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Evidence for metabolic imbalance of vitamin A_2 in wild fish chronically exposed to metals

Michel A. Defo [a], Fabien Pierron [b,c], Philip A. Spear [d], Louis Bernatchez [e], Peter G.C. Campbell [a], Patrice Couture [a,*]

[a] Institut national de la Recherche scientifique (INRS), Centre Eau Terre et Environnement, 490 de la Couronne, Québec, QC, Canada G1K 9A9
[b] Université de Bordeaux, EPOC, UMR 5805, F-33400 Talence, France
[c] CNRS, EPOC, UMR 5805, F-33400 Talence, France
[d] Centre de Recherche TOXEN and Département des Sciences biologiques, Université du Québec à Montréal, C.P. 8888, Succursale Centre-Ville, Montréal, QC, Canada H3C 3P8
[e] Institut de Biologie intégrative et des Systèmes (IBIS), Université Laval, Québec, QC, Canada G1V 0A6

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ABSTRACT

In a recent study on indigenous yellow perch chronically exposed to metals, we reported a negative correlation between liver metal concentration and liver transcription levels of genes encoding for enzymes involved in the metabolism of retinoids. We therefore speculated that metals, and especially the non-essential metal Cd, could alter the metabolism of retinoids in wild fish. Thus the present field study investigates the impact of in situ metal exposure on retinoid storage. A total of 55 yellow perch (*Perca flavescens*) were sampled in six lakes representing a metal contamination gradient ($8 \leq N \leq 10$ per lake). Our results show that yellow perch from Cd-contaminated lakes had significantly higher concentrations of liver dehydroretinol and dehydroretinyl esters than did fish from reference lakes. However, the increase in retinyl ester stores with increasing Cd concentrations was quantitatively much more important than the increase in free dehydroretinol. As a result, a significant decrease in the percentage of hepatic free dehydroretinol with increasing renal Cd concentrations was observed. These results suggest that the enzymes and the binding proteins involved in vitamin A homeostasis are inhibited by the presence of Cd. Alternatively, the increase in tissue vitamin A (antioxidant) levels could serve to better counteract the oxidative stress engendered by Cd exposure. Overall our findings illustrate that vitamin A_2 homeostasis can be altered as a consequence of chronic exposure to low Cd concentrations. Thus, in the context of environmental risk assessment, the percentage of liver free dehydroretinol can be considered as a biomarker of for in situ Cd exposure.

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1. Introduction

Metals released by anthropogenic activities such as mining and smelting have affected many aquatic ecosystems in the world. During the last century, concentrations of trace metals such as cadmium (Cd), copper (Cu) and zinc (Zn) have greatly increased in water, sediment and biota (Klee and Graedel, 2004; Luoma and Rainbow, 2008). The past two decades have seen a major tightening of governmental regulations on smelter emissions. As a result, a decrease in metal releases to the environment has been observed and signs of chemical recovery have been detected both in local ecosystems and regionally (Belzile et al., 2004; Mahler et al., 2006). However, recent research clearly indicates that the physiology, condition and overall health of fish living in metal-contaminated environments are still affected (Couture et al., 2008b; Giguère et al., 2006; Rajotte and Couture, 2002), suggesting that metal contamination continues to exceed levels that are safe for the environment. The essentiality of Cd is still unknown; however, although the biological functions of Cu and Zn are largely accepted, these metals can be toxic at concentrations slightly greater than those required for homeostasis (Atli and Canli, 2011; Niyogi et al., 2007). Yellow perch (*Perca flavescens*) is abundant and widely distributed in North America. In the context of metal ecotoxicology, it is a particularly relevant species because it can tolerate the presence of metals at quite high concentrations; this tolerance explains its wide distribution and abundance in surface waters affected by mining and smelting activities in Canada (Hontela et al., 1992,1995). In addition, because of its sedentary behavior (Aalto and Newsome, 1990), bioaccumulated metals in yellow perch tend to reflect local contamination of ecosystems (Aalto and Newsome, 1990; Campbell et al., 2003).

* Corresponding author. Fax: +1 418 654 2600.
E-mail address: patrice.couture@ete.inrs.ca (P. Couture).

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The use of tissue retinoid concentrations as biomarkers of pollutant exposure has already been discussed in the literature (Marcogliese et al., 2009; Mos et al., 2007; Rodríguez-Estival et al., 2011) and extensively studied in vertebrates including mammals, birds, frogs and fish. The term 'retinoid' refers to a group of compounds having vitamin A biological activity and includes retinol, retinal and retinoic acids. Given their many physiological functions and the inability of fish to synthesize retinoids *de novo* (Garner et al., 2010), these substances and their metabolic precursors are an important resource that can be limited by dietary availability. Dietary retinoids are taken up mainly as retinyl esters from animal prey and are derived from dietary carotenoids, natural plant pigments (D'Ambrosio et al., 2011; Fernández and Gisbert, 2011). In addition to their role as antioxidants (i.e. all-transretinols) (Alpsoy et al., 2009; Dragsted, 2008; Rodríguez-Estival et al., 2011), various retinoids are involved in many essential functions including cell differentiation (Amann et al., 2011b), growth (Rolland, 2000; Tanumihardjo, 2011), vision (D'Ambrosio et al., 2011), immunity (Mora et al., 2008), bone mineralization (Fernández and Gisbert, 2011) and reproduction (Li et al., 2011) *via* the activation of specific transcription factors (Amann et al., 2011a,2011b; Sucov and Evans, 1995). Unlike other vertebrates such as mammals, amphibians and birds, in which all-*trans*-retinol and its esters are typically the predominant forms of vitamin A, some freshwater fish species possess all-*trans*-dehydroretinol and dehydroretinyl esters as the major form of vitamin A (Gesto et al., 2012; Goswami and Barua, 1981). However the tissue distribution, metabolism and roles of these substances are mostly unknown or still poorly known (Gesto et al., 2012). According to the classification of Goswami and Barua (1981), yellow perch can be categorized as a dehydroretinol-type (vitamin A_2) species.

