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Vg mRNA induction in an endangered fish species (*Anguilla anguilla*) from the Loire estuary (France)

Isabelle Blanchet-Letrouvé [a,*], Anne-Gaëlle Lafont [b], Laurence Poirier [a], Sylvie Baloche [b], Aurore Zalouk-Vergnoux [a], Sylvie Dufour [b], Catherine Mouneyrac [c]

[a] Université de Nantes, MMS, EA 2160, 9 rue Bias, F-44035 Nantes, France
[b] Muséum National d'Histoire Naturelle, UMR BOREA, UPMC, CNRS 7208, IRD 207, 7 rue Cuvier, CP 32, F-75231 Paris, France
[c] UCO, MMS, EA 2160, F-49000 Angers, France

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ABSTRACT

Estuarine zones are extremely fragile due to increasing stress from anthropogenic activities. Among those, the Loire estuary (France) is potentially exposed to various contaminants including Endocrine Disruptors Compounds (EDCs) able to impact the reproduction physiology of fish. The European eel (*Anguilla anguilla*), endangered fish species, is apparently not relevant, in its yellow stage, to monitor the effects of endocrine disruption. Despite this weakly responsiveness, this study aimed to investigate whether European eel from the Loire estuary may still be the subject of estrogenic disruption quantifying the hepatic Vg gene expression according to gender and sexual stage. Vitellogenin (Vg) appears as a valuable biomarker of EDCs, as well as for exposure and effects. Quantitative real-time Reverse Transcription Polymerase Chain Reaction (q RT PCR) was used in this study to amplify responses of hepatic Vg transcripts. European eels were sampled in May 2009 ($N=57$) and November 2010 (during the downstream migration, $N=10$) in two sites of the Loire estuary with different ecological conditions and contamination pressures (upstream: Varades; downstream: Nantes). Reproductive (gender, sexual maturity stage) and biometric parameters of collected eels were determined. A laboratory exposure of silver male to steroid hormones (Testosterone (T), 11-KetoTestosterone (11-KT), Estradiol (E2)) was conducted in parallel to validate the q RT PCR approach on hepatic Vg mRNA. Results demonstrated the responsiveness of exposed silver male eels, since hepatic mRNA Vg induction was observed in E2 treated males compared to control specimens. In the field, results of female silver eels reflected large inter-individual differences in the activation of hepatic Vg at silvering. However, while only female silver eels should express hepatic Vg mRNA, quantifiable levels were also detected in a proportion of 38% of the other individuals sampled, normally not inclined to express it, those being undifferentiated eels, yellow females, yellow and silver males. According to each sexual stage, no difference of expression was observed between eels from the two sampling sites. Histological results as well as low Vg mRNA levels detected do not permit a conclusion as to a potential effect of endocrine disruption.

1. Introduction

Estuaries represent ecosystems of high productivity and are crucial in the life histories of many fish, invertebrates, birds, etc. (Mc Lusky, 1989). However, estuarine zones are extremely fragile due to increasing stress from anthropogenic activities. Thereby, complex mixtures of contaminants can enter the aquatic environment *via* effluents or surface runoff, and thus aquatic organisms such as fish are inevitably exposed. Among estuarine fish species, European eels (*Anguilla anguilla*) are in dramatic decline (Dekker, 2003; Robinet and Feunteun, 2002). Overfishing, climate change, habitat reduction and numerous hydraulic barriers to the downstream and upstream migrations, diseases and parasitic infection can in part explain this decline (Dekker, 2003; Elie and Girard, 2009). In addition, this fatty species is characterized by a high bioaccumulation potential for organic pollutants such as Endocrine Disrupting Compounds (EDCs), which may impair eel reproduction and further threaten their population (Geeraerts and Belpaire, 2010) or heavy metals (Pierron et al., 2008a). In this context, there is a growing interest to study the influence of EDCs on biota physiology. Numerous natural and/or chemical compounds can exert endocrine disruptions and the European Community has established a list of more than 500 known or suspected EDCs (Santos et al., 2010). For example, Polychlorobiphenyls (PCBs), dioxin like

* Corresponding author.
E-mail address: isabelle.blanchet-letrouve@ac-nantes.fr (I. Blanchet-Letrouvé).

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compounds (PCDDs, PCDFs), organochlorine pesticides, Polycyclic Aromatic Hydrocarbons (PAHs), alkylphenols, and also plasticizers such as phthalates, are recognized as EDCs which could affect sexual maturation, reproduction and development of organisms living in impacted environments as reviewed by van der Oost et al. (2003). Even if the cause-effect relationship is difficult to establish due to the complexity of the endocrine system and the diversity of modes of action of EDCs, their negative influence has been reported in fish from different countries (Hinfray et al., 2010; Jobling et al., 2005).

So far, different techniques revealing exposure to EDCs, especially in aquatic organisms, have been developed. Among them, vitellogenin (Vg) which is a female-specific yolk protein precursor and energy reserve of eggs in oviparous vertebrates appears as an excellent and valuable biomarker, as well as for both exposure and effects (Matozzo et al., 2008). Vg is synthesized, lipidated, phosphorylated and glycosylated in the liver; then, it is released into the blood and taken up by the oocytes through specific receptors (Barucca et al., 2006). Mature females produce Vg in response to the hormone 17 β-estradiol, while males and juveniles do not normally produce Vg. So, its presence in males, undifferentiated eels or juveniles is largely used as a specific biomarker for endocrine disruption in the aquatic environment in Teleost fish (van der Oost et al., 2003). Different methods such as Enzyme-Linked Immuno Sorbent Assay (ELISA) and Radio Immuno Assay (RIA) allow the Vg quantification in Teleosts but these assays are limited in their use due to the need for species-specific antibodies. Among the Teleosts, the European eel is characterized by weak plasma Vg levels often undetectable in individuals from the field. This is the result of its peculiar biological life cycle and its gonochoristic and semelparous status. Thus, at proteomic level, low responses to estrogenic exposure have been reported in yellow eels by several authors whichever exposure of organisms, in the field or in vivo, and whatever the technique used to measure Vg (ELISA, RIA) (Burzawa-Gerard and Dumas-Vidal, 1991a; Burzawa-Gerard et al., 1991b; Versonnen et al., 2004). Their low responsiveness led several authors to conclude that yellow eels are not suitable sentinels for estrogenic endocrine disruption (for review: Geeraerts and Belpaire, 2010). However, at the transcriptomic level, several studies underlying that the use of eels as bioindicator species of estrogenic exposure is progressively increasing (Barucca et al., 2006; Gorbi and Regoli; 2005). Indeed, Vg mRNA could be detected by PCR in subadult yellow *A. anguilla* injected with 17-β estradiol, confirming their responsiveness to estrogenic EDCs (Barucca et al., 2006). This approach could be more promising than the proteomic level to evaluate EDCs exposure in yellow eels. Furthermore, hepatic Vg mRNA expression already appeared as a suitable approach, in other fish species, since levels of mRNA rise rapidly following Vg gene induction, revealing recent exposure to estrogenic contaminants (Bowman et al., 2000).

