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How Cadmium Could Compromise the Completion of the European Eel's Reproductive Migration

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The European eel (*Anguilla anguilla* L.) is severely threatened with extinction. Surprisingly, even though their unusual life cycle makes them particularly vulnerable to pollution, the possible contribution of contamination remains especially poorly known. Here we have investigated the possible effect of cadmium (Cd), a widespread nonessential metal, on eel reproductive capacities. Both control and Cd precontaminated female silver eels were experimentally matured and forced to swim in metal-free conditions to mimic their reproductive migration. Cd pre-exposure was found to strongly stimulate the pituitary−gonad−liver axis of maturing female silver eels leading to early and enhanced vitellogenesis. This was followed by a strong phenomenon of oocyte atresia and eel mortality. These phenomena occurred before oocytes could reach full maturation and were associated with a large entry of both vitellogenin and Cd into the ovaries. Indeed, a redistribution of previously stored cadmium, even from the low Cd levels of control eels, was observed during sexual maturation. Atresia and mortality phenomena were also associated with an overexpression of the pituitary gene encoding the growth hormone, a marker of physiological stress and energy reserves exhaustion. Significantly, these devastating effects of Cd were observed in organisms that presented liver and kidney Cd concentrations still below those observed in eels from Cd contaminated hydrosystems. Our research shows how common levels of cadmium contamination could disrupt endocrine pathways implicated in gonad maturation and subsequently impair reproductive capacity of eel future genitors.

Introduction

Information collected over the past two decades on the status of the stock and fisheries of the European eel (*Anguilla anguilla*) shows that the population as a whole has dramatically declined. European eel's recruitment would represent now only one tenth of what it was in the early 1980s (*1*) and may even be only one hundredth for the most pessimistic and recent estimations (*2*). The biological cycle of the European eel, which has been well reviewed by several authors (*3, 4*), is especially complex, comprising four life stages: two metamorphoses and two trans-Atlantic migrations. Reproduction of the European eel takes place in the Sargasso Sea from where larvae drift back toward the European coasts following oceanic currents. After metamorphosis of the larvae into glass eels, the organisms reach the juvenile growth phase stage (yellow eel) in continental habitats. This stage can last from several years to more than 20 years, depending on the hydrosystem, and ends with a second metamorphosis called silvering which prepares the future genitors (silver eels) for their transoceanic reproductive migration. However, when silver eels leave the European coasts, their gonads are still immature and maturation is blocked at a prepubertal stage (*5*). This implies that gonad development must occur during their 5500 km migration, i.e., a 5−6 months period marked by swimming activity and starvation that ends with the death of the genitors. In natural conditions, the prepubertal stage is the last known stage, as mature European eels have never been caught in the wild.

From an ecotoxicological point of view, this particular life cycle has two main consequences. First, their long somatic growth phase makes them particularly vulnerable to pollution. Indeed, results from comparative *in situ* studies often show that eels, as compared to other fish species, accumulate large quantities of persistent contaminants such as metals (*6–8*). Second, since silver eels do not eat during their transoceanic migration, contaminants previously accumulated can be remobilized and redistributed, thus triggering potential toxic events (*9*). However, despite this status as a highly sensitive species, few studies have focused on the possible contribution of pollution to the reported eel decline. Consequently, the effects of contaminants on the eels' reproductive success remain especially poorly known (*10*).

Here, we have investigated the potential impact of cadmium (Cd) on the reproductive capacities of the European eel. Cd is a widespread nonessential and highly toxic metal derived from anthropogenic activities such as mining, ore treatment, and used in batteries and paints (*11*). Data available in the literature on Cd effects on fish reproduction are still rather limited. Moreover, according to fish species, exposure conditions and reproductive sate of animals, both inhibitory and stimulatory effects were reported (*12, 13*). In order to mimic reproductive migration, metal-naïve female silver eels were first pre-exposed to waterborne Cd. Thereafter, both precontaminated and unexposed (control) eels were (i) forced to swim in uncontaminated natural seawater and (ii) artificially matured. Although artificial reproduction of European eels still remains a challenge, full gonadal development of female eels can be induced experimentally by long-term gonadotropic treatment with carp pituitary extracts (CPE) (*15, 16*). Organisms of each group were removed immediately after Cd exposure (time 0) and after