The toxic effect of environmental contaminants may be mediated by the disruption of vitamin A transport, metabolism and signaling (Novák et al., 2008). Studies have shown that exposure to organic pollutants (in particular organohalogens and pesticides) can disturb vitamin A homeostasis in animals (Bérubé et al., 2005; Spear et al., 1992,1990). In comparison to such organic pollutants, little is known about the adverse effect of metals on the change in levels of retinoids in populations of animals living in contaminated areas (Novák et al., 2008; Rolland, 2000). Although liver retinoid concentrations of two trout species from selenium-impacted streams in a coal-mining region were species- and gender- dependent, the concentration of total retinyl esters of rainbow trout (RT) decreased with increasing selenium level (Miller et al., 2009). However, selenium did not affect the storage of liver total retinyl esters in brook trout (BT). In the same studies, Miller et al. (2009) showed that liver dehydroretinol levels were higher in RT than in BT, but that immature RT and male BT displayed the highest level of vitamin A_2. After a three-year exposure of European flounder (*Platichthys flesus*) to heavily polluted sludge from Rotterdam harbor, the levels of plasma and liver retinol as well as liver retinyl esters were significantly decreased (Besselink et al., 1998). The authors suggested that PHAHs and PAHs, rather than trace metals, were responsible for the dramatic reductions in plasma and liver retinoid concentrations. By comparing the hepatic retinoid concentrations in seven fish species from a tropical coastal lagoon that receives effluents from iron-ore mining, Pereira et al. (2012) observed a negative correlation between Al, Pb, As, and Cd and hepatic didehydroretinoid concentrations. However, significant and positive correlations were obtained with fish displaying higher levels of hepatic Fe, Cu, and Zn.

A recent study on indigenous yellow perch chronically exposed to metals in lakes of the Rouyn-Noranda region reported a negative correlation between liver Cd concentration and liver transcription levels of genes encoding for enzymes involved in the metabolism of retinoids (Pierron et al., 2011). We therefore speculated that metals, and especially the non-essential metal Cd, alter the metabolism of retinoids in wild fish. Thus the present field study investigates the impacts of in situ metal exposure on retinoid storage in yellow perch sampled in late spring from six lakes representing a metal contamination gradient. We tested the hypothesis that the retinoid transcriptomic response observed in our previous study (Pierron et al., 2011) is translated at the biochemical/physiological level.

2. Materials and methods

2.1. Study area and fish sampling

This investigation was carried out in an area of the Canadian Precambrian Shield that has been subjected to metal emissions from smelters for over 80 years, the Rouyn-Noranda region (Québec). This region presents a broad contamination gradient in Cd, Cu and Zn concentrations in water, sediment and prey (Campbell et al., 2008; Croteau et al., 2003; Perceval et al., 2002) and therefore offers a unique research opportunity to study relationships between metal bioaccumulation and subsequent deleterious effects on indigenous perch populations living in this area (Couture et al., 2008b). For our investigation, we selected six lakes from this gradient (Table 1) that differ substantially in their dissolved and sediment metal concentrations: two pristine lakes, Opasatica and Hélène, located approximately 30 km upwind from the Horne smelter located in the city of Rouyn-Noranda; two intermediate lakes, Vaudray and Bousquet, located approximately 30 km downwind from the smelter; and two highly metal-contaminated lakes, Marlon and Dufault, situated near the smelter.

A total of 55 fish were collected in late spring (mid-June 2011) using a seine net. In order to minimize potential allometric bias, yellow perch were selected within a narrow size range. A total of 8 to 10 adult fish were sampled per lake. Length (11.0 ± 0.3 cm) and weight (16 ± 2 g), (mean $\pm$ SE), were recorded and sex determined. Following the method described in Bérubé et al. (2005), but without anesthesia, blood was collected from the dorsal artery of the caudal peduncle using a heparinised needle. After centrifugation, the plasma was immediately frozen on dry ice and stored at -80 °C in heparinised tubes until analysis. Fish were dissected and the liver (for retinoid analyses) as well as the kidney (for metal analyses) and pyloric caeca (for biometric analyses) were removed and immediately frozen and stored in liquid nitrogen in the field. Upon return to the laboratory, tissues were stored at -80 °C until used for analyses. The fish manipulation protocol was approved by the Ministère des Ressources Naturelles et de la Faune du Québec and the Comité Institutionnel de Protection des Animaux (CIPA) of INRS.

2.2. Calculation of fish condition and corrected pyloric caeca weights

Fulton condition factor (K) is the index commonly used when assessing fish condition. This index is based on the assumption of isometric growth, i.e. fish size increases according to a cubic relationship. However, in most species, growth is

Table 1
Geographical coordinates of the study lakes with their total dissolved (nM) concentrations of the trace metal analyzed.