In this context, the aim of the present study was to quantify the hepatic Vg gene expression in European eels from the Loire estuary according to gender and sexual stage and to validate the method of the q RT PCR for this evaluation. The Loire estuary runs through important urban sites (Nantes, Saint-Nazaire) with shipping, industrial and agricultural activities. Moreover, the Loire basin (117,800 km^2) represents more than 1/5 of the French territory and drains many tributaries. This estuary displays a diffusive pollution including a mixture of contaminants such as heavy metals, pesticides, PAHs and PCBs (Marchand et al., 2004; Robbe et al., 1985). Yellow and silver eels were sampled at two locations, downstream and upstream of the estuary. A set of biometrical measurements was applied to characterize the morphological and physiological stages of sampled eels according to Durif et al. (2005). The hepatic Vg gene expression was evaluated in each individual using quantitative real-time Reverse Transcription Polymerase Chain Reaction (q RT PCR) method to ensure a highly sensitive detection of these transcripts. Histological analyses of gonads were also performed to determine the sex and the maturation stage of all sampled fish and eventually to detect abnormalities. In addition, in vivo experimental exposure of male silver eels to various steroid hormones (Testosterone, T; 11-Ketotestosterone, 11-KT; β-Estradiol, E2) was performed to validate the estrogenic specificity of Vg mRNA induction.

2. Materials and methods

2.1. Field sampling

European eels were caught by local fishermen in the Loire estuary located on the French Atlantic coast. Two sampling sites were selected: Varades (399 155.06-6709048.78) corresponding to the upstream site at the salinity limit, and Nantes (299 429.87-6694726.54), the downstream site, located at 100 km and 50 km from the open sea, respectively (Fig. 1). The Nantes site corresponds mainly to an industrial harbor and urban zone including two incineration factories (Agence de l'eau Loire-Bretagne, 2012) whereas the Varades site is under agriculture pressure. Eels were randomly collected using fyke nets at both sampling sites (Varades, $n=27$; Nantes, $n=30$) in May 2009. In addition, during the downstream run in November 2010, migrant eels ($n=10$) with apparent silver coloration skin were caught with stow nets.

After collection, eels were transported to the laboratory in aerated 200 L tanks containing water from the collection site. At the laboratory, they were maintained in a natural photoperiod (L15/D9) and temperature of 12 ± 2 °C, within the range of the fishing site temperatures.

The purpose of the experimental design of the 2 sampling sites was to compare the two different ecological conditions and contamination pressures according to the Vg m RNA expression. Indeed, even though the European eels can travel long distances, it could suggest than they could settle in a favorable terms area as well. The sampling at 2 locations distant from 50 km and under different pressures permit to evaluate the site effect potentially linking to exposure differences. Indeed, higher levels of persistent organic pollutants such as non dioxin like PCB (congeners # 28, 52, 101, 138, 153 and 180) were observed in yellow eels from the Nantes site (mean of 534 ± 203 ng g^{-1} dw) compared to those of the Varades site (mean of 240 ± 60.6 ng g^{-1} dw) (Blanchet-Letrouvé et al., 2013). Furthermore, Couderc et al. (2013) showed higher polybrominated diphenyl ether (PBDE) and perfluorinated compound (PFC) levels in eels from Nantes ($\sum$PBDEs$=12.9 \pm 6.15$ ng g^{-1} dw and $\sum$PFCs$=123 \pm 104$ ng g^{-1} dw) than individuals from Varades ($\sum$PBDEs$=2.18 \pm 2.28$ ng g^{-1} dw and $\sum$PFCs$=88.4 \pm 71.9$ ng g^{-1} dw).

Animal manipulations were performed according to the guidelines of the French Ethical Committee and under the supervision of authorized investigators. Eels were anaesthetized in a 10 L water bath to which was added 1.5 or 2 mL of clove oil solution dissolved in ethanol (70%), in function of water temperature and eel size (Palstra et al., 2007).

2.2. Morphometric and biometric parameters

Eel body length (mm) and weight (g) were measured. A sample (around 50 mg) of liver was quickly dissected with sterile scalpel on RNase free drainboard, stored in sterile Eppendorff containing 1 mL of RNA Later solution (Ambion Inc., Austin, TX, USA), and kept frozen at −20 °C until extraction.

Gonads and digestive tract were removed delicately and three pieces from the anterior, medium and posterior sections of the gonads were fixed in Bouin solution for histological analysis. Pectoral fin length (PFL mm), horizontal (EDh mm) and vertical eye diameters (EDv mm) on both eyes, were measured using a digital calliper. The following indices were calculated according to Pankhurst and Lythgoe (1983):

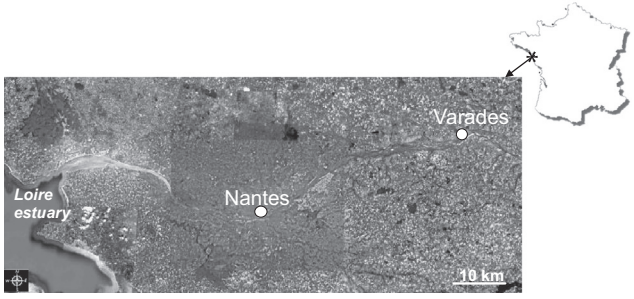

Fig. 1. Collection sites.

Ocular Index: $OI = 100 \times (((EDh+EDv) \times 0.25)^{2\square} \times (10 \times BL)^{-1})$
Gonado Somatic Index: $GSI = 100 \times (\text{gonad weight} \times (\text{total BW})^{-1})$
Pectoral Fin Index: $PFI = 100 \times (PFL \times (\text{total BL})^{-1})$
Digestive Tract Index: $DTI = 100 \times (\text{digestive tract weight} \times (\text{total BW})^{-1})$

Using the biometric data of the studied organisms, a condition factor K (Fulton Index) was calculated according to Ricker (1975):

$$K = (BW \times 10^5)/BL^3$$

where BW and BL respectively expressed as g and mm. This index allows the evaluation of the health condition of eels.

The Silver Index (SI) has been calculated according to Durif et al. (2005). This index consisted of five stages for female eels, represented by stages FI and FII (corresponding to the growth phase), stage FIII (a premigrant stage characterized by the beginning of gonad development), stage FIV (marked by the onset of the starvation and the gonadotropin production) and stage FV (corresponding to the digestive tract regression, pectoral fin elongation and higher gonadotropin levels, characteristic of migrating stages for female eels). Two stages were proposed for males, MI (a resident sexually undifferentiated stage) and MII (a migratory stage). This classification allows a more precise and ecological description of the stages "yellow" and "silver" and could be used for the quantification of potential spawners from a catchment basin (Durif et al., 2009).