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8, 18, and 22 weekly CPE injections. At each sampling time, different reproductive end points in fish were assessed. The expression level of three pituitary genes encoding the pituitary luteinizing hormone (*lh*-β and *gp*-α) and the pituitary growth hormone (*gh*) as well as the liver vitellogenin (*vg*) was determined by quantitative PCR. Vg is an egg yolk precursor protein providing energy and metabolites to embryogenesis. It is synthesized in the maternal liver to be secreted into the blood circulation and finally internalized in oocytes via receptor-mediated endocytosis (*17*), thus triggering the increase in gonadic mass. LH is a necessary promoter of steroidogenesis, Vg incorporation into gonads, and subsequently oocyte growth (*5, 18*). In addition to its well-known effect on growth, GH is also recognized as a pleiotropic hormone acting on various functions in fish such as osmoregulation, reproduction, and stress. GH promotes lipid and glycogen breakdown as well as gluconeogenesis during prolonged starvation or stress (*19, 20*). Molecular analyses were reinforced by the determination of plasma Vg concentrations, by morphometric measurements such as the gonado-somatic index (GSI), and by histology of the gonads. Analysis was completed by the Cd content determination in various fish organs.

Materials and Methods

Experimental Design. *First Step: Pre-Exposure to Cd.* Fifty-two female silver eels averaging 553.5 ± 6.7 mm long and 292.9 ± 9.4 g in weight (mean ± SE) were caught in December 2005 in the Loire river (northwest of France) during their continental downstream migration, which represents the start of their reproductive migration. The animals were transferred to the laboratory (Marine station, Arcachon, France) and equally distributed into a flow-through system consisting of two separate 300 L swim tanks devoid of a water current and constantly supplied with aerated brackish water (salinity 10 ‰, natural seawater diluted with aerated tap water) at a rate of 600 mL·min^{-1}. After two weeks acclimation period, half of the individuals (i.e., animals in one of the two tanks) were exposed over 30 days to dissolved Cd at a nominal concentration of 15 μg·L^{-1}. The other half was maintained in the same physicochemical conditions in Cd-free water thus constituting a group of control eels. Concerning the contaminated tank, metal exposure was initiated by adding Cd to the water in the form of $CdCl_2$ from a stock solution (1 g·L^{-1}, Merck, Darmstadt, Germany). To maintain constant Cd contamination over time, the tank was fitted with a peristaltic pump (Gilson Miniplus2; Villier-le-Bel, France) which added Cd at the desired concentration at a rate of 400 μL·min^{-1} from a stock solution at 22.5 mg·L^{-1}. The water column was permanently monitored for temperature (11.5 ± 0.5 °C and 11.1 ± 0.5 °C for the control and the contaminated tank, respectively), pH (7.78 ± 0.02 and 7.77 ± 0.02 °C for the control and the contaminated tank, respectively), and salinity (9.6 ± 0.5 and 9.9 ± 0.5 ‰ for the control and the contaminated tank, respectively; mean ± SE, $n = 20$). Water samples were collected three times a week. After acidification and dilution, they were checked for Cd concentration. Cd concentrations were non detectable in the control tank and reached in mean 14.8 ± 0.4 μg·L^{-1} (mean ± SE, $n = 12$) in the contaminated tank. At the end of this step, ten eels, five per tank, were removed and dissected for analyses.