Lake	Longitude	Latitude	[Cd] nM	[Cu] nM	[Zn] nM	References
Hélène	48°12′44″N	79°10′18″W	0.15	36.4	8	(Hare and Tessier, 1998)
Opasatica	48°04′58″N	79°17′35″W	0.3	33	19	(Giguère et al., 2006)
Bousquet	48°12′59″N	78°36′46″W	2.37	43.5	117	(Hare and Tessier, 1998)
Dufault	48°18′30″N	78°59′58″W	6.7	180	1100	(Giguère et al., 2006)
Vaudray	48°05′09″N	78°40′47″W	0.5	42	60	(Giguère et al., 2006)
Marlon	48°15′51″N	79°03′55″W	1.43	169	57	(Hare and Tessier, 1998)

allometric. The relative index (Kn) removes biases related to size and allows the comparison of fish from the same population or different populations, irrespective of size (Baigún et al., 2009). The equation $Kn=(W_f/L^{SC})\times 100$ (where "W_f" is fish weight, "L" is the fish length and "SC" is the scaling coefficient) was used to calculate "Kn", a calculation used when fish size varies appreciably within a given dataset (Pyle et al., 2008). SC is the slope of the logarithmic relation between the mass and the size of perch in the same region (Gauthier et al., 2009), which corresponds to 3.23 in our case.

Laboratory studies have demonstrated the existence of a positive correlation between pyloric caeca (PC) weights and feeding rate (Gauthier et al., 2008), and with food conversion efficiency in fish (Bergot et al., 1981). Due to the non-linearity of the relation between fish wet weight and wet weights of PC, we standardized the PC weights by applying an allometric correction described elsewhere for yellow perch (Gauthier et al., 2011). The pyloric caeca weights were corrected to a standard fish weight of 16 g with an allometric exponent of 0.960, calculated as the slope of the logarithmic relationship between fish weight and pyloric caeca weights. The correction was done using the following equation: corrected pyloric caeca weights$=((16/\text{fish wet wt})^{0.96})$ PC wet weights.

2.3. Kidney metal concentration analysis

Tissue metal concentrations (Cd, Cu and Zn) were determined in the kidney according to Pierron et al. (2009). Briefly, lyophilized samples were transferred to acid-washed (15% HNO_3) Eppendorf polypropylene tubes and digested in trace metal grade nitric acid at room temperature over 5 d. To monitor analytical accuracy and recovery, 5 replicates of certified reference material from the National Research Council of Canada (TORT2) were also analyzed. In parallel, for each batch of 10 samples, blanks (trace metal grade HNO_3) were subjected to the same treatment in order to control for potential contamination during the digestion and analysis procedures. After 5 d, hydrogen peroxide was added to the digests; after 24 h the digests were diluted with deionized Milli-Q water (10% of HNO_3) and stored at 4 °C until analysis. Metal concentrations were measured by inductively couple plasma-atomic emission spectrophotometry (ICP-AES), or by inductively coupled plasma-mass spectrometry (ICP-MS) when the concentrations were too low for detection by ICP-AES. Internal standards were within 10% of nominal values in all cases and recoveries averaged for metal analyses was 90 ± 3% for Cd, 78 ± 3% for Cu, 78 ± 6% for Zn (mean ± S.E.).

2.4. Retinoid analysis in plasma and liver

2.4.1. Retinoid extraction

To prevent isomerization, the analyses were conducted under yellow incandescent lights. The plasma retinoid extraction procedure was modified from that used by Spear et al. (1988). Briefly, 50 μL of plasma was vortexed with an equal volume of ethanol in order to achieve protein precipitation, and with an equal volume of ultrapure water to dissociate retinoid–protein complexes. Extraction was achieved by adding 200 μL of hexane followed by phase separation by centrifugation (13,000g × 2 min). A 160-μL aliquot of the organic phase was evaporated to dryness (5 min at 30 °C) using a vacuum centrifuge (Vacufuge plus, Eppendorf, Hamburg, Germany). The extract was resuspended in 100 μL of ethanol and to prevent retinoid loss was stored at −20 °C until analysis. Once prepared, the extracts were held for maximum 2 h at −20 °C prior to analysis. An 80-μL volume was injected into the high-performance liquid chromatography (HPLC) system.

The method for liver retinoid extraction was modified slightly from Spear and Moon (1986). Specifically, 0.05 g of partially thawed yellow perch liver was dehydrated by grinding with 0.5 g of anhydrous Na_2SO_4 and the resulting powder extracted with 1.25 mL hexane for 10 min using a rotary mixer. After centrifugation (2 min at 2000 g), an 800-μL aliquot was transferred to an Eppendorf tube, evaporated to dryness at 30 °C during 10 min using a vacuum centrifuge and the residue dissolved in 100 μL of acetonitrile. A volume of 80 μL was injected into the HPLC.

2.4.2. High-performance liquid chromatography

All solvents used were HPLC grade. All-*trans*-retinol (ROH) and all-*trans*-retinyl palmitate (ROH-palmitate) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other retinoids were synthesized by methods routinely used in our laboratory. The standard all-*trans*-3,4-dehydroretinol (dROH) was synthesized as described previously (Bérubé et al., 2005). All-*trans*-3,4-dehydroretinyl esters of palmitate (dROH-palmitate), myristate (dROH-myristate) and stearate (dROH-stearate) were produced by condensing dROH with fatty acid anhydrides following the method of Doyon et al. (1998).

