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Impairment of lipid storage by cadmium in the European eel (*Anguilla anguilla*)

Fabien Pierron [a], Magalie Baudrimont [a,*], Angélique Bossy [a], Jean-Paul Bourdineaud [a], Daniel Brèthes [b], Pierre Elie [c], Jean-Charles Massabuau [a]

[a] *UMR CNRS 5805 EPOC, Team GEMA, Université Bordeaux 1 and CNRS, Place du Dr Peyneau, 33120 Arcachon, France*
[b] *IBGC, UMR CNRS 5095, Université Bordeaux 2 and CNRS, 1, rue Camille Saint Saëns, 33077 Bordeaux Cedex, France*
[c] *Cemagref, Unité Ecosystèmes Estuariens et Poissons Migrateurs Amphihalins, U.R. EPBX, Cemagref, 50 Avenue de Verdun, 33612 Cestas, France*

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Abstract

Because European silver eels (*Anguilla anguilla*) fast during their reproductive migration to the Sargasso Sea, the successful completion of their unusual life cycle depends on quantity of lipids stored beforehand. These lipids are mainly accumulated during the growth phase stage of the animals, called yellow eel, as triglycerides in muscle. They are then catabolized to provide sufficient energy to enable migration, gonad maturation and spawning. In the laboratory, we investigated the possible impact of cadmium on the lipid storage efficiency of yellow eels in order to evaluate the possible contribution of this pollutant to the reported decline of European eel populations. Eels were exposed to dissolved cadmium at nominal concentrations of 0 and 5 $\mu g\,L^{-1}$ for 1 month. Cd toxicity was then examined by studying the activity and expression level of several enzymes involved in liver lipolysis and lipogenesis and by determining lipid content in muscle. Contaminated eels showed a lower body weight growth with a lower efficiency of lipid storage compared to controls. Using two complementary approaches, genetic and enzymatic, it was possible to conclude that this impairment is mainly explained by an increased utilisation of triglycerides since cadmium contamination did not trigger a reduced fatty acid synthesis. These observations suggest an increased fat consumption in presence of cadmium, which could compromise successful reproduction.

Keywords: *Anguilla anguilla*; Cadmium; Lipid storage; Yellow eel stage

1. Introduction

European eel populations (*Anguilla anguilla*) are in a dangerous state of decline. Glass eel recruitment has been decimated since the early 1980s throughout the whole European repartition area (Feunteun, 2002). The causes of this decline are still largely unknown. The biological cycle of the European eel is especially complex, with four life stages and two metamorphoses, which have been well described by Tesch (2003). Reproduction takes place in the Sargasso Sea from where larvae drift back towards the European coasts following oceanic currents, such as the Gulf Stream. 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, silver eels are still sexually immature when they leave freshwater systems. Moreover, they fast during their transoceanic migration (∼5500 km). This unusual life cycle means that European eels must accumulate energy reserves during their growth phase in the form of lipids, mainly as triglycerides. Lipids are then catabolized to provide the energy necessary for migration to the Sargasso Sea, gonad maturation and spawning. Consequently, the success of this migration is heavily dependent on the quantity of lipids stored during their growth phase (Boëtius and Boëtius, 1985). It has been estimated that the total stored lipids must exceed 20% of the body weight to cover migratory needs (Boëtius and Boëtius, 1980). In the literature, some authors consider that the second metamorphosis (silvering) does not occur as long as this 20% limit is not reached. Should this be the case, silvering could be delayed to

* Corresponding author. Tel.: +33 556 223 927; fax: +33 556 549 383.
E-mail address: m.baudrimont@epoc.u-bordeaux1.fr (M. Baudrimont).