The otoliths enabled a determination of the age (A) of the organisms according to Lecomte-Finiger (1985). The largest pair of otoliths named sagitta were removed from the eel's head. After extraction, otoliths were cleaned of all organic membranes using distilled water, dried with ethanol, and then stored in Eppendorf tubes. The otoliths were later embedded in synthetic resin (Synolithe), and then

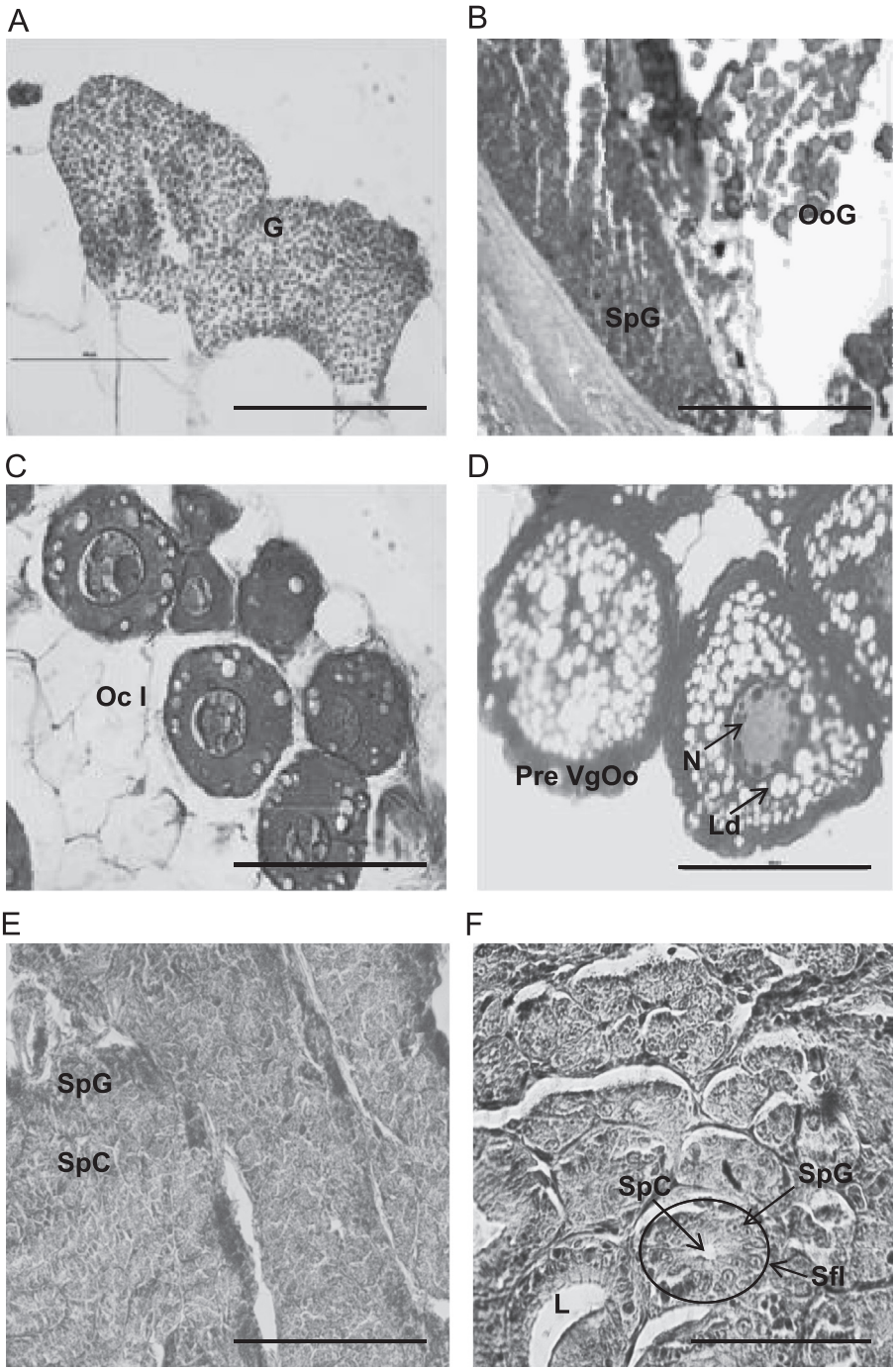

Fig. 2. Photomicrographs of histological sections stained with hematoxylin and eosin of testis and ovaries from European eels of the Loire estuary. **A:** Syrski organ with gonia (G): undifferentiated eel. **B:** intersexual gonad with spermatogonia (SpG) and oogonia (OoG): undifferentiated yellow eel. **C:** Primary Oocytes (Oc I) of yellow female with few lipid droplets (Ld). **D:** growing previtellogenic oocytes (Pre VgOo): silver female. **E:** Testis at stage I with spermatogonia and spermatocytes (SpC): yellow male. **F:** Testis at stage II, silver male. N : nucleoli; L: Leydig cell; Sfl : seminiferous lobule. Scale bars: 100 μm (A; B; C; D; F); 400 μm (E).

polished to the nucleus with a polishing wheel (Streuers Rotopol-35) using two different grits sand paper (grade 1200 and 2400). Fine polishing was done by hand with alumina (1 μm grain) on a polishing cloth. Etching was done using 10% EDTA. A drop of this solution was applied on the mold during fifteen minutes. The otoliths were then rinsed with distilled water and stored in dry condition. Yearly increments were revealed by staining with a drop of 5% Toluidine blue on the otolith and letting it dry. Growth rings were then counted under binocular magnifier. The age of each eel was determined by the number of increments starting from the nucleus which was considered as year 1 of the eel's life. The age used in the statistical analysis was determined from three readings by three different researchers. The otolithometry was performed in partnership with the IRSTEA, Cestas, France.

2.3. Gonad histology

Fixed sections of gonads were dehydrated, paraffin-embedded and cut (5 μm) with a microtome. Thin sections were colored with haemalin-eosin before observation with a light microscope coupled to an image analyzer to determine gender, reproductive development stage (undifferentiated, yellow, silver) according to previous studies (Huertas et al., 2006).

Sexual gender determination and gonad stages were evaluated macroscopically and microscopically (Fig. 2). The absence of oocytes and the presence of clusters of spermatogonia were used as criterion to discriminate the male gonad from late intersexual gonads considered in this study to correspond to the undifferentiated stage.

2.4. In vivo steroid hormones exposure

For the steroid exposure experiment, thirty-six male European silver eels (body length (BL) 400.2 ± 3.7 mm, body weight (BW) 85.14 ± 2.40 g) were purchased from Rungis International Market, France, transferred to the laboratory (MNHN, Paris, France) and kept in running aerated freshwater tanks under natural photoperiod and temperature (15 ± 2 °C). They were not fed, as they are naturally fasting at the silver stage.

Testosterone (4-Androsten-17β-ol-3-one), 11-ketotestosterone (Teleost-specific non aromatizable androgen, 4-Androsten-17β-ol-3,11-dione) and β-Estradiol (1,3,5 [10]-Estratriene-3,17β-diol) were purchased from Sigma-Aldrich (Lyon, France).

The thirty-six male silver eels were divided into four different groups ($n=8$–10 per group; 4 or 5 eels per 100 L tank fitted with a water recirculation system) and acclimated during four weeks before treatment. Each eel received one perivisceral injection per week of T, 11-KT or E2 (2 μg/g) BW, suspended in saline (0.9% NaCl) or of saline alone (controls) according to our previous protocol (Aroua et al., 2007). This protocol was previously used to investigate steroid feedback mechanisms on various brain and pituitary targets in eels (Aroua et al., 2007, Montero et al., 1995; Weltzien et al., 2006). This protocol and this level of injection give a stable and physiologically relevant plasma concentration (from 10 to 80 ng ml^{-1}) of the injected steroids after 6–8 weeks. On the sixth week, eels were killed by decapitation. Liver samples were quickly removed and stored in RNA Later at −20 °C until extraction.

