Pesticides Inhibit Retinoic Acid Catabolism in PLHC-1 and ZFL Fish Hepatic Cell Lines

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ABSTRACT: The population of yellow perch (*Perca flavescens*) in lake Saint-Pierre (QC, Canada) has been dramatically declining since 1995 without any sign of recovery. Previous studies have shown disrupted retinoid (vitamin A) metabolic pathways in these fish, possibly due to the influence of pesticides. Our study aimed to evaluate the impact of some herbicides and neonicotinoids on retinoic acid catabolism in the fish hepatic cell lines PLHC-1 and ZFL. We hypothesized that pesticides accelerate the catabolism of retinoic acid through oxidative stress that exacerbates the oxidation of retinoic acid. Results obtained with talarozole, a specific CYP26A1 inhibitor, and ketoconazole, a generalist inhibitor of cytochrome-P450 enzymes, revealed that CYP26A1 is mainly responsible for retinoic acid catabolism in ZFL but not PLHC-1 cells. The impacts of pesticides on retinoic acid catabolism were evaluated by incubating the cells with all-*trans*-retinoic acid and two herbicides, atrazine and glyphosate, or three neonicotinoids, clothianidin, imidacloprid, and thiamethoxam. Intracellular thiols and lipid peroxidation were measured following pesticide exposure. The possible causal relation between oxidative stress and the perturbation of retinoic acid catabolism was investigated using the antioxidant *N*-acetylcysteine. The data revealed that pesticides inhibit retinoic acid catabolism, with the involvement of oxidative stress in the case of atrazine, imidacloprid, and thiamethoxam but not with clothianidin and glyphosate. Pesticides also affected the isomerization of all-*trans*-retinoic acid over time, leading to an increased proportion of active isomers. These results hint at a possible perturbation of retinoic acid catabolism in fish living in pesticide-contaminated waters, as suggested by several *in vivo* studies. Such a disruption of retinoid metabolism is worrying, given the numerous physiological pathways driven by retinoids.
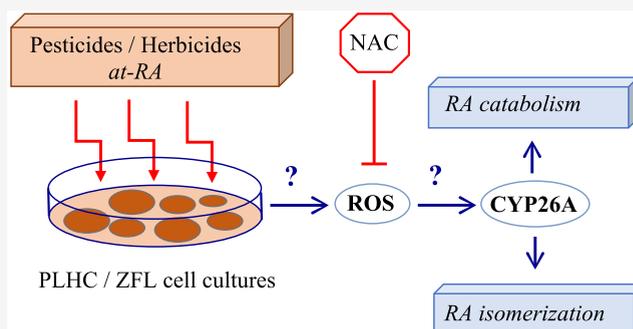
INTRODUCTION

Yellow perch (*Perca flavescens*) is an emblematic fish of lake Saint-Pierre (QC, Canada), quite popular among commercial and recreative fishers. However, around 1995, the population's abundance dropped to reach an alarmingly low number of individuals in the early 2000s, and the population has not recovered since.[1,2] Several measures have been adopted between 1997 and 2012 to stop the decline, including a 5 year moratorium, which was renewed, of all forms of yellow perch fishing, but to no avail.[2,3]

Many causes for the abrupt decline were identified, including agricultural exploitation of the lake's floodplain, essential to perch's spawning, and the resulting poor water quality.[1] Emerging contaminants, pharmaceuticals, and personal care products are also present in the St. Lawrence River, and up to 21 pesticides were detected in lake Saint-Pierre and in its tributaries, especially the southern ones, which go through Quebec's largest agricultural area.[4] Concentrations of clothianidin, imidacloprid, and thiamethoxam exceeded their CVAC value of 8.3 ng/L (criteria for the protection of aquatic life—chronic effect used by the Ministère de l'Environnement et de la Lutte contre les changements climatique, Quebec, to

estimate the quality of surface water), and atrazine water concentration reached the CVAC (1.8 μg/L) established for this herbicide.[4] Additionally, the levels of glyphosate have been increasing in the lake's watershed.[5] Of note, the concentrations of pesticides measured in lake Saint-Pierre peaked in June,[4] a few weeks after eggs hatching,[6] which may impair the development of larvae and juvenile yellow perch. First-year yellow perch are experiencing a growth deficit that prevents them from attaining the minimal size required for survival through their first winter.[2] Consequently, the population fails at recruiting juveniles into the higher age layers, a failure that prevents its growth and maintains it in a state of critically low abundance.[2] Given the primordial role of retinoids in

development and growth, we suspect that pesticides may impact their metabolism.