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enable eels to store a sufficient amount of lipids (Larsson et al., 1990; Feunteun et al., 2000). Other authors, on the contrary, consider that silvering may occur independently of the amount of lipids stored (Svedäng and Wickström, 1997) which could in some instances lead to spawning failure owing to lipid content depletion. The relationship is not clear but it undoubtedly illustrates the importance of lipid storage during the growth phase. Surprisingly, few studies have focused on the impact of pollution on eel lipid metabolism. Moreover, those that have, are restricted to the impact of pesticides (Gimeno et al., 1995; Ceron et al., 1996; Fernandez-Vega et al., 1999) whereas eels are also recognized to be highly contaminated by heavy metals, especially by cadmium, in several European estuaries compared with other species (Barak and Mason, 1990; Usero et al., 2004; Durrieu et al., 2005). In this context, we investigated the possible impact of cadmium (Cd) on lipid storage by the European eel. Yellow eels were experimentally submitted to two water Cd concentrations ($[Cd]_w = 0$ and 5 $\mu g\,L^{-1}$) over an 1-month period. The liver was selected for our analysis because it is a main site of Cd bioaccumulation in fish (Durrieu et al., 2005) and a main site of lipid metabolism in eels, including lipogenesis and lipolysis (Aster and Moon, 1981). Cd impacts were evaluated by determining lipid content in muscle, the main site of triglyceride storage in eels (Boëtius and Boëtius, 1985), and by studying the activity of various liver cytosolic enzymes involved in lipogenesis and lipolysis. The activity of acetyl-CoA carboxylase (ACC), the key limiting enzyme in lipid storage transforming acetyl-CoA into malonyl-CoA which is required for fatty acid biosynthesis, was analysed. Activities of glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH), belonging to the pentose phosphate pathway, were investigated because they are implicated in the liver NADPH production. NADPH is an indispensable co-factor of fatty acid biosynthesis. For the same reasons, the activity of malic enzyme (ME), the key enzyme of the citrate-malate-pyruvate shuttle, and isocitrate dehydrogenase (IDH), which transforms the isocitrate into α-ketoglutarate, were studied. Finally, the liver lipase activity involved in triglyceride mobilization was investigated.

The expression level of genes encoding some of these lipogenic enzymes (*g6pdh*, *6pgdh*, *acc*) and the liver triglyceride lipase was analysed by quantitative real time RT-PCR. To do this, as a prerequisite we cloned and sequenced partial cDNA stretches of the corresponding genes.

2. Materials and methods

2.1. Animals and biometric measurements

Thirty immature yellow eels (*Anguillla anguilla*), averaging 32.2 ± 1.3 cm in length and 40.4 ± 1.8 g in weight (mean ± S.E., $n = 30$) were used. They were captured in June 2005 in the Grandlieu lake (Northwest France), one of the largest lakes of plain in France situated at the downstream section of a mainly agricultural basin and partly classified in natural reserve. The animals were transferred to the laboratory (Marine Biology Station, Arcachon, France) and kept in running aerated brackish water (salinity 5‰, natural seawater dilution with aerated tap water) thermostatted at 20 °C over a 1-month maintenance period, prior to experimentation. During this period, fish were fed every day with mussels and no lethality was observed.

Total body length and mass were recorded before and after experimentation to evaluate the Fulton condition factor ([weight (g)] × 10^5)/([length (mm)]^3).

2.2. Exposure to cadmium

The experiment was performed on acclimated yellow eels placed in a flow-through system consisting of two separate 215 L experimental units (EU). Each tank contained 15 organisms and was supplied with artificial brackish water (Instant Ocean, salinity = 5‰) by two water flowmeters at a rate of 150 mL h^{-1}. Fish in the first EU were in uncontaminated water and constituted control animals. In the second tank, fish were exposed to Cd at a dissolved metal concentration of 5 $\mu g\,L^{-1}$. Metal exposure was initiated after 1 week of adaptation to the experimental conditions by adding Cd to the water in the form of $CdCl_2$ from a stock solution. 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 500 $\mu L\,min^{-1}$ from a stock solution at a nominal concentration of 1.5 mg L^{-1}. During the experiment, the water column was permanently monitored for temperature (20 ± 1 °C), pH (8.25 ± 0.11) and salinity (5.2 ± 0.5‰). Water samples were collected three times a week. After acidification and dilution, they were checked for Cd concentration and flows were adjusted if necessary. The average concentrations were 0.02 ± 0.01 and 5.09 ± 0.37 $\mu g\,L^{-1}$ (mean ± S.E., $n = 12$) in the control and contaminated tanks, respectively. During the month of exposure, eels were fed every evening to an excess (7.5% wet weight animal/day) with mussels ([Cd] = 115.9 ± 15.2 ng g^{-1} (dw), mean ± S.E., $n = 5$). Every morning unconsumed food was removed, dried and weighed.

At time 0 (i.e. just before addition of Cd in water), 10 eels, five per EU were removed after the adaptation week to determine the Fulton condition factor and to perform lipid analysis at T_0. After 1 month of exposure (30 days), the yellow eels ($n = 10$ per EU) were removed, weighed and measured to determine the Fulton condition factor. Five eels per experimental condition were dissected and their liver was divided into two parts. The first part was immediately stored at −80 °C until enzymatic and genetic analyses. The second part was dried and used for Cd determination.

2.3. Metal determination

Biological samples were dried (48 h at 45 °C) and then digested with 3 mL of pure nitric acid (Fluka; Buchs, Switzerland) at 100 °C for 3 h.