2.5. Tissue RNA extraction and cDNA synthesis

RNA extraction and cDNA synthesis were performed as previously described (Aroua et al., 2007). Total RNA from liver samples was extracted using Trizol reagent (Invitrogen SARL, Cergy Pontoise, France) according to the manufacturer's instructions. Samples were homogenized using the TissueLyser system (Quiagen, Hilden, Germany). Following extraction, samples were submitted to desoxyribonuclease I (Roche Ltd., Meylan, France) treatment. First strand cDNA was synthesized using 1 μg of total RNA and a Superscript III First Strand cDNA Synthesis Kit (Invitrogen), according to the manufacturer's instructions. cDNA was stored at −20 °C for use in q RT PCR studies.

2.6. Quantitative real-time PCR (q RT PCR)

Gene specific primers (Table 1) previously designed for European eel beta-actin and Vg (Pierron et al., 2009) were purchased from Eurofins MWG Operon (Ebersberg, Germany).

Quantitative assays of eel beta-actin and Vg gene expression were set up using the Light Cycler 2.0 Platform (Roche) with Light Cycler FastStart DNA MasterPlus SYBR Green I sequence-unspecific detection kit (Roche). The PCRs were prepared with 4 μL of diluted cDNA template, 2 μL of PCR grade water, 2 μL of SYBR Green master mix and 1 μL of each forward and reverse primer (500 nM each at final concentration). q RT PCRs for both genes were performed as follows: an initial step of polymerase activation for 10 min at 95 °C; then 41 cycles with 10 s at 95 °C for denaturation, 5 s at 60 °C for annealing, 10 s at 72 °C for primer extension. Directly after the amplification phase, a melting curve analysis was carried out to confirm the presence of a single PCR product. Serial dilutions of a pool of cDNAs from liver samples were used as a standard and individual samples were assayed in duplicate. Each PCR run contained a non-template control and a calibrator to adjust the assay variations. Relative expression levels were calculated as previously described (Weltzien et al., 2005). The efficiency of all primers was tested and the specificity of each reaction was assessed by melting curve analysis to ensure the presence of only one product, and by sequencing. The stability of the expression of beta-actin was checked and validated between the different groups for the in vivo experiment and according to each sexual stage for feral individuals (ANOVA, p-value≥0.23). Therefore, Vg transcript levels were normalized to the reference gene (beta-actin) RNA levels. Vg/actin ratios were expressed as Arbitrary Units (AU).

2.7. Statistical analysis

Results from the steroid-treatment experiment are given as mean ± SD. Means were compared by Kruskall–Wallis non parametric ANOVA followed by Dunn's multiple comparison test, using Instat (GraphPad Software Inc., San Diego, CA, USA).

Mean Vg mRNA expression of feral individuals were compared by sexual stage and sampling site using Kruskall–Wallis non parametric ANOVA. Correlations with biometric parameters were analyzed by principal component analysis. XLSTAT and STATISTICA softwares were used for these statistical analyses.

3. Results

3.1. Effect of steroid treatment on liver Vg mRNA levels in male silver eels

No mortality occurred during the five weeks of experiment. Vg transcripts were not detectable by q RT PCR in the liver of all control (saline injected) male silver eels. Low Vg mRNA levels could be detected in the liver of two T-treated males (0.5 and 1.4 AU) and one 11-KT treated male (16AU), while Vg transcripts remained undetectable in the other androgen-treated males. No significant changes in mean Vg mRNA levels were induced by T nor by 11-KT treatments (0.25 ± 0.52 and 2.08 ± 5.89 AU compared to controls 0.00 ± 0.00 AU, $p > 0.265$ and $p > 0.509$ respectively). In contrast, E2 treatment induced a large and significant increase in mean Vg mRNA levels (22534 ± 42142 AU; $p < 0.0001$ compared to controls), but with striking individual variations (from undetectable levels (< 0.4 AU) to more than 120000 AU). A statistical analysis (PCA, not shown) was performed to analyze eventual relationships between Vg mRNA levels and morphometrics of the individuals, such as BL, BW, GSI, DTSI and OI but no correlation of variables could explain this variability.

3.2. Biometric indices, sex sexual stage and silver index of eels sampled in the Loire estuary

Sixty seven European eels were sampled in two locations of the Loire estuary (Varades and Nantes). Their BL ranged between 258

Table 1
Sequences of the primers (actin and Vg) and accession number of the fragment amplified.

Gene	Primers	5′-3′ Sequence	Tm (°C)	Amplicon size (bp)	Reference
Actin-b	Actin-b F	AGTATTTGCGCTCGGGTG	58.25	225	Aroua et al., 2007
	Actin-b R	CAGCCTTCCTTCCTGGGT	58.60		
Vg	Vg F	CCTACCACCAGCTTACCTTATG	62.67	220	Pierron et al., 2008a, 2008b
	Vg R	CGCTGGGAGTGCGGAA	61.84		

and 1014 mm (Table 2). Among these, 30 yellow females, 4 silver females, 16 yellow males and 7 undifferentiated eels were caught in May 2009 while 8 silver females and 2 silver males were caught in November 2010 during the downstream migration. The body length of silver males was smaller than that of silver females in agreement with the strong sexual dimorphism of the species.

Sexual gender determination and gonad stages were evaluated macroscopically and microscopically (Fig. 2).

Concerning testis, the stage I corresponds to single or clusters of spermatogonia and the tubular structure appears not yet developed (Fig. 2E), whereas at stage II, clusters of spermatogonia are grouped into tubules with large luminae (Fig. 2F). In the present study, the stage I corresponds to the yellow male eels caught in May 2009, the stage II to the male silver eels running down the estuary and caught in November 2010.

At stage III, tubules have enlarged and begin to break down; lumen of some tubules are filled with spermatozoa, intertubular connective tissue is partly reduced and only spermatocytes, spermatids and spermatozoa are present. However, these maturation sexual silver stages are only known at date according to an artificial induction of gonad maturation by hormonal injections (Palstra and van den Thillart, 2009).

Concerning ovaries, several oocyte stages were observed. At stage I, the primary oocytes are present without lipid droplets whereas at the stage II, the first lipid vesicles become apparent. In the stage III, the ovary is dominated by growing oocytes with a nucleus in late perinucleolus stage of development and a first ring of lipid vesicles become apparent (Fig. 2C).

Growing oocytes become clearly previtellogenic oocytes, with lipids incorporated in vesicles; these lipid droplets cover less than 50% of the cytoplasm and form a complete ring around the circumference of the developing oocyte. This stage is more and less evolved and could be observed for yellow female individuals. Later, the oocyte diameter enlarges (100–200 μm) and the whole cytoplasm was recovered by lipid vesicles. These early vitellogenic oocytes have a nucleus with numerous nucleoli, a granulosa and a theca and could be observed for silver female eels (Fig. 2D). The stage IV corresponds to the Vg incorporation in oocytes, which cannot be observed in individuals collected in the framework of this study as it is supposed that it is gradually realized during the journey towards the spawning site. No abnormalities such as necrosis or unusual oocyte diameters were observed at optical microscopic level for the sampled individuals.

Biometric parameters and gonad histology allowed to further assessment of the classification of the sampled eels (Table 2). GSI values were in agreement with histological observations and stage classification, GSI of yellow and silver female eels being respectively lower and higher than 1.2. Ocular Index was also in agreement with the stage classification, high OI values being exhibited only by silver female and male eels.

