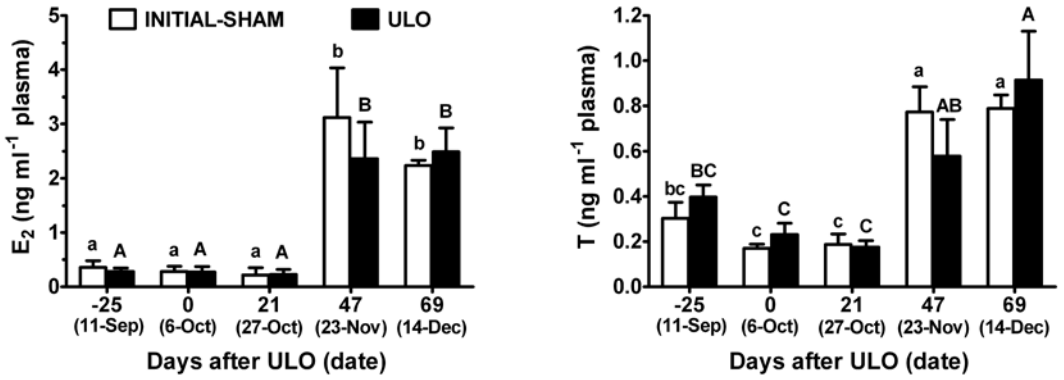
Figure 5 Expression of target genes (shown in the individual panels) in the brain of European sea bass in response to unilateral ovariectomy (ULO). For each gene, the expression levels at 0 and 69 days after the operations are indicated in arbitrary units (mean ± SEM). White bars represent data from INITIAL group at 0 days and from SHAM group at 69 days. An asterisk denotes a significant difference compared to the respective parameter in SHAM operated females at 69 days after the operations ($P<0.05$).



711

712 Figure 1

713



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715 Figure 2

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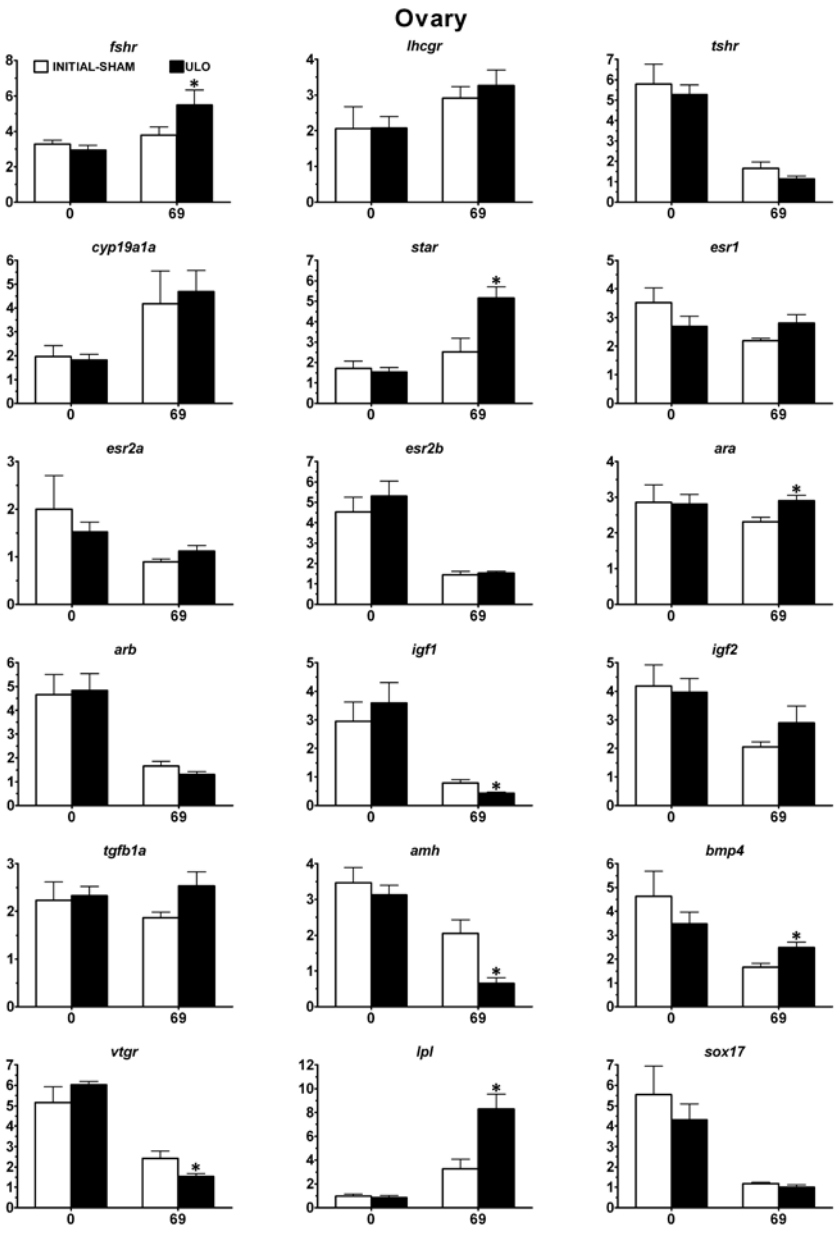
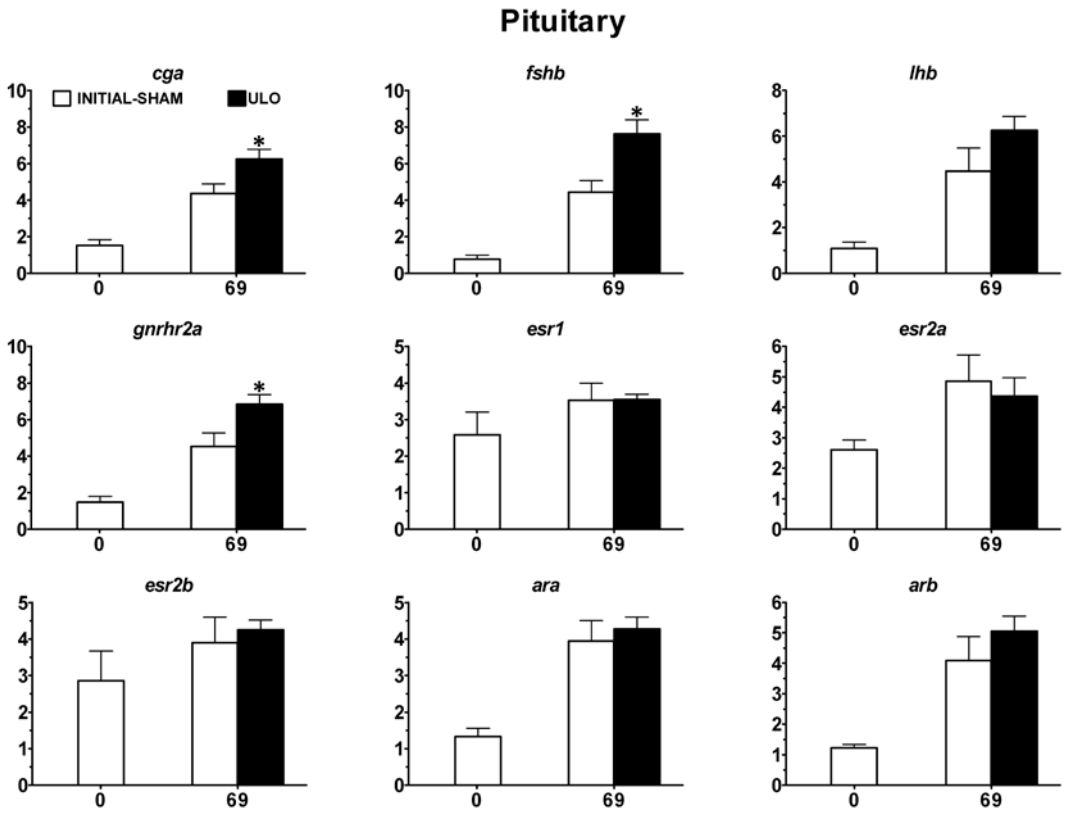