After a six-fold dilution of the digestates with ultrapure water (Millipore, Bedford; MA, USA), Cd concentrations were determined by electrothermic atomic absorption spectrophotometry with Zeeman correction, using a graphite furnace tube (EAAS Thermoptec M6Solaar). Samples of 20 μL were mixed before atomisation with 4 μg of Pd (analyte modifier) and

3 μg of $Mg(NO_3)_2$ (matrix modifier). The detection limit was 0.1 μg L^{-1} Cd. All metal concentrations were expressed as ng g^{-1} of dry weight tissue (dw). The validity of the method was checked periodically against certified biological reference materials (Tort-2: lobster hepatopancreas and Dolt-2: dogfish liver from NRCCC-CNRC; Ottawa, Canada). Values were consistently within the certified ranges (data not shown).

For Cd determination in EUs, water samples were diluted, acidified at 2% HNO_3 and mixed with Pd and $Mg(NO_3)_2$.

2.4. Enzyme assays

The triglyceride lipase activity of the liver and the activity of $NADP^+$ dependent enzymes were assayed spectrophotometrically on the same sample used for Cd determination. Liver pieces (0.1 g) were homogenised in a tissue grinder fitted with a teflon pestle in ice-cold buffer (1:5, w/v) consisting of Na_2HPO_4, 20 mM, $MgCl_2$, 10 mM, and PMSF, 1 mM, adjusted to pH 7.8. The resulting homogenate was centrifuged for 12 min at 12,000 rpm at 4 °C (Sigma 3K12, Bioblock scientific). The clear supernatant fraction was removed with a Pasteur pipette from beneath the upper fat layer and used for protein and enzyme activity determination. Protein concentration was determined using the BCA protein assay kit (Pierce, USA). Bovine serum albumin was used as standard. For $NADP^+$ dependent enzymes β-mercaptoethanol 5 mM, glycerol 0.5 M, and 1 μL of aprotinin (10 mg mL^{-1}) were added to the supernatant after protein determination. Reaction rates were assayed spectrophotometrically at 340 nm following the appearance of NADPH. All assays were performed at 20 °C under saturating substrate conditions in phosphate buffer (Na_2HPO_4, 20 mM, $MgCl_2$, 10 mM, pH 7.8, final volume = 800 μL). The reaction mixtures were selected to give optimal activities with liver homogenates (final protein concentration = 0.1 μg L^{-1}) and were as follows:

- G6PDH (E.C. 1.1.1.49): glucose-6-phosphate 1 mM, $NADP^+$ 0.3 mM.
- 6PGDH (E.C. 1.1.1.44): 6-phosphogluconate 0.5 mM, $NADP^+$ 0.3 mM.
- ME (E.C. 1.1.1.40): malate 5 mM, $NADP^+$ 0.3 mM.
- IDH (E.C. 1.1.1.42): isocitrate 0.5 mM, $NADP^+$ 0.3 mM.

The lipase activity of the liver was determined using the Lipase-PS kit (Trinity biotech, USA) according to the manufacturer's instructions.

Acetyl-CoA carboxylase (E.C. 6.4.1.2) activity was assayed using [^{14}C]-bicarbonate (specific activity 3.7 MBq $mmol^{-1}$, Amersham Biosciences, UK) according to a previously described method (Rollin et al., 2003).

2.5. Lipid analysis

A whole section containing skin, subcutaneous fat, and red and white muscle was excised from an area 2–8 cm posterior to the anus. Lipid content was determined using the method of Folch et al. (1957). The biological pieces were homogenised in 20 volumes of methanol–chloroform (1:2). After 10 min pre-incubation in methanol, samples were homogenised for 5 min using Ultra-Turrax (T25 Janke & Kunkel IKA Labortechnik). Chloroform was then added and the mixture was once again homogenised for 5 min. The homogenate was filtered through a glass microfibre paper (Whatman GFA). The crude extract was mixed with 0.2 volume of salt solution (NaCl 0.58%) for 20 min and allowed to settle overnight. The lower organic phase was then separated and evaporated (SpeedVAc, Savant SC110A+) and lipids were weighed. Results are expressed as lipid percentage (g of lipids/100 g of sample).

Table 1
Sequence of primer pairs used to clone glucose-6-posphate dehydrogenase, 6-phosphogluconate dehydrogenase, triglyceride lipase and acetyl-CoA carboxylase cDNA

Gene name	Unspecific primers (5′–3′)
g6pdh	TACGCCAAGATGATGAGCAAGAAACC[a] GATGAAGGAGAGGGGTGAAGATCC[b]
6pgdh	CTGATGTGGAGAGGAGGCTGCATCATC[a] CCGTGACCCGTCCAGTTAGTGTGG[b]
tgl	TTGACTTCTACCCTAACGGAGG[a] GATCTTCTCAGCGCCGAGCTTGG[b]
acc	CAGCTCGCAAGACCACC[a] CGGCTCAGCTCCAGCTGG[b]

[a] Forward primer.
[b] Reverse primer.