The SI calculation of individuals indicated 56.7% of FV, 3% of FIII, 34.3% of FI and 6% of MII in the sampled population fraction from the Loire estuary.

3.3. Liver Vg mRNA levels of eels sampled in the Loire estuary

The relationships between Vg expressed in hepatocytes and GSI of European eels collected in both sites of the Loire estuary are illustrated in Fig. 3.

Silver females ($n=8$) captured during the downstream migration in November 2010 presented Vg mRNA levels ranging from 2.5 to 9034.5 AU (Fig. 3 A). Low Vg mRNA levels (1 and 1.7 AU) were respectively detected in the liver of the 2 silver males (Fig. 3B).

Concerning non migrant individuals collected in May 2009, 3 out of 8 yellow females from Varades presented detectable Vg levels (ranging between 0.4 and 45.5 AU), whereas Vg transcripts were below or close to detection level (≤0.4 AU) in the other yellow females and in the 2 collected silver females (Fig. 3C). Concerning yellow males, 6 out of 14 exhibited detectable Vg mRNA levels (between 0.4 and 26.24 AU) (Fig. 3D). One undifferentiated eel out of 3 depicted expressed Vg (0.6 AU). In the samples from Nantes, Vg was detectable in 7 out of 22 yellow females (0.5–21.4 AU) and in one of the 2 collected silver females (2.4 AU) (Fig. 3E). One yellow male out of 2 and one undifferentiated eel out of 4 also revealed Vg mRNA expression (1.6 and 1.2 AU respectively) (Fig. 3 F).

The comparison of mean Vg mRNA levels according to each sexual stage do not show a difference between the two sampling sites. To conclude, 10 out of 25 yellow or undifferentiated eels collected at the upstream site and 9 out of 28 on the downstream site of the estuary, exhibited a quantifiable hepatic Vg mRNA expression.

The mean Vg mRNA level expressed by migrant silver females was significantly higher than mean levels calculated for yellow females, yellow males and undifferentiated eels (Kruskall–Wallis, p-value < 0.0206). No statistical difference was observed on mean Vg mRNA levels between these last three groups of individuals.

Principal component analysis (PCA) was performed in order to evaluate correlation between Vg mRNA levels and biometric parameters (BL, K, A, OI, GSI, PFI, DTI) of all the sampled

Table 2
Sexual stage and biometric indices determined in sampled eels from both studied sites, upstream and downstream of the Loire estuary.

Sampling date		May 2009				November 2010	
Sex		**U**[a]	**f**[b]	**F**[c]	**m**[d]	**F**	**M**[e]
n[f]	Up	3	8	2	14	8	2
	Down	4	22	2	2		
BL[g]	Up	291 ± 36	416 ± 94	815 ± 48	328 ± 46	615 ± 58	384 ± 11
	Down	430 ± 97	528 ± 139	732 ± 270	328 ± 20		
GSI[h]	Up	0.55 ± 0.11	0.42 ± 0.39	1.80 ± 0.16	0.35 ± 0.16	1.73 ± 0.24	0.74 ± 0.74
	Down	0.42 ± 0.15	0.54 ± 0.25	1.40 ± 0.09	0.30 ± 0.24		
OI[i]	Up	3.16 ± 1.07	4.09 ± 1.28	9.32 ± 0.68	3.39 ± 0.71	8.69 ± 1.24	9.40 ± 0.82
	Down	4.51 ± 1.25	5.42 ± 1.69	9.38 ± 1.49	3.09 ± 1.19		

[a] Undifferentiated.
[b] Yellow female.
[c] Silver female.
[d] Yellow male.
[e] Silver male.
[f] Number of samples.
[g] Body length.
[h] Gonado somatic index.
[i] Ocular index.

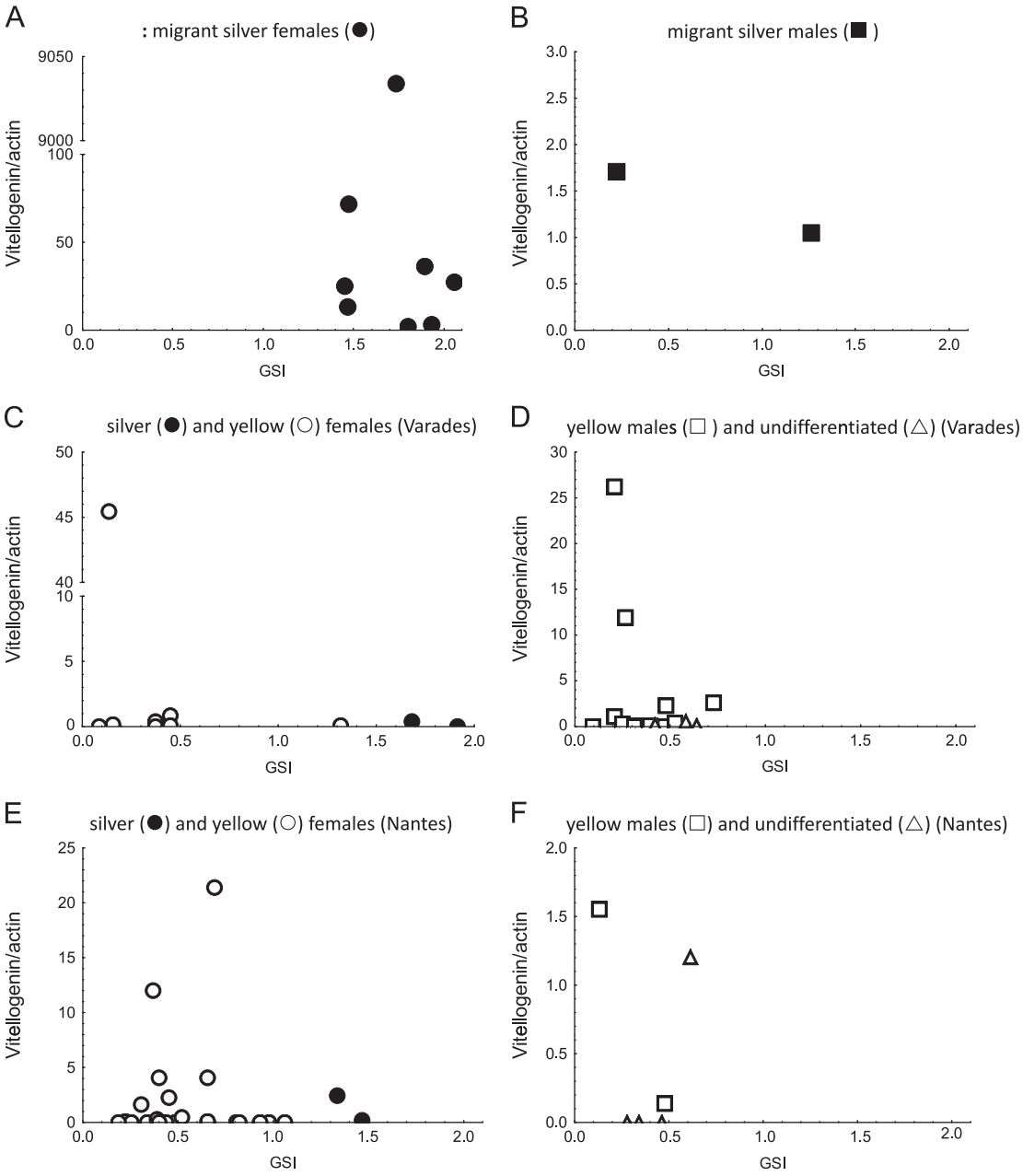

Fig. 3. Liver Vg mRNA levels of European eels *A. Anguilla* measured by q RT PCR on both sites of Loire estuary (dowstream: Nantes; upstream: Varades) function of GSI.

individuals (Fig. 4). Two qualitative variables (gender and sexual maturity, and Silver Index) have been added as supplementary variables to plot the individuals.