Second Step: Swim Activity and Gonad Maturation. Subsequently, all animals were placed in natural uncontaminated seawater (water from Arcachon basin, Cd concentration inferior to detection limit, salinity 30.5 ± 0.2 ‰, temperature 13.5 ± 0.4 °C, mean ± SE, $n = 110$) and submitted to a water current to force their swimming at a speed of about 30−40 cm·s^{-1} representing a distance of 5500 km covered in 6 months (corresponding to 0.5−0.7 body length·s^{-1}) (*4, 21*). In our experimental conditions, the velocity of the water flow was maximum at the periphery of the tank and decreased toward the center. However, access of eels to the zone where the current was inferior to 30 cm·s^{-1} was blocked by a plastic mesh. After one week, the time required for the water Cd concentration in the previously contaminated tank to become undetectable, all animals, both precontaminated and control eels, were individually marked with colored elastomer tags (Northwest Marine Technology, Shaw Island, WA) and randomly mixed in the two 300 L swim tanks to prevent effects of experimental units. To induce gonad maturation, eels received, without previous anesthesia, one perivisceral injection a week of carp pituitary extract (CPE, Catvis BV, Den Bosch, Netherlands) at a dose equivalent to 20 mg pituitary powder/kg body weight, according to a method previously described (*16*). Five animals per tank were removed for analysis after 8, 18, and 22 CPE injections. However, due to excess mortality, only four pre-exposed eels could be analyzed at the last sampling time.

Sampling Procedure. At each sampling time, fish were weighed, measured, and killed by severing the medulla oblongata. Blood samples were prepared as previously described (*22*) and frozen (−20 °C) until vitellogenin immunoenzymatic assays. Pituitaries were quickly removed and immediately frozen (−80 °C) until RNA extraction. For ovaries, samples needed for histology and Cd determination were collected according to a standardized method, 4 cm in front of the anus. These samples and the rest of the ovaries were weighed to calculate the gonadosomatic index (GSI expressed as a percentage: (gonad weight/total body weight) × 100). For the liver, one part was immediately frozen (−80 °C) until genetic analysis. The other part was dried until Cd determination. In the same way, the muscle, the kidney, the digestive tract, and the gills were entirely dissected and dried.

The body condition factor was calculated as follows: [((total body weight (g) − gonad weight (g)) × 10^5)/(body length (mm))^3]. Gonad weight was not taken into account in this calculation to assess the physiological state of animals turning down the Cd effect on gonads, this effect being approached by GSI determination.

Cd Determination. Metal concentration was determined by electrothermic atomic absorption spectrophotometry. The methodology applied to biological samples was the same as previously described for eels (*23*). Before dosage, water samples were diluted, acidified at 2% HNO_3, and mixed with 4 μg of Pd, 3 μg of $Mg(NO_3)_2$, and 1.2 g of NH_4NO_3. The detection limit was 0.1 μg·L^{-1}.

Quantitative Real Time RT-PCR. For each gene, specific primers were determined (see Supporting Information). Extraction and reverse transcription of mRNA as quantitative PCR reactions and subsequent controls were carried out on three replicates as previously described (*23*).

Vitellogenin Determination. Plasma Vg levels were determined using an ELISA previously developed for the European eel (*22*).

Histology. Ovary samples were immediately fixed in Bouin fluid, dehydrated, embedded in paraffin, cut in sections (thickness 10 μm), rehydrated, and stained by the Cleveland−Wolf method as described by Gabe (*24*).

Data Treatment. All values are presented as mean ± SE. Comparisons among groups were performed using analysis of variance (ANOVA), after checking assumptions. If significant effects were detected, Least Square Deviation test was used to separate means. When assumptions were not met, we used Box−Cox data transformation (*25*). For all the statistical results, a probability of $P < 0.05$ was considered significant.