2.6. Cloning and sequencing of glucose-6-posphate dehydrogenase, 6-phosphogluconate dehydrogenase, triglyceride lipase and acetyl-CoA carboxylase cDNA.

Total RNAs were extracted from 40 mg of fresh liver (wild organism from unpolluted site) using the Absolutely Total RNA Miniprep kit (Stratagene, Netherlands), according to the manufacturer's instructions. RNA quality was evaluated by electrophoresis on a 1% agarose gel and concentration was determined by spectrophotometry. First-strand cDNA was synthesized from 5 μg of total RNA using the Stratascript First-Strand Synthesis System (Stratagene, Netherlands) according to the manufacturer's instructions. To obtain a partial cDNA fragment of *glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, triglyceride lipase* and *acetyl-CoA carboxylase*, amplifications by PCR were carried out (50 amplification cycles at 95 °C for 1 min, 48 °C for 1 min and 72 °C for 1 min) using oligonucleotide primers previously deduced from alignment (using Clustalw software, Infobiogen) of corresponding sequences available in library from different fish and mammalian species (Table 1). Amplified products of expected size were then cloned into pGEM-T easy vector according to manufacturer's instructions (Promega, A1360, Madison, USA) and sequenced (Millegen Biotechnologies, France).

2.7. Quantitative RT-PCR

For each cDNA sequence, specific primers were determined (Table 2) using the Light-Cycler® probe design software (Ver-

Table 2
Accession number and specific primer pairs for the five genes from *A. anguilla* used in quantitative real time PCR analysis

Gene name	Accession number	Specific primers (5′–3′)
β-actin	DQ493907	CAGCCTTCCTTCCTGGGT[a] AGTATTTGCGCTCGGGTG[b]
g6pdh	DQ493914	CTGGACCTCACCTACGGC[a] GCTTCTTTCAGTTCATCACTGCG[b]
6pgdh	DQ493912	AGTGTTTTCTTGGGAAAGATCAAGG[a] CATGTTGTGTCTGTACCCGT[b]
acc	DQ493915	CCATCAAGACGGACAGC[a] GTGCCTGTAGATGCGG[b]
tgl	DQ493916	CTGAACACCGCCGACG[a] TTTTGGTCACTTCGCCCG[b]

[a] Forward primer.
[b] Reverse primer.

sion 1.0, Roche). After extraction and reverse transcription (see above), amplification of cDNA was monitored using the DNA intercaling dye SyberGreen I. Real-time PCR reactions were performed in a Light-Cycler® (Roche, Switzerland) following the manufacturer's instructions (one cycle at 95 °C for 10 min and 50 amplification cycles at 95 °C for 5 s, 60 °C for 5 s and 72 °C for 20 s). Each 20 μL reaction contained 2 μL reverse transcribed product template, 1 μL of master mix including the SyberGreen I fluorescent dye (Roche, Switzerland), and the specific primer pairs at a final concentration of 300 nM each. The reaction specificity was determined for each reaction from the dissociation curve of the PCR product. The dissociation curve was obtained by following the SyberGreen fluorescence level during a gradual heating of the PCR products from 60 to 95 °C. The relative quantification of each gene expression level was normalized according to the *β-actin* gene expression. In this way, for each gene expression level, the mean value and the associated standard deviation ($n = 5$) were determined. From this comparison, induction or repression factors were obtained for each gene by comparing each mean value observed in the contaminated eels with that of the corresponding control eels.

2.8. Data treatment

One-way analysis of variance was used to determine the effects of water Cd concentration on food intake, Cd bioaccumulation, Fulton condition factor, lipid content, enzyme activities and gene expression levels. For all the statistical results, a probability of $P < 0.05$ was considered significant. Assumptions necessary for using this parametric test were checked (normality, independence and homoscedasticity of the error term), based on analysis of residues, both graphically and using ad hoc tests (Kolmogorov–Smirnov and Bartlett test). If these assumptions were not fulfilled, Box–Cox data transformation was performed (Peltier et al., 1998) using the method of likelihood estimator.

Table 3
Cadmium concentrations (mean ± S.E., $n = 5$) in different tissue compartments of yellow eels after 30 days of exposure to cadmium: control, no cadmium added; contaminated, 5 μg L^{-1}

Organ	[Cd] (ng g^{-1}) (dw)	
	Control	Contaminated
Kidney	2627 ± 852	4914 ± 1366
Liver	335 ± 105	2341 ± 264*
Gills	48.6 ± 8.6	7480 ± 1001*
Digestive tract	471 ± 185	715 ± 138
Muscle	10.7 ± 1.4	7.1 ± 1.2

For each organ, * denotes significant difference (one-way analysis of variance, $P < 0.05$).