The first two components of the analysis represented 52.8% and 13.4% respectively of the total inertia (Fig. 4A). Morphometric parameters such as BL, OI, A, PFI, and also GSI and K are highly and significantly correlated together on the first axis ($r=0.759$, $r=0.777$, $r=0.464$, $r=0.624$, $r=0.517$ respectively, $p<0.05$), whereas the second axis is explained by Vg mRNA expression and the Fulton Index K. The first axis underlined also that the sexual maturation stage "silver female" is strongly and positively correlated to BL ($r=0.576$), A ($r=0.525$), OI ($r=0.697$), PFI ($r=0.387$) and GSI ($r=0.872$) whereas it is negatively correlated to DTI ($r=-0.636$). Thus, the first axis characterized the silvering process by the development of gonads (GSI) and the regression of the digestive tract (DTI), both in negative correlation ($r=-0.620$, $p<0.05$), corresponding to the silver eel starvation. Relationships between the SI FV and the silver prepubertal eels and the SI FI and immature eels can be underlined. The SI MII and FIII, which did not contribute to the components of the analysis, have been removed from the figure in order to clarify the representation. Vg mRNA levels were only correlated to the GSI and to the silver female stage. Contrarily, Vg expression was not significantly correlated to the stage migrant FV. This result could probably be explained by a misclassification for stage FV eels. The analysis clearly separated three groups corresponding to the sex and/or sexual maturation (Fig. 4B). The addition of SI as a qualitative variable permitted confirmation that apparent silver eels were migrants eels ($r=0.408$, $p<0.05$). Concerning undifferentiated and yellow males, the PCA showed significant negative correlation with morphometric parameters. Indeed, males do not have the same growth and development as females in the European eel species, which present a strong sexual dimorphism. The silver males were correlated significantly to OI ($r=0.297$, $p<0.05$) and PFI ($r=0.259$, $p<0.05$) which is characteristic of the silvering process.

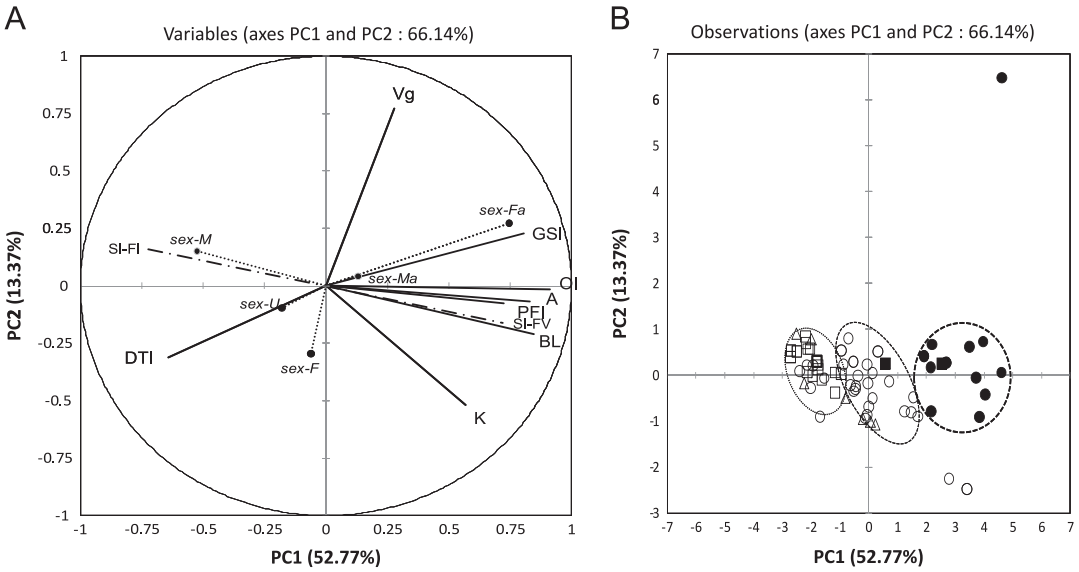

Fig. 4. Principal Component Analysis of biometric parameters, sexual maturation stage, silver index and vitellogenin mRNA expression of European eels from Loire estuary (both sampling sites, $n=67$). A: correlation loadings (BL: body length; K: Fulton index; LW: lipid weight; DTI: Digestive tract Index; PFI: Pectoral fin Index; GSI: Gonado Somatic Index; A: Age; Vg: Vg mRNA expression; SI: Silver Index); B: sample representation (Δ = undifferentiated individuals ; □ = yellow males; ■ = silver males; ○ = yellow females; ● = silver females).

The second axis is characterized by a significant positive correlation between Vg and GSI ($r=0.245$) but any other biometric parameter was correlated to Vg expression levels. This result was explained by silver female eels collected in May 2009 and November 2010.

4. Discussion

4.1. Hepatic Vg mRNA expression as an indicator of estrogenic exposure in the European eel A. anguilla

Vg is a precursor protein of yolk synthesis, representing energy reserves required for development of embryos in Teleosts as in other oviparous vertebrates. Transcription, translation and biosynthesis of this large phospholipoglycoprotein (200–700 kDa) take place in the liver under the regulation of ovarian estrogens (Burzawa-Gerard and Dumas-Vidal, 1991a; Burzawa-Gerard et al., 1991b). Vg is expressed specifically in female hepatocytes during vitellogenesis under the effects of endogenous estradiol. In males and juveniles of both sex, Vg gene is present but stays silent except in the presence of xeno-estrogen, so that its expression signs a potential endocrine disruption by xenobiotics (Kime et al., 1999). Differently from the majority of Teleosts, European yellow eels are in juvenile and immature stage during most of their continental life cycle. Just before the downstream stage of the only reproductive migration towards the spawning oceanic areas, they began a progressive and important metamorphosis usually known as silvering. At this time, silver eels are in a prepubertal stage and the females presented previtellogenic oocytes for which no inclusion of yolk was observed (Burzawa-Gerard et al., 1994). Thus, Vg expression could be detected in silver females but should not be observed in any other continental stages (undifferentiated eels, yellow females, yellow and silver males).