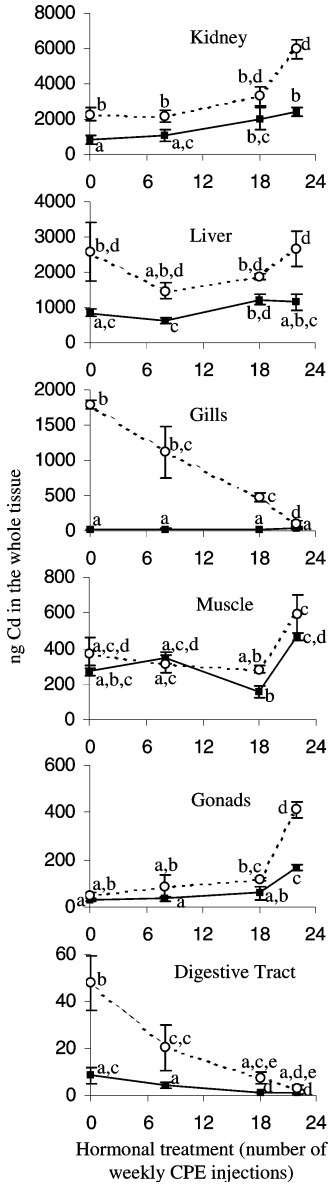

FIGURE 1. Change in Cd content of kidney, liver, gills, muscle, gonads, and digestive tract of control (solid line, black squares) and precontaminated eels (dotted line, white circles) experimentally matured by weekly injection of carp pituitary extract (CPE). All results are expressed as mean ± SE (n = 5/group except for the last sampling time where only 4 cadmium precontaminated eels could be studied due to excess mortality). For each organ, means labeled with different letters (a, b, c, d, e) are significantly different (ANOVA P < 0.05).

Results and Discussion

After 30 days of Cd exposure (time 0), a significant metal accumulation was observed in the kidney, the liver, the gills and the digestive tract of Cd exposed eels (Figure 1). At this time, average Cd concentrations (data not shown) in the main organs of Cd bioaccumulation, the liver and the kidney, reached means of 1.7 ± 0.4 and 10.6 ± 2.7 $\mu g \cdot g^{-1}$ (dry weight, dw), respectively, in the case of Cd-exposed eels versus 0.9 ± 0.1 and 5.9 ± 0.2 $\mu g \cdot g^{-1}$ (dw), respectively, in the controls, i.e., in eels from the Loire river (France). For comparison, Cd concentrations in the liver and the kidney of yellow eels (aged 6−14 years) inhabiting the Gironde estuary (*7*), which is characterized by a historic Cd pollution, reach means of 5 ± 0.8 and 34.2 ± 5.1 $\mu g \cdot g^{-1}$ (dw), respectively. Cd concentrations found in internal organs of animals experimentally