3. Results

3.1. Cd bioaccumulation

Results of Cd bioaccumulation in the five organs studied are presented in Table 3. No significant differences in Cd bioaccumulation were found in muscle ($P = 0.11$) or digestive tract ($P = 0.35$) between contaminated eels and controls. Despite a marked increase in Cd concentrations, similar results were found in kidneys ($P = 0.2$) which represented the main site of internal Cd accumulation in contaminated as well as in control eels. However, our results highlighted a significant Cd bioaccumulation in gills ($P = 0.00007$) and liver ($P = 0.0001$) ([Cd]contaminated/[Cd]control = 154 and 7, respectively).

3.2. Cd impacts on the behaviour and physiological state of eels

In order to exclude any effects of Cd on the food intake by eels, unconsumed food was removed daily, dried and weighed (data not shown). Results showed no Cd effect on food intake (ANOVA, $P < 0.05$) after 1 month of metal exposure. Both the control and contaminated eels had regularly taken food at a rate of 3.65 ± 0.42% (mean ± S.E., $n = 60$) of their fresh total body mass per day. Surprisingly, while Cd did not seem to influence food intake, the Fulton condition factor, which measures the relative plumpness of fish, was significantly affected by Cd exposure (Fig. 1). After 30 days of exposure, control organisms had a Fulton condition factor similar to that of eels removed at the beginning of the experiment. However, after 1 month of exposure, contaminated eels had a condition factor which was 12% lower than that of the control animals.

3.3. Biochemical responses to Cd exposure

The first result concerns the determination of lipid content in the muscle of yellow eels (Fig. 2). Before the start of the experiment, eels presented a lipid content of 2.9 ± 0.3%. After 30 days, increase in lipid content was only statistically significant for control eels (4.2 ± 0.3%), but not for contaminated eels (3.5 ± 0.6%).

Table 4 shows the results for enzyme activities measured in control and contaminated liver extracts. Whereas no signifi-

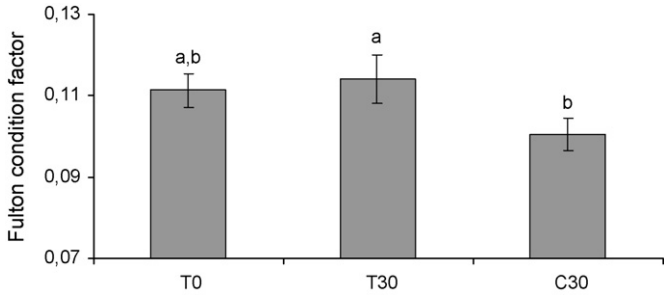

Fig. 1. Fulton condition factor (mean ± S.E., $n = 10$) of yellow eels removed at 0 day and after 30 days of experiment. T0: organisms removed before the start of the experiment. T30: control organisms ($[Cd]_w = 0\ \mu g\ L^{-1}$) removed after 30 days of experimentation. C30: contaminated organisms ($[Cd]_w = 5\ \mu g\ L^{-1}$) removed after 30 days of experimentation. Means designated with different letters (a and b) are significantly different (one-way analysis of variance, $P < 0.05$).

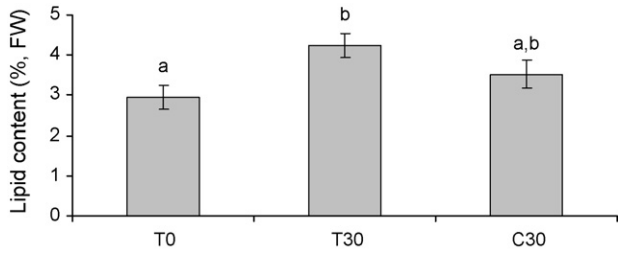

Fig. 2. Lipid content (mean ± S.E., $n = 3$) expressed as lipid percentage (g of lipids/100 g of sample, fresh weight) in muscle of yellow eels removed at 0 day and after 30 days of experiment. T0: organisms removed before the start of the experiment. T30: control organisms ($[Cd]_w = 0\ \mu g\ L^{-1}$) removed after 30 days of experimentation. C30: contaminated organisms ($[Cd]_w = 5\ \mu g\ L^{-1}$) removed after 30 days of experimentation. Means designated with different letters (a and b) are significantly different (one-way analysis of variance, $P < 0.05$).

cant difference was observed in the total protein concentration between control and contaminated extracts, Cd exposure triggered an increase in all enzyme activities involved in NADPH production and lipolysis. On the contrary, no effect on the ACC was observed.