An increasing number of investigations and international programs are being performed around the world in various aquatic ecosystems (marine, fresh or brackish water) using Vg as biomarker of estrogenic disruption underlined by reviews (EELREP, 2005; Matthiessen, 2003). However, in eels, differently from other investigated Teleosts, low or undetectable levels of Vg have been reported by ELISA, RIA and PCR following an estrogenic exposure (Barucca et al., 2006; Burzawa-Gerard et al., 1991b; Versonnen et al., 2004). For instance, field studies in Flanders showed no enhancement of yellow eel Vg plasma levels despite a very high internal pollution load with organochlorine and PCBs (Versonnen et al., 2004). From the river Thames, the hypothesis of an endocrine disruption has however been depicted in immature yellow eels, since abnormal elevated Vg plasma levels were recorded in individuals with body length less than 45 cm which can normally not correspond to female silver eels (Peters et al., 2001).

The proteomic level does not seem to be a valuable indicator of exposure to estrogen compounds. Indeed, the hepatic synthesis of Vg is controlled by nuclear receptors E2 dependent but the maximal capacities of E2 binding as Vg exocytose process are lower in *A. anguilla* compared to other fish species (Burzawa-Gerard et al., 1994). These authors showed, during a seven weeks experimental induction of sexual maturation that significant Vg plasmatic levels could be reached only after induction of the number and the binding capacities of hepatocytes E2 receptors. Furthermore, the specificity of the European eel sexual maturation (semelparous status, long oceanic migration without feeding) and particularly its dopaminergic inhibition of vitellogenesis, at hypothalamus and pituitary levels in earlier steps of gametogenesis, do not lead to significant Vg plasmatic levels in feral prepubertal individuals (Weltzien et al., 2009). Laboratory experiments showed thus that the induction of full sexual maturation requires 3 to 6 months of weekly injections with pituitary extracts (Palstra et al., 2005).

RT PCR method appeared as a promising approach to monitoring estrogenic exposure since Vg expression rises rapidly and indicates recent exposure to EDCs, as already shown in other various fish (Bowman et al., 2000). Moreover, according to Marohn et al. (2008), measuring mRNA concentrations excluded a variety of environmental influences and was more relevant than investigations based on protein contents or enzymes activities. In European eel, Barucca et al. (2006) underlined that treated juvenile individuals showed basal Vg gene expression during experimental induction by steroid hormones, using specific designed primers, and no amplification of Vg m RNA was observed in juvenile *A. anguilla* from the field.

The present paper based one's argument on these previous studies to measure the Vg induction only at the transcriptomic

level. The q RT PCR, higher sensitive than RT PCR, was used to amplify responses of hepatic Vg transcripts in eels. To firstly validate the approach, a laboratory exposure of silver males to various steroids was conducted and demonstrated the responsiveness of exposed silver males, since a significant hepatic mRNA Vg induction was observed for E2 treated males compared to controls or androgen-treated specimens.

The q RT PCR was then performed in eels sampled from the Loire estuary according to gender (undifferentiated, male or female) and sexual stage (yellow or silver) of each individual. In female silver eels sampled at the time of the downstream migration, hepatic Vg mRNA levels ranged from 2.5 to 9035 AU. This is within the range of the large individual variations previously observed in Vg plasma levels in silver females, ranging from undetectable (less than 100 ng/ml) to 10 μg/ml as measured by RIA (Burzawa-Gerard and Dumas-Vidal, 1991a; Burzawa-Gerard et al., 1991b) or ELISA (Sbaihi et al., 2001). These results reflected large inter-individual differences in the activation of hepatic Vg at silvering, as already highlighted by Sbaihi et al. (2001). Quantifiable Vg expression was also depicted in some individuals of the other sex and stages: in undifferentiated eels, in yellow previtellogenic females and in yellow and silver males. In these categories, Vg mRNA values ranged from undetectable (less than 0.4 AU) to 26 AU. Furthermore, this unusual expression of Vg was exhibited by a large proportion of the sampled eels (38% of the 55 individuals) and was found similarly at both sampling sites.

In our study, the highly sensitive q RT PCR method thus allowed the measurement of different levels of hepatic Vg mRNA transcripts in individuals from the field or experimentally exposed to estrogenic compounds. According to these results, detectable levels of hepatic Vg mRNA expression could suggest a potential estrogenic exposure of individuals for which no Vg is expressed under normal conditions. This work validates the suitability of q RT PCR and its value to monitoring endocrine exposure in this species.

4.2. Statistical relationship between Vg levels and measured variables

The PCA demonstrated that Vg mRNA levels were only correlated to the GSI and to the silver female stage. Contrarily, Vg expression was not significantly correlated to the stage migrant FV. This result could probably be explained by a misclassification of stage FV eels. Indeed, according to Durif et al. (2009), these misclassifications can be influenced by the ecological conditions or individual specificities. In our study, there is an excellent correlation between SI and gonad histological results on the upstream site (Varades) whereas all the yellow specimens collected on the downstream site (Nantes) were classified as migrant eels (SI FV). This could suggest that these immature eels prepare their further silvering for the next autumn. Indeed, K and the biometric parameters BL, A and PFI are positively correlated to the stage SI FV, contrarily to the silver eels for which K is not correlated, indicating the starvation whereas yellow eels are already in the growth phase.

On the other hand, it could be also hypothesized that a reversal of silvering or an interruption of the silvering process for bigger and aged individuals which became sedentary because of favorable trophic conditions in this area. However, the addition of SI as a qualitative variable permitted confirmation that apparent silver eels were migrants eels.

The second axis could expressed the pubertal process with the expression of Vg mRNA, indicating a further incorporation of Vg in oocytes. Finally, the PCA cannot demonstrate a relationship between silver index and Vg m RNA expression neither correlation with biometric parameters other than GSI. Concerning relationships with the sexual maturation stages, only silver eels collected in May 2009 and November 2010 were significantly correlated to the Vg levels, confirming a real status of migrants and potential genitors.

4.3. Relation between hepatic mRNA Vg induction and gonad histology

In parallel to the quantification of Vg mRNA expression, gonad histology allows the investigation of links between induction of Vg and potential abnormalities as oocytes atresia (Pierron et al., 2008b) or early incorporation of yolk globuli. Indeed, European eel gonads contained oocytes representing stages I, II and III according to Palstra and van den Thillart (2009). Stage III was characterized by the presence of lipid droplets, where yolk globuli, characteristic of vitellogenesis, were not found. The enlargement of the oocyte size (≥250 μm) associated to the decrease of lipid droplets number indicates the incorporation of vitellogenin into vesicles (Palstra, 2007). In the present study, no abnormality was observed on histological section examinations, despite a Vg gene induction observed in undifferentiated, male or yellow female eels from the Loire estuary. This result confirms the suitability of the quantification of Vg mRNA expression compared to histological studies of gonads which are time-consuming and particularly awkward in eels. Moreover, contrary to the intersexuality observed in various fish in the French rivers (Hinfray et al., 2010; Sanchez et al. 2008), gonad histology cannot indicate to potential intersex in eel because juvenile hermaphroditism or intersexual stage (Beullens et al., 1997). However, our histological observations allow determination of the sexual stage (size or number of oocytes, presence of lipid droplets, potential precocity of yolk vesicle apparition, testicular development stage) (Burzawa-Gerard, 1994). The absence of oocytes presenting a diameter superior to 250 μm validated the prepubertal stage of silver eels. No abnormalities such as necrosis or unusual oocyte diameters were observed at optical microscopic level for the sampled individuals. Our histological studies as well as low Vg mRNA levels detected do not permit a conclusion as to a potential effect of endocrine disruption.