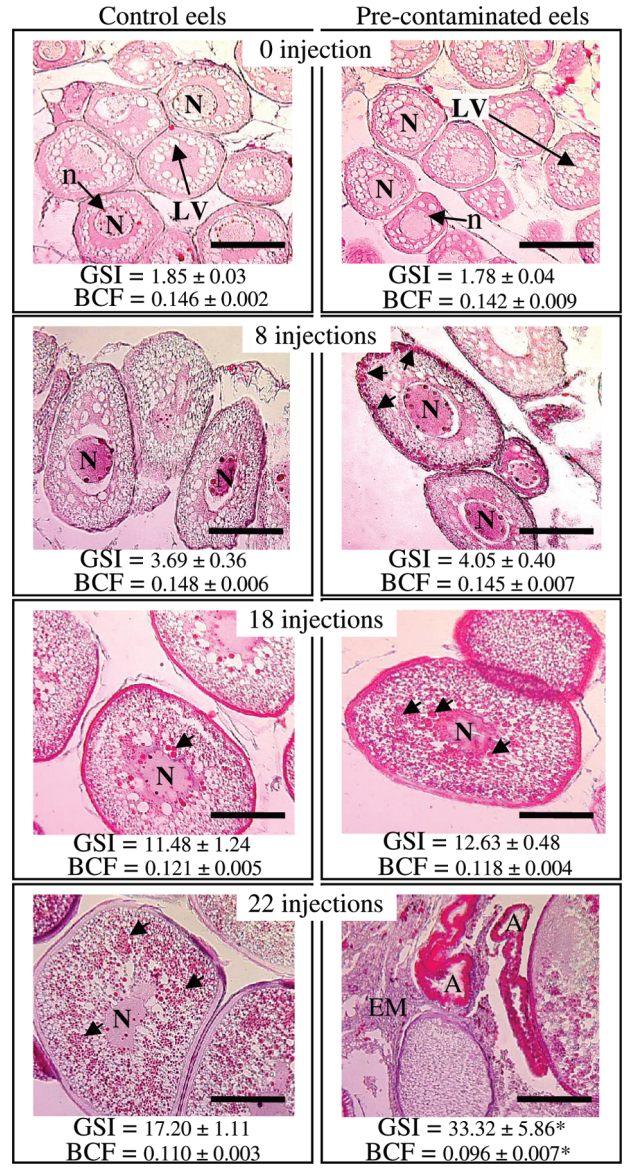

FIGURE 2. Change in gonado-somatic index (GSI), body condition factor (BCF), and ovarian histology of control (left panels) and Cd precontaminated (right panels) female silver eels treated for up to 22 weeks with weekly injections of carp pituitary extract. Before hormonal treatment (0 week), oocytes of both control and Cd precontaminated eels showed small nucleoli (n) at the periphery of the nucleus (N) and contained numerous lipid vesicles (LV) in the ooplasma, a characteristic feature of the immature oil droplet stage. After 8 weeks, only in precontaminated eels, yolk granules (black arrow) appeared at the periphery of a few oocytes. After 18 weeks, oocytes of both control and precontaminated eels presented a few yolk granules dispersed in the ooplasma and a visible zona radiata (ZR), a feature typical of the vitellogenic stage. However, this process was more advanced in the case of precontaminated eels as their oocyte, in comparison to those of controls, contained more yolk granules and had a larger zona radiata. After 22 weeks, oocytes of control eels reached the midvitellogenic stage. At the same time, numerous atresic oocytes (A) associated with extruded material (EM) were observed in Cd precontaminated eels. Such atresic oocytes were never observed in control eels. Only one scale, bar = 150 μm. For GSI, results are expressed as mean ± SE (n = 5/group except for the last sampling time where only 4 cadmium precontaminated eels could be studied). For each sampling time, * denotes a significant effect of cadmium pre-exposure (ANOVA, P < 0.05).

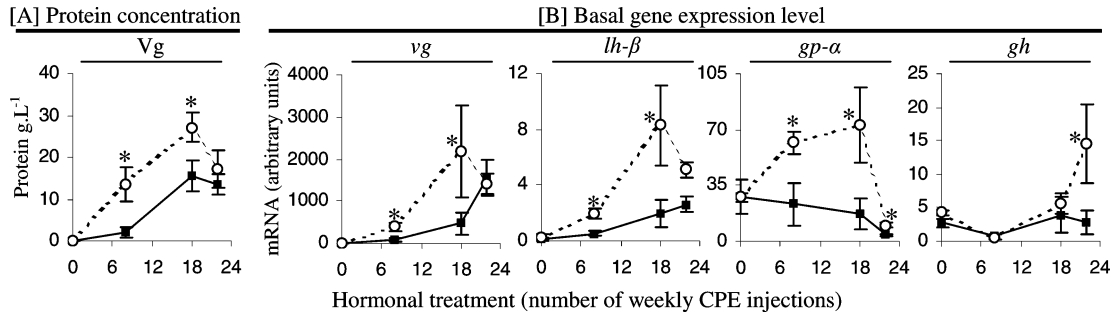

FIGURE 3. Change in [A] vitellogenin plasma concentration (Vg; mean ± SE, *n* = 5/group except for the last sampling time where only 4 cadmium precontaminated eels could be studied) and [B] basal expression level (mean ± SE, *n* = 3) of genes encoding for vitellogenin (*vg*), luteinizing hormone (*lh-β*, *gp-α*), and growth hormone (*gh*) in control (solid line, black squares) and cadmium precontaminated (dotted line, white circles) eels experimentally matured by weekly injection of carp pituitary extract (CPE). For each sampling time, * denotes a significant effect of cadmium pre-exposure (two ways analysis of variance, *P* < 0.05).