The HPLC system consisted of dual model 510 pumps, a model 150A injector, and a model 486-absorbance detector with data acquisition and instrument control by the Millenium 32 computer program (all Waters Associates, Milford, MA, USA). Retinoids were separated on a CSC-Inertsil C-18 analytical column (5 μm particle size, 150 Å pore, $4.6\times 250\ mm^2$; Chromatography Sciences, Montréal, QC, Canada). The detector was set at 354 nm, corresponding to the maximum light absorbance of all-*trans*-3,4-dehydroretinol (dROH) and dROH esters, the major retinoids. Extremely small peaks of other retinoids were apparent in yellow perch tissues, but were not quantified. Plasma dROH eluted at 10.6 min under isocratic conditions of 90% methanol and 10% water and a flow rate of 1 mL/min. The analyte dROH was separated from ROH, the latter having a retention time of 13.2 min. In the case of the hepatic retinoids, the method of Doyon et al. (1998) was adapted to improve peak resolution. At a constant flow rate of 1 mL/min, the initial mobile phase (tetrahydrofuran/methanol/water (18:75:7)) changed linearly between 2 and 6 min to the second mobile phase composed of the same solvents in different proportions (36:57:7). Retention times under these conditions were dROH 5.4 min, ROH 5.7 min, dROH-myristate 17.1 min, dROH-palmitate 20.3 min, ROH-palmitate 22.3 min and dROH-stearate 24.5 min. Allowing for the elution of retinoid esters and column solvent re-equilibration, samples could be injected every 28 min. Peak identification was based on Doyon et al. (1998). No attempt was made to separate the palmitate and oleate esters, the peak being described here as dROH-palmitate. The major retinoids were quantified using a dROH external standard and adjusting for the mass differences of the different esters. The mass sum of individual retinoids was used to estimate 'total vitamin A', whereas the mass sum of esterified forms was taken to represent 'total retinyl esters'. The recovery of the different retinoids from spiked fish liver ranged from 86–103%. The unesterified all-*trans*-3,4-dehydroretinol is referred to as 'free dehydroretinol' in this paper.

2.5. Data analysis

Due to the limited number of males (12/55), statistical analyses were performed without considering sex as an explanatory variable. Differences in vitamin A_2 levels, biometric parameters, and metal concentrations among sites were performed by analysis of variance (ANOVA), after checking assumptions of normality and homoscedasticity of the error terms (Levene test, $p>0.05$). When the assumptions were not met, we used $\log_{10}$ and Box-Cox (Peltier et al., 1998) data transformations or the non-parametric Wilcoxon/Kruskall–Wallis tests. If significant effects were detected, the Least Square Deviation test (LSD or Tukey test) and the non-parametric *U*-Mann–Whitney test were used to determine whether means between pairs of samples were significantly different from one another. Computations were performed using JMP 8.0 program and STATISTICA version 6.1 software (StatSoft, USA). The relationships between individual metal concentrations in kidney and retinoid concentrations or biometric parameters were investigated using the non-parametric Spearman rank correlation test due to the non-normality of the data.

3. Results

3.1. Kidney metal concentrations in yellow perch

Ratios of renal metal concentrations between the most and the least contaminated fish, i.e. $[M]_{max}/[M]_{min}$, increased in the order Cu (1.44) < Zn (2.72) < Cd (36.7). Renal Cd concentrations presented a clear concentration gradient in the lakes sampled (Fig. 1A) and varied from 1.35 to 49.5 μg/g dry weight (12 to 440 nmol/g) from Lake Hélène to Lake Marlon, the latter lake being very close to the smelter. Renal Cu concentrations in yellow perch from all lakes sampled were similar except in the most pristine lake (Hélène) where values were significantly lower than in fish from lakes Dufault and Marlon (Fig. 1B). Mean kidney Zn concentrations ranged from 345 to 939 μg/g dry weight (5.3 to 14.4 μmol/g; Fig. 1C). Fish from lakes Dufault and Marlon showed the highest Zn concentrations, significantly higher than those of the Bousquet and Vaudray fish.

3.2. Biometric parameters

Fish from Lake Opasatica displayed lower condition factors that did fish from the other lakes, but this difference in condition was only significant when compared to fish from lakes Marlon and Bousquet (Table 2). Corrected pyloric caeca wet weights were not significantly different among most of the populations sampled (Table 2), except for fish from Lake Bousquet, the pyloric caeca of which were significantly heavier than those of from Lake Marlon. Significant negative relationships were observed between corrected pyloric caeca wet weights and renal Cd and Zn concentrations (Table 3).