Table 4
Enzyme activity (mean ± S.D., $n = 5$) in the liver extracts of uncontaminated (control) and contaminated yellow eels ($[Cd]_w = 5\ \mu g\ L^{-1}$) after 1 month of exposure and Cd effect compared to control (enhanced activity (×) or no effect (–))

	Control	Contaminated	Cd effect
IDH	76.0 ± 10.3	110.8 ± 8.3*	×1.5
G6PDH	17.2 ± 3.0	38.7 ± 7.6*	×2.2
6PGDH	47.8 ± 5.5	71.7 ± 8.7*	×1.5
ME	4.62 ± 0.73	11.92 ± 2.11*	×2.6
ACC	3.64 ± 0.3	3.06 ± 0.25	–
Lipase	3.24 ± 0.64	5.74 ± 0.29*	×1.8
[Protein]	49.8 ± 2.5	47.7 ± 4.2	–

All enzyme activity is expressed in nmol min^{-1} mg per protein. Protein concentration is expressed in microgram of protein per milligram of liver, fresh weight. For each enzyme activity, * denotes significant difference (one-way analysis of variance, $P < 0.05$).

Table 5
Basal gene expression (mean ± S.D., $n = 5$) as compared to *β-actin* in liver from yellow eels after 1 month of exposure to cadmium: control, no cadmium added; contaminated, 5 $\mu g\ L^{-1}$ and variations in gene expression as compared to control (induction (×) or repression factor (/)) in liver of yellow eels after 1 month of dissolved Cd exposure at 5 $\mu g\ L^{-1}$

Functions	Genes	Control	Contaminated	Cd effect
NADPH production	*g6pdh*	0.002 ± 0.0005	0.006 ± 0.001	×3.4[a]
	6pgdh	0.001 ± 0.0005	0.003 ± 0.0008	×2.2
Lipogenesis	*acc*	0.005 ± 0.006	0.001 ± 0.00008	1/4.5[a]
Lipolysis	*tgl*	0.0007 ± 0.0002	0.09 ± 0.01	×130.5[a]

[a] Significant effect of Cd on gene expression (one-way analysis of variance, $P < 0.05$).

3.4. Gene regulation by Cd

A partial *glucose-6-phosphate dehydrogenase* cDNA of 161 bp was amplified and sequenced. The resulting partial protein (53 amino acids (aa)) sequence was compared with the Genbank database using the BLASTX program, and the most significant alignments were found to correspond to G6PDH protein from fish *Danio rerio* (96% identity, 98% similarity) and *Takifugu rubripes* (92% identity, 96% similarity). In the case of *6-phosphogluconate dehydrogenase*, a partial cDNA was amplified and sequenced (779 bp; 229 aa). As detailed for G6PDH, the most significant alignments correspond to 6PGDH protein from fish *Danio rerio* (90% identity, 93% homology) and mammal *Mus musculus* (81% identity, 90% homology). For *acetyl-CoA carboxylase*, a partial cDNA was obtained (872 bp), coding for a 290 aa. This partial sequence showed extensive identities with corresponding proteins from *Macaca mulatta* (83% identity, 88% homology) and *Ovis aries* (82% identity, 87% homology). A partial cDNA of the *triglyceride lipase* (551 bp) encoding for a 183 aa was sequenced. This partial protein sequence presents the highest identity score with triglyceride lipase of *Anguilla japonica* (96% identity, 98% similarity) and *Rattus norvegicus* (57% identity, 74% similarity). All these sequences are available on the Genbank database, corresponding accession numbers are reported in Table 2. These sequences enabled us, subsequently, to study their expression levels in control and contaminated eels. All results obtained by quantitative RT-PCR are presented in Table 5. Results showed marked differences in genetic response to Cd exposure. Cd triggered an induction of genes encoding G6PDH, 6PGDH and TGL. Cd exposure, on the other hand, triggered a down-regulation of the gene encoding the ACC.

4. Discussion

Analysis of Cd bioaccumulaltion showed that gills were the main target organs of Cd bioaccumulation after 30 days of dissolved metal exposure. In these exposure conditions, gills represent the organ at the interface of the fish with the contaminated water. Gills are thus the tissue from which the metal loading of the organism will proceed. The liver was found to be an important target of internal accumulation, reinforcing the interest of studying Cd impacts on this organ. However, concerning inter-

nal accumulation, as described in literature for different fish species (Durrieu et al., 2005), highest Cd concentrations were found in kidneys in both eel groups. Cd concentrations found in controls are comparable to those found in different freshwaters fish species inhabiting uncontaminated field sites (Andres et al., 2000). Authors reported kidney Cd concentrations varying from 390 to 6284 ng g^{-1} wet weight according to fish species (compared to 627 ng g^{-1} wet weight in control individuals used during our study). For comparison, kidney Cd concentrations found in European eels inhabiting the Gironde estuary, characterized by old poly-metallic contamination, reach 34,100 ng g^{-1} dry weight.