exposed to the metal were thus even lower than levels likely to occur in field eels. However, because contamination was carried out via dissolved Cd, metal concentrations in gills were higher in our experimental conditions compared to concentrations found in eels from the Gironde estuary, reaching 2.7 ± 0.08 and 0.8 ± 0.1 $\mu g \cdot g^{-1}$ (dw), respectively (*7*). We must add, however, that in terms of quantity (ng Cd in the whole tissue), liver and kidneys represented the main metal accumulation sites, as Cd distribution in experimentally contaminated eels was as follows: liver > kidneys > gills. Thereafter, during the maturation phase, which unfolded in Cd-free seawater, a significant increase in the Cd content of gonads and kidney of Cd precontaminated eels was observed. This was associated in these animals with a significant decrease in the Cd content of gills and digestive tract. Interestingly, such a redistribution of previously accumulated Cd, probably related to the mobilization of metabolite and ion stores for vitellogenesis in these fasting animals (*26*), was also observed, to a lesser extent, from the low Cd contamination levels of controls. Indeed, as for Cd precontaminated eels, a significant decrease in the Cd content of the digestive tract, probably related to the well-known regression of this tissue during artificial maturation (*16*), was also observed in controls. Moreover, as previously described for Cd precontaminated eels, the gonads and the kidney of controls were the main target organs of this redistribution. Metal content of these two tissues increased significantly during the maturation phase. The accumulation of Cd in the gonads could be explained by the incorporation of Vg into the oocytes. Indeed, Vg is a phosphoglycolipid−protein that is very rich in calcium (Ca) (*26*). Moreover, Ca and Cd ions are very similar elements as they present similar load and ionic radius (0.97 and 0.99 Å, respectively) (*27*). This is supported by the fact that in our experiment, the massive accumulation of Cd into the gonads was concomitant with the massive internalization of Vg in the oocytes as shown by histological and morphological investigations (Figure 2).

In both control and Cd precontaminated eels, the hormonal treatment with CPE increased the secretion of Vg (Figure 3 A). Surprisingly, despite the proven toxicity of Cd, the phenomenon appeared to be more precocious and significantly more important in the case of Cd precontaminated eels. Indeed, after only 8 weeks of hormonal treatment, precontaminated eels presented a mean Vg plasma concentration that was significantly 6.5-fold higher than that of controls. This effect of Cd on vitellogenesis, which was also observed after 18 CPE injections, consecutively led to a larger ovarian growth. At the end of the experiment, the mean GSI reached by precontaminated eels was significantly (1.9-fold) higher than that of controls (Figure 2). However, this potentiating effect, which could *a priori* appear profitable to the future progenitor, was also associated with a strong phenomenon of oocyte atresia (Figure 2) and mortality affecting only precontaminated eels. Indeed, from the 20th week of maturation to the end of the experiment (i.e., 3 weeks), whereas no control organisms died, four precontaminated eels of the eight remaining died. The extent of this mortality forced us to bring the end of the experiment forward, in such a way that oocytes could only reach a midvitellogenic stage. Due to the low number of test animals, it is difficult to link unambiguously this mortality to Cd pre-exposure. However, it is significant to underline that during the first 20 weeks of hormonal treatment only two organisms died in each group. The hormonal treatment itself seems thus to have exerted a low and equitable stress to the two groups of animals, at least during the first 20 weeks of treatment.

To understand some basic aspects of the underlying mechanisms, we followed throughout the maturation phase the gene expression level of different inducers of gonad growth (Figure 3B). In accordance with the results obtained at the protein level, Cd pre-exposure significantly triggered an up-regulation of the gene encoding *vg*. Its expression level was increased 4.9- and 4.7-times after 8 and 18 CPE injections, respectively. This could bear witness to a direct effect of Cd on the liver metabolism via a possible interaction with estradiol (E_2) signaling pathway, E_2 being the primordial promoter of vitellogenesis at the hepatic level (*17, 28*). Previous studies developed to investigate such an effect of Cd have led to contradictory results. *In vitro* Cd exposure of recombinant yeast expressing E_2 receptor ([Cd] = 50−500 μM, [E_2] = 10 nM) (*29*) or of mammal cells ([Cd] = 10^{-12} to 10^{-6} M, without E_2) (*30*) was found to decrease in the first case or inversely increase the estrogenic activity of cells in the second one. Alternatively, as we simultaneously observed a similar pattern at the pituitary level and more precisely for the two genes encoding the *lh* subunits, the stimulating effect of Cd on vitellogenesis could be indirect. Indeed, the expression level of *lh-β* and *gp-α* was, in mean, increased 3.1- and 4.3-times after 8 and 18 CPE injections, respectively. Cd pre-exposure seems to have triggered an early and enhanced stimulation of the pituitary−gonad axis. Due to the play of feedback mechanisms characterizing the hypothalamus−pituitary−gonad axis, it appears rather difficult to determine the main Cd action site. Studies undertaken on the fish *Micropognias undulates* could suggest that Cd mainly acts at the pituitary level. *In vitro* spontaneous gonadotropin (GTH) secretion by hemipituitaries from Cd-treated fish was found to be 3 times higher than that in controls (*12*). Similarly, it has been shown that Cd exposure induces dose-dependent stimulation of GTH secretion by perfused carp pituitary cells (*31*).