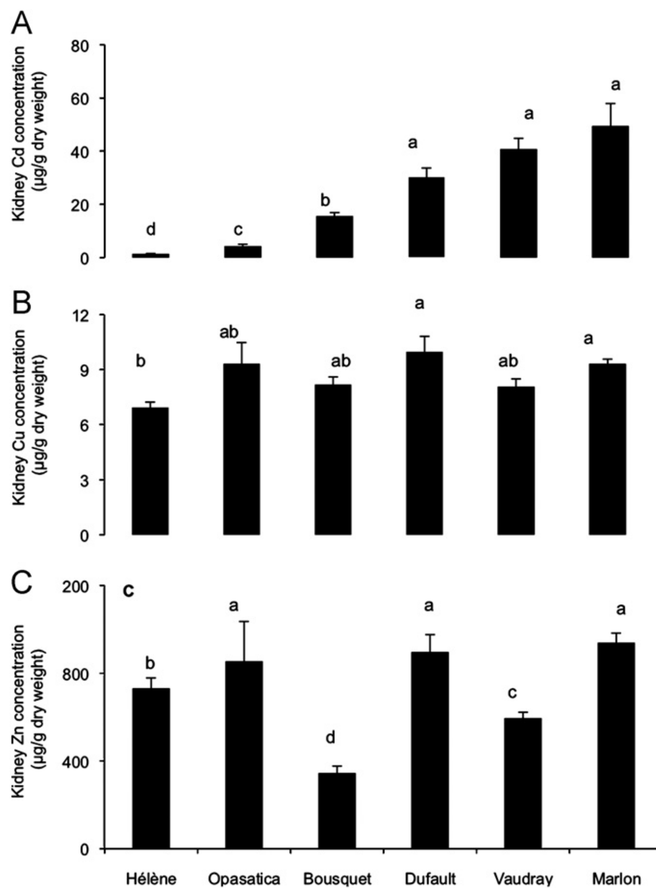

Fig. 1. Total cadmium (A), copper (B) and zinc (C) concentrations in the kidney of yellow perch collected from six lakes along a metal concentration gradient. Values are means ± SE ($8 \le N \le 10$/lake). Means designated with different letters are significantly different (LSD test, $p < 0.05$).

Table 2
Number of fish in lake sampled (N), relative condition index (Kn) and corrected pyloric cæca weight of wild yellow perch used for vitamin A analysis. Values are mean ± SE. ($8 \le N \le 10$/lake). Values within columns sharing at least one letter are not significantly different from each other (LSD test, $p < 0.05$).

	N	Relative index condition (Kn)	Corrected pyloric cæca wet weight
Hélène	9	0.55 ± 0.02^{bc}	0.034 ± 0.006^{ab}
Opasatica	8	0.50 ± 0.01^{c}	0.024 ± 0.001^{ab}
Bousquet	9	0.63 ± 0.01^{a}	0.038 ± 0.004^{a}
Dufault	9	0.550 ± 0.005^{bc}	0.025 ± 0.003^{ab}
Vaudray	10	0.55 ± 0.01^{bc}	0.026 ± 0.003^{ab}
Marlon	10	0.58 ± 0.01^{ab}	0.022 ± 0.002^{b}

3.3. Dehydroretinoid concentrations in liver and plasma

Analysis of liver samples by means of HPLC revealed that all-*trans*-3,4-didehydroretinoids (vitamin A_2) are the predominant form of the vitamin in liver and plasma of yellow perch. The major vitamin A_2 stored in yellow perch liver was dROH palmitate, with lesser amounts of dROH myristate, dROH stearate and other unidentified forms. Hepatic concentrations of free (i.e. unesterified) dROH, specific retinyl esters, total retinyl esters and total vitamin A differed significantly among the most Cd contaminated lakes (Dufault, Vaudray, Marlon) and the least Cd contaminated lakes (Hélène, Opasatica and Bousquet) (Table 4). A similar trend was observed for stores of dehydroretinyl ester. Concentrations of dROH stearate, dROH myristate and dROH palmitate were 3-, 235- and 3.56×10^5 times greater, respectively, in fish from Lake Marlon Lake in comparison to fish from Lake Hélène.

Although hepatic dehydroretinoid levels increased in fish inhabiting the most contaminated lakes, the percentage of liver free dROH decreased significantly with increasing contamination (Fig. 2). The percentage of free dROH was 9.3-fold, 24-fold and 806-fold lower in fish from lakes Dufault, Vaudray, and Marlon respectively, in comparison to fish from Lake Opasatica.

The analyses of plasma extracts revealed significant differences in dROH concentrations among sites (Fig. 3). The highest concentrations were detected in perch from lakes Marlon, Vaudray and Bousquet, while significantly lower levels were measured in fish from Lake Opasatica. Fish from Lake Hélène had similar plasma concentrations of dROH to those in fish from Lake Dufault. Fig. 4 represents the plasma dROH:liver dROH ratio in fish from the lakes sampled. The ratios were generally higher in fish from lakes less impacted by Cd, except in Lake Opasatica fish. However, the differences were generally non significant, except in the most Cd impacted lake (Marlon) (Fig. 4). No significant correlation was observed between plasma and liver dROH concentration.

There was no significant correlation between renal Cu and hepatic retinoids except for liver dROH stearate (R=0.28; p=0.045). All forms of hepatic dehydroretinoids were significantly and positively correlated with kidney Cd and to a lesser extent with Zn concentration (Table 3). In contrast, plasma dROH was significantly and negatively correlated with kidney Zn (R=−0.37; p=0.0097) whereas no significant correlations were observed between plasma dROH levels and renal Cu (R=−0.41; p=0.78) or Cd (R=0.26; p=0.072) concentrations.

4. Discussion

Previous studies have reported a good correlation between liver and kidney metal concentrations, notably Cd concentration, in yellow perch from this region (Kraemer et al., 2005a,b). Among the six lakes sampled, a gradient in kidney Cd was observed, with an 36-fold difference between fish from the cleanest and the most contaminated lake (Fig. 1A). This result is in agreement with those reported by Couture et al. (2008a), who also observed a kidney Cd gradient in yellow perch populations living in this mining region (Couture et al., 2008a). The same results were also obtained in liver (Bourret et al., 2008).

The relative index Kn was used to assess fish condition. Kn is associated with recent feeding activity; this metric is used as an estimate of somatic energy reserves accumulated in fish. Fish from Lake Opasatica displayed lower condition factors when compared to fish from lakes Marlon and Bousquet (Table 2). Studies reported that yellow perch living in metal impacted sites had lower indicators of physical condition than fish from cleaner lakes (Laflamme et al., 2000; Pyle et al., 2008). Moreover these fish were smaller for a similar age, indicating slower growth rate. Furthermore the relative condition factor and scaling coefficients were lower (Pyle et al., 2008).