Surprisingly, whereas the follow-up of food intake by eels through the 30 days of Cd exposure did not show any significant differences between control and contaminated eels, contaminated organisms showed a lower Fulton condition factor than controls. This result is consistent with field investigations. Indeed, several authors reported lowest condition factors in different fish species, including the European eel, subjected to metals such as Cd, Cu or Zn (Kearns and Atchinson, 1979; Munkittrick and Dixon, 1988; Linde et al., 1996; Levesque et al., 2002; Maes et al., 2005). In the present work, Cd significantly affected weight growth of eels. This is confirmed by the lowest efficiency of lipid storage observed in the muscle of contaminated eels. Indeed, after 30 days of Cd exposure, contaminated eels, unlike controls, did not significantly accumulate lipids in their muscle. Another paradoxical effect results from enzyme activities involved in the production of liver NADPH. Indeed, IDH, G6PDH, 6PGDH and ME are considered as lipogenic enzymes since NADPH is a pivotal cofactor for lipogenesis. It implies that these enzymes must be activated when lipogenesis is stimulated (Kather et al., 1972; Rho et al., 2005). Our results showed that fish exposure to Cd triggered a significant increased in *in vitro* activities of all enzymes implicated in liver NADPH production (IDH, G6PDH, 6PGDH, ME). Moreover, it is significant to note that this apparent increased activity is directly correlated with the up-regulation of genes encoding the 6PGDH and the G6PDH. Cd seems to strongly stimulate the total potential of liver NADPH production by increasing the amount of IDH, G6PDH, 6PGDH and ME in liver of contaminated eels. However, this stimulation was not correlated with an increase in lipid storage. Despite a down-regulation of the gene encoding the ACC, no effects of Cd were observed on its *in vitro* activity. However, since ACC is chronologically the first enzyme involved in fatty acid biosynthesis, it is considered as the rate-limiting enzyme of this process (Rollin et al., 2003; Wakil et al., 1983). The potential increase in the liver pool of $NADP^+$ linked enzyme under Cd exposure and the absence of Cd effect on ACC activity do not enable us to explain the Cd impact on lipid storage in muscle. For a better understanding, it is necessary to take into account the effect of Cd exposure on the lipase activity. Indeed, our results showed an increased activity (amplification factor = 1.8) and gene expression of the TGL (induction factor = 130) under Cd exposure, suggesting an increase in lipid mobilization. The aim of this triglyceride mobilization could have several origins. First, it is now well established that Cd induces lipid peroxidation of hepatocyte membrane (Stohs and Bagchi, 1995). Lipolysis that we observed could contribute to the renewal of damaged lipids by releasing diacylglycerol and fatty acids for membrane phospholipid synthesis (Unger et al., 1999). Second, because triglyceride is an energy-dense substance, its utilisation could indicate an increased energy need in contaminated organisms. In this way, Sherwood et al. (2000) have shown that yellow perch living in lakes polluted by heavy metals (Cd, Cu and Zn) have greater total energy costs and lower specific growth rates than yellow perch inhabiting reference lakes, while these populations present similar food consumption rates. Another argument suggesting a stronger energy need under Cd exposure is the increased plasma cortisol observed in fish experimentally exposed to metals such as Cd (Hontela et al., 1996; Monteiro et al., 2005). Cortisol is a gluconeogenic and lipolytic hormone stimulating glucose release from liver. In the European eel, an increase in plasma cortisol and glucose was observed after short-term exposure to copper (Teles et al., 2005). When eels are exposed to Cd, however, the response appears to be different. Gill et al. (1993) described an increase in plasma cortisol in the Americen eel, *Anguilla rostrata*, after long-term exposure to Cd (16 weeks; $[Cd]_w = 150\ \mu g\ L^{-1}$). However, this increase in hormone secretion was not correlated with an increase in plasma glucose. On the contrary, plasma glucose levels were significantly lower after 2 weeks of Cd exposure until they returned to levels close to control. This effect was supported by *in vitro* studies (Fabbri et al., 2003) undertaken on European eel hepatocytes. The authors have shown an inhibitory effect of Cd on glucose release from cells stimulated by epinephrine, a glycogenolytic hormone, with an IC_{50} of 0.04 μM (approximately 4.5 $\mu g\ L^{-1}$). In agreement with the literature, our results tend to show that Cd exposure triggered an increased energy cost. The apparent inability of eels to increase their plasma glucose level in response to Cd contamination could be an aggravating factor. Lipids would then constitute a main energy source which could explain the potential increase in lipolysis and the lower lipid storage that we observed in contaminated eels.