The term retinoids refers to vitamin A and its derivatives.[7] Animals, being unable to synthesize retinoids *de novo*, take them up from their alimentation, either as vegetal carotenoids or as animal retinyl esters.[8] During digestion, carotenoids are oxidized into retinaldehyde (RAL) or 3,4-didehydroretinaldehyde (DRAL). These products bind to cellular retinol-binding protein II (CRBP II) and are reduced into retinol (ROH) or 3,4-didehydroretinol (DROH) by a microsomal retinaldehyde reductase. Retinol and DROH are then esterified by lecithin:retinol acyltransferase (LRAT) or acyl-CoA: retinol acyltransferase (ARAT) into retinyl esters or dehydroretinyl esters, such as retinyl palmitate (PAL) or 3,4-didehydroretinyl palmitate (DPAL). These retinyl esters are then transported into chylomicrons to the liver, where they are stocked alongside other carotenoids, vitamin E, and dietary lipids.[8,9] When the plasma level of ROH is low, retinyl esters are hydrolyzed into ROH by the retinyl ester hydrolase (REH) and sent into the bloodstream toward the tissues (Figure 1).
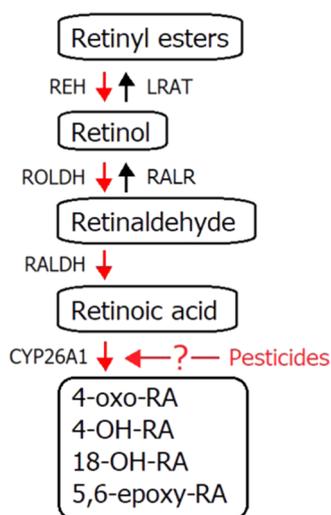

Figure 1. Cellular metabolic pathway of retinoids and the hypothetical action of pesticides on the step mediated by CYP26A1. Lecithin:retinol acyltransferase (LRAT), retinyl ester hydrolase (REH), retinol dehydrogenase (ROLDH), retinal reductase (RALR), and retinaldehyde dehydrogenase (RALDH).

Inversely, when the plasmatic level of ROH is high, ROH is esterified by LRAT in the liver and stocked there.[8−10] In the plasma, ROH is transported by the retinol-binding protein (RBP), which delivers it to the cells via binding to the RBP receptor.[11] In mammals, the RBP affinity for retinoic acid (RA) is almost as much as that for ROH *in vitro*.[12] However, *in vivo* RA appears to be mainly bound to albumin,[13] and RA bound to albumin might be transferred to the cells.[11] Once in the cell, ROH is oxidized into RAL by the retinol dehydrogenase (ROLDH), which may be reduced back to ROH by the retinal reductase (RALR). Retinaldehyde is then irreversibly oxidized into RA by retinaldehyde dehydrogenase (RALDH) (Figure 1).[8,14] Retinoic acid is the active form of retinoids; it penetrates the cell's nucleus and activates the transcription of target genes.[15] It has three isomers: all-*trans*-RA (*at*-RA), 9-*cis*-RA, and 13-*cis*-RA. The isomerization process can be spontaneous in the presence of compounds, with a radical group or mediated by an isomerase.[14,16,17] Both *at*-RA and 9-

cis-RA are active forms of RA; the nuclear receptors of the retinoic acid receptor (RAR) family are activated by both isomers, whereas the nuclear receptors of the retinoid X receptor (RXR) family are activated by 9-*cis*-RA exclusively.[15] The isomer 13-*cis*-RA has a very low affinity to either nuclear receptor, but its role remains unclear.[8] When activated, the RAR and RXR nuclear receptors bind to target genes and induce their transcription.[8] Cytotoxic when in excess, the RA concentration is finely regulated. The major catabolic pathway of RA involves an initial hydroxylation into 4-hydroxy-retinoic acid (4-OH-RA) and a subsequent dehydrogenation into 4-oxo-retinoic acid (4-oxo-RA) by the cytochrome-P450 (CYP450) oxidative enzymes, especially CYP26A1.[8,18] The metabolites, being more polar, are more easily excreted, although they still possess some biological activity, especially all-*trans*-4-oxo-RA (*at*-4-oxo-RA).[18−20]