Although tissue concentrations of dehydroretinoids of fish samped were high, values obtained for fish living in pristine lakes were well within the range reported in other studies. For example, values of 0.24 µmol/µL and 0.17 µmol/µL of plasma retinol content were analyzed in flounder exposed for 3 years to the reference sediment and contaminated sediment respectively (Besselink et al., 1998). Those concentrations are comparable to the plasma dROH obtained in this study (0.1 µmol/µL–0.3 µmol/µL). In the same manner, values of liver dROH or ROH and ester forms

Table 3
Spearman correlations between kidney metal concentrations, biometric parameters and plasma and liver retinoids in yellow perch ($49 \le N \le 52$) sampled in the mining region of Rouyn-Noranda, Québec, Canada (Spring, 2011).

	Kidney Cu		Kidney Cd		Kidney Zn	
	R Spearman	*P*	*R* Spearman	*P*	*R* Spearman	*P*
Relative index condition	0.141	0.319	0.046	0.748	−0.253	0.071
Corrected pyloric caeca	0.040	0.777	−0.280[a]	0.045	−0.373[a]	0.006
Plasma dehydroretinol	−0.041	0.777	0.259	0.072	−0.366[a]	0.010
Liver dehydroretinol	0.114	0.419	0.413[a]	0.002	0.414[a]	0.002
Liver dehydroretinyl myristate	0.212	0.132	0.696[a]	< 0.0001	0.374[a]	0.006
Liver dehydroretinyl palmitate	0.177	0.214	0.678[a]	< 0.0001	0.357[a]	0.010
Liver dehydroretinyl stearate	0.279[a]	0.045	0.668[a]	< 0.0001	0.399[a]	0.003
Liver total vitamin A esters	0.176	0.213	0.678[a]	< 0.0001	0.382[a]	0.005
Liver total vitamin A	0.175	0.216	0.665[a]	< 0.0001	0.399[a]	0.003
Liver percentage of free dehydroretinol	−0.207	0.141	−0.718[a]	< 0.0001	−0.283[a]	0.042
Ratio of plasma dehydroretinol/Liver dehydroretinol	−0.089	0.542	−0.318[a]	0.026	−0.506[a]	0.0002

[a] $P < 0.05$.

Table 4
Vitamin A levels (nmol/g liver wet weight) determined in livers of yellow perch sampled in Rouyn-Noranda region. Québec, Canada. Values are mean ± SE. ($8 \le N \le 10$/lake). Values within lines sharing at least one letter are not significantly different from each other (LSD test, $p < 0.05$).

Retinoids	Hélène	Opasatica	Bousquet	Dufault	Vaudray	Marlon
Free dehydroretinol	308 ± 175[ab]	47 ± 10[ab]	22.2 ± 6.5[b]	419 ± 228[a]	4171 ± 3693[a]	9537 ± 5187[c]
Dehydroretinyl palmitate	5772 ± 3211[a]	918 ± 622[a]	298 ± 134[a]	(1.6 ± 1.0)E+7[b]	(4.3 ± 2.1)E+9[b]	(2.1 ± 1.4)E+9[b]
Dehydroretinyl myristate	17.0 ± 5.7[a]	10.2 ± 3.2[a]	11.2 ± 3.6[a]	1218 ± 909[b]	1806 ± 1128[b]	4003 ± 1873[b]
Dehydroretinyl stearate	3.4 ± 0.3[a]	3.0 ± 0.1[a]	3.0 ± 0.1[a]	7.0 ± 0.9[bc]	5.4 ± 0.6[b]	10.7 ± 1.7[c]
Total vitamin A esters	5793 ± 3410[a]	931 ± 625[a]	267 ± 130[a]	(1.4 ± 1.1)E+7[b]	(4.3 ± 2.1)E+9[b]	(2.1 ± 1.4)E+9[b]

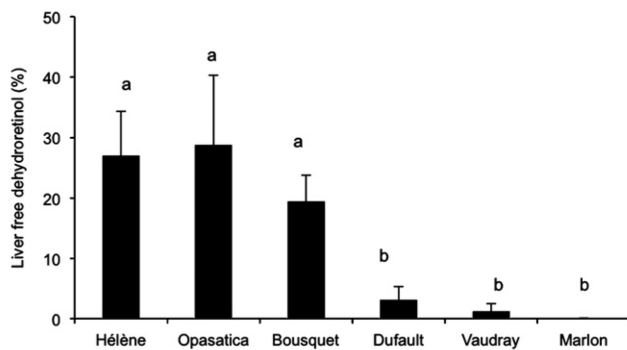

Fig. 2. Percentage of hepatic free dehydroretinol. Bars represent means and standard errorr. Bars that do not share a common letter are significantly different (LSD test, $p < 0.05$).

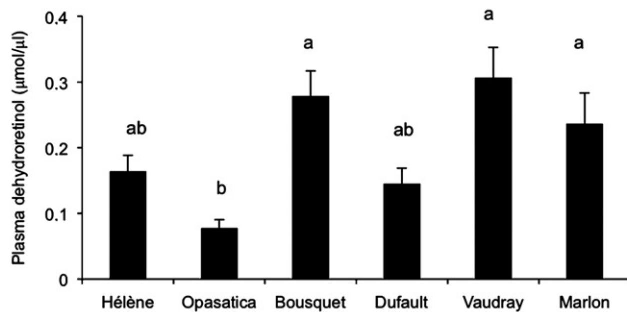

Fig. 3. Plasma dehydroretinol concentrations in yellow perch sampled in the mining region of Rouyn-Noranda, Québec, Canada. Bars indicate means and standard errors ($7 \le N \le 10$). Bars that do not share a common letter are significantly different (LSD test, $p < 0.05$).

reported by Miller et al. (2009), Besselink et al. (1998), Gesto et al. (2012), and Pereira et al. (2012) are similar to values reported in this study in the cleanest lake (Opasatica).