Another interesting point from our results is the confrontation between enzyme activities and their corresponding expression levels. Two groups of enzymes seem to stand out. The first group, consisting of G6PDH and 6PGDH, shows similar increases in their expression levels and their activities under Cd exposure (3.4 and 2.2 respectively for G6PDH, 2.2 and 1.5 respectively for 6PGDH). This tends to show that these enzymes are mainly regulated at the transcriptional level. On the other hand, ACC and TGL showed no comparable variations. In the case of ACC, while there was no Cd effect on its activity, there was a 4.5-fold decrease in gene expression. In the case of TGL, there was a 130-fold increase in gene expression under Cd exposure but lipase activity was affected by a positive factor of only 1.8. It is significant to note that Cd triggered an up-regulation of G6PDH and 6PGDH while, at the same time, it triggered a down-regulation of ACC. Stimulation of enzymes involved in the pentose phosphate pathway does not seem to be involved in fatty acid synthesis. We suggest that the aim of this stimulation might be to provide (i) nucleotides to repair DNA damage generated by Cd (Risso-de Faverney et al., 2001), (ii) intermediate metabolites to the glycolysis in order to enhance ATP production, and (iii) NADPH

which represents a crucial source of reducing equivalents needed to reduce oxidized gluthatione and to neutralize reactive oxygen species generated by Cd (Stohs and Bagchi, 1995; Xu et al., 2003).

The regulation of ACC and TGL in fish is not well documented. Nevertheless, in fish as in mammals, it is well established that these enzymes are hormone sensitive, since they are regulated both by a variety of hormones such as insulin, glucagon, growth hormone, leptin and by a variety of mechanisms (Khoo et al., 1974; Wakil et al., 1983; Mommsen and Moon, 1989; Harmon et al., 1993). They are controlled by hormones in two ways. The first, called short-term control, involves allosteric or metabolic regulation (polymerisation/depolymerisation) and covalent modification (phosphorylation/dephosphorylation) of the enzyme. Long-term control, on the other hand, involves change in the amount of the enzyme notably by modifying its expression level. Even if 30 days of experiment are sufficient to induce long-term control mechanisms, due to the short-term regulation, enzyme activities could not directly reflect their accumulation levels in cells. Differences between increases in their mRNA quantities and activities could be due to these fast regulation mechanisms. Furthermore, a possible effect of Cd exposure or Cd-induced hormones on the post-transcriptional regulation of these sequences could not be excluded. Another hypothesis can be put forward. Cd could directly poison these enzymes or enzymes implicated in their signal transduction pathway. Indeed, as previously mentioned, Fabbri et al. (2003) showed an impairment of the adenylate cyclase/cAMP transduction pathway by Cd in freshly isolated European eel hepatocytes stimulated by epinephrine. Epinephrine is a stress response hormone involved in the hydrolysis of glycogen to glucose and in the breakdown of lipids in fish. This inhibitory effect of Cd may contribute to the gap between the strong induction of the gene encoding the TGL and the much lower increase in lipase activity. Indeed, there are several ways in which hormones can induce gene expression. However, the AMPc way, *via* pyruvate kinase A, is at present the only one known to activate TGL activity.

5. Conclusions

Our results showed that Cd triggers an impairment of lipid storage in the muscle of yellow eels. This impairment appeared to be mainly explained by a Cd effect on lipid mobilization. Indeed, at the genetic level as well as the enzymatic level, Cd seems to trigger an increased lipolysis. This effect could have severe consequences. First, if silvering occurs independently of the quantity of lipids stored, as described by Svedäng and Wickström (1997), it would imply an unsuccessful transoceanic migration. This assumption obviously needs further investigation, notably for much longer exposure periods, including studies in several seasons. Second, if silvering does not occur as long as sufficient energy quantities are stored, it could not only delay the metamorphosis as described in literature (Larsson et al., 1990) but also compromise successful spawning. Indeed, if this effect on lipolysis is not restricted to the yellow stage, it could involve excessive energy consumption in contaminated migrants (silver eels) and result in an unsuccessful transoceanic migration. Due to their uncommon life cycle and the extreme performance they have to produce, European eels could then be especially sensitive to pollution. This study clearly illustrates the need for further toxicological investigations taking into account their very particular life cycle.

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