Another pertinent question is how Cd pre-exposure could trigger oocyte atresia and mortality, and why is this happening 19 weeks after animals had been effectively exposed to metal?

When following Cd intake in the ovaries throughout the 22 weeks of hormonal treatment, we found the occurrence of atresic oocytes to be correlated with a massive entry of Cd into the gonads (cf. Figures 1 and 2). This likely leads to the occurrence of toxic events including apoptosis. Surprisingly, even though the pro-apoptotic effect of Cd has already been studied and described in different cell types including fish male gametes (*32, 33*), such a phenomenon has only been evoked in the case of fish oocytes (*12*). Alternatively, as we previously evoked, since we observed a strong stimulation of *lh* subunit genes expression (Figure 3B), Cd could stimulate atresia indirectly via alterations in hormone levels. For example, in humans, whereas LH is a promoter of steroidogenesis and oogenesis, excessive levels have been found to adversely affect oocyte development, leading to premature oocyte maturation and oocyte atresia (*34*). Finally, since the energy regulation of fecundity by atresia has already been described in both captive (*35*) and wild fish (*36*), it appears significant to mention previous studies carried out on energy stores of immature female silver eels. It has been shown that 38.5% of their initial energy stores are allocated to swimming, thus leaving 60% of stores for gonad development, which, in theory, would allow the eel to reach a GSI of 22 (*21*). In the present work, while the last four remaining precontaminated eels presented a GSI higher than 22, no control eels could reach a GSI of 22 after 22 CPE injections. In this view, it is important to underline that the resulting precontaminated eels presented significantly lower body condition factors than the respective controls. For information, for the three precontaminated eels which died during the last 16 days of the experiment, this parameter reached a mean of 0.088 $\pm$ 0.002 (for a GSI mean of 30.25 $\pm$ 0.69). As the remaining precontaminated eels were also distinguishable from controls by an overexpression of the gene encoding *gh* (Figure 3B, factor of 5.2), a marker of energetic stress (*19, 20*), it could be hypothesized that the enhanced/accelerated gonad growth induced by Cd triggered the exhaustion of the animals' energetic reserves. This phenomenon could limit the final gonad maturation process and subsequently trigger oocyte atresia. It could at the same time also lead to the death of the puniest precontaminated eels.

To conclude, our work clearly shows that Cd is a strong endocrine disrupter for eels. By stimulating the hypothalamus−pituitary−gonad axis, it could not only alter the quality and/or number of eggs but also lead to the exhaustion of migrating female silver eels. However, it must be underlined that an important limitation of our work relies on the use of hormonal treatment. In this view, it appears important to mention that prepubertal blockage of European silver eels is due to a deficiency in pituitary GTH secretion, resulting from both a deficiency in the gonadotropin-releasing hormone (GnRH) function of hypothalamic neurons and a strong dopaminergic inhibition exerted at the pituitary level, counteracting GnRH-stimulated LH synthesis and release (*5*). As Cd involved an enhanced expression of both *lh* subunits during our experiment, this could imply an enhanced metal effect in natural conditions, i.e., when the dopaminergic inhibition should probably be relieved. However, the question that remains unresolved is whether the chronology and nature of morphological and hormonal phenomena that occur during artificial treatment are representative of those occurring during the natural reproductive migration of European eels. Another important limitation comes from the regime of Cd contamination. However, the fact that we observed a significant remobilization and redistribution of Cd during the course of the experiment, and also in control individuals, reinforces the potential occurrence of phenomena that we observed under natural conditions. Finally, as we already evoked, since the effect of Cd on fish reproduction has been described to vary significantly, notably according to the exposure conditions (*12–14*), different metal doses should be tested to assess the repeatability of our results.

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Supporting Information Available

Precise accession number and specific primer pairs used in quantitative real time PCR analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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