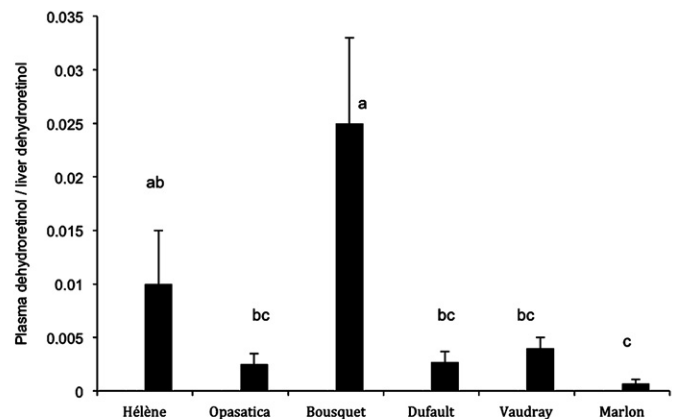

Fig. 4. Ratios of $[\text{dehydroretinol}]_{\text{plasma}}/[\text{dehydroretinol}]_{\text{liver}}$. Bars represent means and standard errors. Bars that do not share a common letter are significantly different (LSD test, $p < 0.05$).

Yellow perch from Cd-contaminated lakes had significantly higher concentrations of liver dROH and dROH esters than fish from reference lakes. However, the increase in dROH ester stores with increasing Cd concentrations was quantitatively much more important than the increase in free dROH. Thus, a significant decrease in the percentage of liver free dROH with increasing Cd concentrations was observed (Fig. 2). These findings differ from those of Branchaud et al. (1995), who reported that white suckers, *Catostomus commersoni*, living in organohalogen- and PAH-contaminated sites had lower levels of both ROH and ROH palmitate in liver compared with those from their reference site. Similarly, hepatic concentrations of major retinoids including dROH and its esters, in lake sturgeon, *Acipenser fulvescens*, captured in a organohalogen contaminated river-lake system were 40 times lower than in lake sturgeon from a reference site (Doyon et al., 1998). These observations suggest that trace metals (Cd) and organic micropollutants (PAHs, PCBs, polychlorinated

pesticides) have different modes of action on vitamin A homeostasis in fish living in polluted areas.

In a previous study we reported that the transcription level of the epidermal retinol dehydrogenase 2 gene, thought to be involved in the oxidation of retinol to retinaldehyde for retinoic acid biosynthesis (Lee et al., 2009), was down-regulated in response to chronic Cd exposure (Pierron et al., 2011). Retinol acts as an essential precursor for the biosynthesis of these two critical retinal metabolites. Retinaldehyde is involved in rhodopsin metabolism and vision (D'Ambrosio et al., 2011). Retinoic acid is an extremely active form of vitamin A, also known as a potent hormone that controls the expression of numerous genes (Ross and Zolfaghari, 2004) through its binding to nuclear receptors, RA receptors (RARs) and retinoid X receptors (RXRs), which act on the genomic targets. Our results suggest that cadmium may inhibit the synthesis of dehydroretinoic acid, leading to higher concentrations of dROH and its esterified forms. This hypothesis needs to be confirmed by measuring dehydroretinal and dehydroretinoic acid in liver and plasma of yellow perch.

Dehydroretinoids are normally esterified to inactive forms, such as dROH palmitate, that are stored in tissues, mainly the liver, and can be mobilized depending on dietary vitamin intake level and physiological demands (Branchaud et al., 1995). Although all the lakes sampled are located in the same region with a similar natural macroenvironment, the availability and the quality of the prey available to yellow perch may vary among lakes depending, notably, on the contamination level of the lakes (Iles and Rasmussen, 2005). Several authors have reported a food web simplification in the most metal-contaminated lakes of the Rouyn-Noranda region (Iles and Rasmussen, 2005; Kövecses et al., 2005). Namely, Kövecses et al. (2005) found that the density of yellow perch prey, notably benthic macroinvertebrates, in Cd- and Cu-contaminated lakes was significantly lower than in uncontaminated lakes. Prey types were less diverse and fish inhabiting these contaminated lakes did not switch to larger prey size as they became older (Kövecses et al., 2005). Adult perch living in pristine lakes are predominantly piscivorous whereas chironomid and zooplankton are dominant prey items for yellow perch inhabiting metal-polluted lakes (Sherwood et al., 2000). As intake of vitamin A is dietary, the consumption of diets with different levels of vitamin A or its precursors likely influences liver vitamin A stores. One might speculate that the prey available in contaminated lakes could be richer in vitamin A or in β-carotene than in the available dietary items in clean lakes, thus explaining the increase in hepatic dehydroretinoids in metal-contaminated fish. However, if dietary availability was the principal factor explaining the higher levels of vitamin A in Cd contaminated fish, these fish would be expected to have a higher feeding rate or a higher food conversion efficiency than those inhabiting pristine lakes. In contrast, Sherwood et al. (2000) reported that yellow perch populations from polluted lakes had consumption rates that were similar to those for fish from reference lakes in the region of Rouyn-Noranda, and that their food conversion efficiency was lower than that achieved by fish from the clean lakes. We conclude that dietary factors are unlikely to be responsible for the higher levels of vitamin A in Cd-contaminated yellow perch.