In previous studies, we have shown lower levels of liver DPAL in yellow perch from lake Saint-Pierre compared to those in yellow perch populations from upstream lakes Saint-Louis and Saint-François.[21] Since the plasmatic DROH levels were similar, the authors suggested that yellow perch from lake Saint-Pierre overmobilized their hepatic stocks of DPAL to maintain their level of plasma DROH constant, which could be related to an increased RA catabolism.[21,22] Therefore, pesticides are suspected of playing a role in some perturbation of retinoid metabolism in yellow perch but this still needs to be clarified.[23] The present study aims at evaluating the impact of herbicides and neonicotinoid insecticides on RA catabolism in two fish hepatic cell lines widely used in toxicology studies, testing the hypothesis that pesticides stimulate the catabolism of RA through oxidative stress that exacerbates the oxidation of RA. Increased catabolism of RA would explain the over-mobilization of hepatic 3,4-didehydroretinol esters by yellow perch in lake Saint-Pierre (Figure 1). The effect of atrazine, glyphosate, clothianidin, imidacloprid, and thiamethoxam on the cellular RA catabolism was investigated *in vitro* in the fish liver cell lines PLHC-1 (*Poeciliopsis lucida* hepatocellular carcinoma-1) and ZFL (zebrafish nontransformed normal liver cells) focusing on four research questions: (1) What is the relative contribution of CYP26A1 in the catabolism of RA in PLHC-1 and ZFL cells? (2) Do pesticides perturb RA catabolism? (3) Do pesticides induce oxidative stress in PLHC-1 and ZFL cells? and (4) Would a perturbation in RA catabolism be related to oxidative stress?

■ MATERIALS AND METHODS

Chemicals. Commercial formulations of pesticides were used. Aatrex 480 (atrazine) and Credit Xtreme 540 (glyphosate) were purchased from Les Moulins Mondou (Mirabel, QC, Canada). Titan 600 (clothianidin), Admire 240 (imidacloprid), and Actara 240 (thiamethoxam) were obtained from Synagri S.E.C., St-Hyacinthe, QC, Canada. Retinoid standards (all-*trans*-retinoic acid (*at*-RA), 9-*cis*-retinoic acid (9-*cis*-RA), 13-*cis*-retinoic acid (13-*cis*-RA), and 13-*cis*-4-oxo-retinoic acid (13-*cis*-4-oxo-RA)), amino acids, epidermal growth factor, tetrazolium salt MTT, reduced glutathione (GSH), 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB, also known as Ellman's reagent), 1,1,3,3-tetramethoxypropane, thiobarbituric acid, talarozole, ketoconazole, *N*-acetylcysteine, methanol, acetonitrile, tetrahydrofuran, high-performance liquid chromatography (HPLC)-grade water, ethyl acetate, acetic acid, butylated hydroxytoluene (BHT), and bovine serum albumin were from Merck KGaA Millipore Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's minimum essential medium (DMEM) with high glucose (25 mM), Earle's salt minimum essential medium (EMEM), F-12 nutrient mixture (F-12), Leibovitz's L-15 medium (L-15), penicillin−streptomycin, and trypsin−EDTA were

purchased from Gibco Life Technologies Co. (Grand Island, NY). Fetal bovine serum (FBS) and human insulin came from Wisent Inc. (St-Bruno, QC, Canada). Rainbow trout serum was obtained from Cedarlane (Burlington, ON, Canada). Dimethyl sulfoxide (DMSO) was from Caledon Laboratories (Georgetown, ON, Canada). Hexane and trifluoroacetic acid were from Thermo Fisher Scientific (St-Laurent, QC, Canada). Trichloroacetic acid was obtained from Anachemia (Montreal, QC, Canada). Coomassie Brilliant Blue G-250 dye (Bradford reagent) was from Bio-Rad Laboratories (Mississauga, ON, Canada). The EnzyChrom Triglyceride Assay Kit (Catalog No. ETGA-200) was purchased from BioAssay Systems (Hayward, CA). HPLC-grade solvents were used.