Ovary
fshr
INITIAL-SHAM
ULO
lhcgr
tshr
cyp19a1a
star
esr1
esr2a
esr2b
ara
arb
igf1
igf2
tgfb1a
amh
bmp4
vtgr
lpl
sox17
0
69

Figure 3


Pituitary
cga
INITIAL-SHAM
ULO
fshb
lhb
gnrhr2a
esr1
esr2a
esr2b
ara
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0
69
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Figure 4

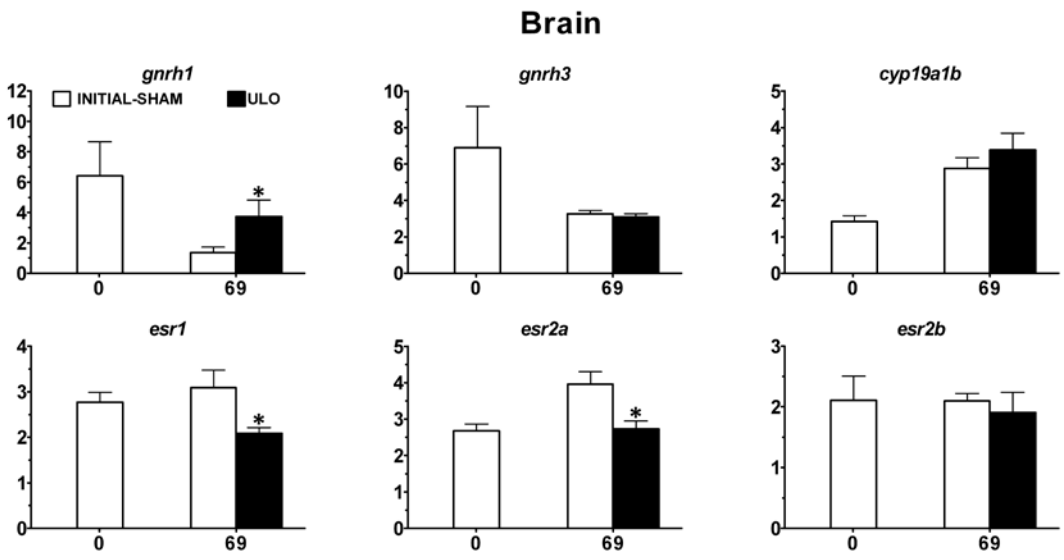
Brain
gnrh1
INITIAL-SHAM
ULO
gnrh3
cyp19a1b
esr1
esr2a
esr2b
0
69

Figure 5