Laboratory investigations have established links between pyloric caeca weights and feeding rate (Gauthier et al., 2008) and between pyloric caeca weight and food conversion efficiency (Bergot et al., 1981), with increases in pyloric caeca weight reflecting higher feeding rates and food conversion efficiencies. However, in our study, there were no significant differences in pyloric caeca weight among populations. Moreover, fish from the most contaminated lake (Lake Marlon) had lower pyloric caeca weights than fish from Lake Bousquet, which also had higher relative condition. Fish from Lake Bousquet also had lower hepatic concentrations of vitamin A than fish from Lake Marlon. Although there was a positive relationship between plasma dROH and fish condition factor, in our study, as in earlier work (Audet and Couture, 2003; Couture and Pyle, 2008; Eastwood and Couture, 2002), fish living in more Cd-contaminated lakes did not show higher condition as would be expected if greater food availability was the main mechanism explaining their elevated liver dehydroretinoid concentrations. Thus these results also argue against a dietary explanation for the higher levels of vitamin A in Cd-contaminated yellow perch. However this conclusion needs to be confirmed by determining the vitamin A content in fish diets.

Highly significant and positive correlations were observed between kidney Cd concentrations and all forms of vitamin A examined in liver. Since they act as antioxidants, vitamins A are molecules capable of inhibiting the oxidation of other substances present in cells (Alpsoy et al., 2009; Dragsted, 2008). Subcellular antioxidant systems are known to be sensitive to metal exposure and they have been proposed as indicators of metal toxicity (Atli and Canli, 2010). Laboratory experiments have shown that Cd exposure can induce the symptoms of oxidative stress (Shi et al., 2005), but yellow perch living in metal contaminated areas seem to have developed several protective mechanisms to scavenge reactive oxygen species (ROS) before detrimental effects occur in cells. For example, Giguère et al. (2005) found little evidence of oxidative stress in the livers of yellow perch collected from lakes representing a metal contamination gradient (Cd, Cu, Ni, Zn); they speculated that the observed increase in metallothionein concentrations with increasing accumulated metals might protect fish against ROS. Our present results suggest an alternative explanation, namely that the increase in hepatic vitamin A concentrations in fish most impacted by Cd may provide protection against oxidative stress. However, it is noteworthy that strong and positive relationships between liver vitamin A and kidney burdens of cadmium were identified in birds (*Melanitta perspicillata*) (Harris et al., 2007). Similarly, Rodríguez-Estival et al. (2011) observed a relatively high proportion of free liver retinol in wild ungulates exposed to mine pollution. Vitamin A is also known to be involved in cell growth. The higher level of retinoids in the tissues of fish living in more cadmium-impacted lake could accelerate cell differentiation and growth. This finding is in accordance with the study of Couture and Pyle (2008), which proposed that yellow perch living in metal impacted lakes in the region of Sudbury (Canada) grow faster but die younger (Couture and Pyle, 2008).

The six populations of yellow perch were selected to cover a range of metal exposure concentrations, especially for Cd. The ratio of free dROH over dROH palmitate, the predominant ester, or over total esters, is considered to be an index of the metabolism of stored dehydroretinoids in the liver. A lower ratio of liver ROH/ROH palmitate in bullfrogs was related to polluted conditions (Boily et al., 2005). In our study, the percentage of free dROH was significantly and negatively correlated with kidney Cd concentrations. Likely, ester hydrolysis was affected by Cd contamination. We speculate that enzymes involved in the hydrolysis of liver dROH esters were inhibited by the presence of Cd. This finding is in agreement with the study of Tsin and Malsbury (1991), who reported that divalent cations such as Cd inhibited the activities of retinyl ester hydrolases in bovine ocular tissues.

Plasma dROH concentrations were analyzed in the same fish, to verify whether the increase in liver dROH concentrations accelerated its mobilization from liver to blood. Interestingly, no significant correlation was observed between plasma and liver dROH concentrations. In the liver, carrier proteins such as retinol binding proteins (RBPs) and transthyretin (TTR) are

activated to transfer this molecule from the liver to target organs. Liver secretion of these proteins is modulated by the vitamin A concentration and by the animal's stress status (D'Ambrosio et al., 2011). The decrease in the plasma dROH:liver dROH ratio in the most Cd impacted fish (Fig. 4) could be attributed to the inhibition of the synthesis of RBP and/or TTR protein or to the inhibition of the binding of dROH to RBP by the presence of Cd. Interestingly, in a previous study we reported an increase in the transcription level of the gene encoding for TTR with increasing Cd concentrations in yellow perch. In this context, an increase at the transcription level could suggest a compensatory mechanism aiming at counteracting the direct effects of Cd at the protein level.

5. Conclusions

Our study showed (1) that yellow perch living in contaminated lakes in the Rouyn-Noranda region display high liver vitamin A_2 levels, potentially providing protection against oxidative stress induced by metal exposure; (2) that the proportion of liver free dROH decreases along a gradient of increasing Cd contamination, suggesting a negative effect of Cd on enzymes and binding proteins involved in vitamin A homeostasis. Clearly then, vitamin A_2 homeostasis can be altered as a consequence of exposure to environmentally relevant Cd concentrations. Our results also support the hypothesis that down-regulation of genes involved in retinol metabolism can be reflected at the biochemical level.

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