As such, what are the potential sources of EDCs from the Loire estuary which could partly and potentially explain the hepatic mRNA Vg induction?

4.4. Likely origins of eel contamination with EDCs in the studied area

The Loire estuary (largest French estuary draining 110,000 km^2) receives several effluents streams (wastewater treatment plants, atmospheric, industrial and agricultural leaching products), and has suffered from a higher urban development and environmental constraints (Perraudeau and Després, 2009).

Literature data indicates that the Loire estuary is impacted by several types of contaminants such as organochlorine pesticides, polybromodiphenylethers, PCBs, PAHs, heavy metals both in physical compartments (waters and sediments) and in aquatic organisms (Bragigand et al., 2006; Marchand et al., 2004; Robbe et al., 1985). Eels from the river Loire exhibited moderate PCB, PBDE and PFC contaminations (Babut et al., 2009; Blanchet-Letrouvé et al., 2013; Couderc et al., 2013). The marker-PCB (congeners #28, 52, 101, 118, 138, 153 and 180) were notably analyzed in individuals sampled in the framework of this study. According to the development stage, marker PCB contamination significantly increased from glass eel stage (3.49 ± 0.16 ng g^{-1} ww) to other life stages (193 ± 101 ng g^{-1} ww for yellow eels from Nantes and 82.3 ± 27.4 ng g^{-1} ww for yellow eels from Varades; 229 ± 130 ng g^{-1} ww for silver eels). Concerning the 2 sampling sites, eels from Varades appeared less contaminated by

PCBs than those from Nantes. Overall, eels from the Loire estuary showed an intermediate contamination level compared to other international/national areas. Indeed, at the international scale, eels from the Loire estuary appear to be more contaminated than those from some other sites in Poland, Ireland, Spain, Italy and the UK (Bordajandi et al., 2003; Corsi et al., 2005; McHugh et al., 2010; Santillo et al., 2005). However, other sites are more contaminated than the Loire estuary (twice to 10 times higher), i.e. the River Elbe in Czech Republic and Germany, the Tevere and Gagliarino rivers in Italy, Flanders in Belgium and different lakes in Finland (Belpaire et al., 2011; Maes et al., 2008; Tulonen and Vuorinen, 1996; van der Oost et al., 1996). Throughout France, eels from the Loire estuary are slightly more contaminated than those from the Vacares lagoon and about three times more than those from the Thau pound (Oliveira Ribeiro et al., 2008; Santillo et al., 2005), whereas they are less contaminated than eels from the Rhone River (about ten times less) and the Gironde estuary (about two times less, whatever the life stage and the size class) (Tapie and Le Menach, 2011).

These PCBs are particularly suspected because of their potential of estrogenic and anti-estrogenic disruptors (Geeraerts et al., 2011) and their neuroendocrine effects (Kodavanti and Curras-Collazo, 2010), endangering several fish species and notably the eel population (van Ginneken et al., 2009). In the Loire estuary, endocrine disruption has recently been shown in the bivalve *Scrobicularia plana* (Fossi Tankoua et al., 2012) which is a prey for the flat fish (*Plathychtys flesus*) (Masson, 1987), itself a potential prey for the top predator *A. anguilla*, an opportunist species.

Finally, European eels can be contaminated by several possible ways such as sediments, water or/and trophic chain (Pierron et al., 2008b). Indeed, juvenile eels are in direct contact with the sediment during their continental life (from 5 to 22 years for the Loire eels, personal communication) and thus potentially exposed to contaminated sediments for a long time compared to other Teleosts (Tesch, 2003). So, depending to their maturation stage and gender, European eels can be contaminated by multiple pollutants through different biologic or metabolic ways.

What potential risk do pollutants for represent the European eel health, its reproductive function and eventually its reproductive success?

4.5. Potentials impacts of Vg induction and estrogenic disruption on eel reproductive function

Concerning the possible adverse effects in male and immature organisms, the production of Vg probably interferes with other pathways diverting metabolic resources necessary for growth and spermatogenesis as described in other Teleosts (Ankley et al., 2010). In the case of eel, an induction of Vg could interfere with the lipid metabolic pathway. Lipids represent pivotal molecules for the success of reproduction, related to the vitellogenic yolk globuli, the lipid droplets, the steroid and hormone metabolism, the energetic reserves in muscles necessary to provide the energy required for physiological modifications in marine water and reproductive migration. This energetic cost has been well-documented by Van Ginneken et al. (2009), who showed that PCB exposure of migrant eels significantly reduced oxygen consumption during swimming. These authors suggested also that the current levels of PCBs and other dioxin-like compounds may seriously impair the reproduction of the European eel. The swimming animals lost about 75% more weight compared to resting animals and had about 50% lower plasma fat content.

Recent studies on muscles lipid contents of yellow eels, using time-trend monitoring in Belgian and Netherlands rivers showed that fat contents decreased from 21% to 13%, coinciding with the strong reduction of the European eel stock (Belpaire et al., 2009). Furthermore, impairment of lipid storage by cadmium was shown in the European eel (Pierron et al., 2007). Lipid stores are also necessary to ensure the metabolic challenge of gonad growth and maturation, occurring during the ultimate oceanic phase. This unique reproduction migration of about 6000 km as well as the gonad maturation can be achieved because of the large amount of accumulated lipid stores, which attract lipophilic chemical substances such as EDCs (van Ginneken and Maes, 2005). Moreover, swimming appears to be a trigger which stimulates fat deposition in the oocytes but suppresses vitellogenesis. That suggested these events are separated in the field and occurred sequentially. This implies that wild eels undergo vitellogenesis and final maturation near or at the spawning grounds (Palstra and van den Thillart, 2011) and could explain the low responsiveness of the eel concerning Vg induction at the onset of the migration, when they leave the Loire estuary. This also validates the interest of our results relative to endocrine disruption.

In the eel, a specific threat could also result from the redistribution of contaminants bioaccumulated during the continental juvenile stages in the lipid stores. These contaminants are released in the blood and reallocated to other tissues during the lipid mobilization required for reproductive migration and sexual maturation, while the eels have ceased feeding. Pierron et al. (2008a) demonstrated that cadmium is redistributed from the fat tissue to various organs including liver, kidney, and ovaries, during experimental sexual maturation in the eel. Future studies should aim to further investigate potential links between the presence of EDCs and lipid store depletion in eels comparing two major fat tissues (muscle and gonad) and their own PCBs levels.

5. Conclusions

In conclusion, this study performed in the Loire estuary constitutes a preliminary report validating the use of q RT PCR as a highly sensitive approach to quantify Vg m RNA transcripts in European eel. While only female silver eels normally express hepatic Vg mRNA, Vg transcripts were also quantified by q RT PCR in a proportion of 38% of the other individuals sampled, normally not inclined to express it, those being undifferentiated, yellow females, yellow and silver males. However, our findings are not fully conclusive due to the very low Vg expression and the absence of impact on gonad histology. Today, no evidence for Vg endocrine disruption in European eels was reported so far by previous field studies even in heavily contaminated hydrosystems (for review, Geeraerts and Belpaire (2010)). As such, the present work consisted on a preliminary field study and need further investigations to evaluate the potential of hepatic Vg mRNA expression as biomarker of endocrine disruption in this species of great economical and ecological interest.

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