Cell Culture. Cell lines PLHC-1 (ATCC CRL-2406) and ZFL (ATCC CRL-2643) were maintained in 75 cm^2 flasks at 28 °C in a 5% CO$_2$-humidified atmosphere. PLHC-1 cells were grown in EMEM containing 0.1 mM nonessential amino acids (L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, glycine, L-proline, L-serine), supplemented with 5% FBS. ZFL cells were grown in a mixture of L-15, DMEM, and F-12 (50%−35%−15%) supplemented with 5% fetal bovine serum (FBS), 0.5% rainbow trout serum, 0.01 mg/mL insulin, and 50 ng/mL epidermal growth factor. Both cell lines were maintained in the presence of penicillin−streptomycin (50 000 U/L to 50 mg/L). The medium was changed every 2 days, and cells were passed once a week by trypsinization (0.05% trypsin−0.53 mM EDTA). ZFL and PLHC-1 cells were plated at a density of 1.33×10^4 and 2.00×10^4 cells/cm^2, respectively. PLHC-1 and ZFL cells were maintained for 10 and 6 days, respectively, to ensure the confluence.

Retinoid Extraction and High-Performance Liquid Chromatography. Extraction of retinoids was adapted from the procedure of Solari et al., and was performed under yellow light to prevent the oxidation of retinoids.[24] On the day of analysis, cell samples that were frozen at −70 °C after treatments were thawed on ice and mixed with 200 μL of 1 mg/mL BHT diluted in methanol to prevent oxidation. One milliliter of an extraction solvent mixture composed of 50% ethyl acetate and 50% acidified hexane containing 0.068% acetic acid was added to the sample, which was then vortexed for 1 min and centrifuged at 1625g for 8 min. Afterward, 800 μL of the organic phase was transferred into a glass tube and evaporated in a Vacufuge Plus evaporator (Eppendorf, Mississauga, ON, Canada) for 15 min at 45 °C. The same steps were performed with the medium samples, with the following modifications: 400 μL of BHT was added to the sample, the first centrifugation lasted 15 min, and only 700 μL of the organic phase could be collected after the first centrifugation. The precedent procedures, from adding the extraction solvent onwards, were repeated twice, but 1000 μL of the organic phase was collected and evaporated, always in the same glass tube, to pool the retinoids extracted. After the third evaporation, 100 μL of acetonitrile was added to the glass tube and vortexed for 20 s. To ensure the optimal preservation of the retinoids, the glass tubes were stored at −20 °C and processed one by one. All samples from a set of experiments were injected the same day (using a volume of 90 μL per sample) in reverse-phase high-performance liquid chromatography (HPLC), as described by Solari et al. (2010).[24] Intracellular and extracellular at-RA contents are expressed relative to cellular protein contents (ng/mg protein).

Total Protein Content Determination. Total protein contents were estimated according to Bradford[25] on cell samples previously homogenized with the Kinematica Polytron PT 1600 E Homogenizer using the Coomassie Brilliant Blue G-250 dye (Bradford reagent) in 96-well plates: 200 μL of the diluted Bradford reagent was added to 50 μL of diluted homogenates. Bovine serum albumin at concentrations ranging from 0 to 100 μg/mL was used as the calibration standard. The optical density was measured at 595 nm using a Tecan SpectraFluor Plus microplate spectrophotometer (Esbe Scientific Industries Inc., St. Laurent, QC, Canada). In some cases where the volumes of the samples were too low for mechanical homogenization, cell digestion was performed with a 1 h incubation in NaOH 1 N, and protein determination was conducted with the appropriate standard curve performed in the presence of 0.040 M NaOH to a final concentration.

Cell Coexposure to at-RA and CYP450 Inhibitors. All of the following experiments, except cell culture and maintenance, were performed under yellow light to prevent the oxidation of retinoids. PLHC-1 and ZFL cell monolayers grown in 100 mm Petri dishes were exposed to 50 nM at-RA dissolved in 95% ethanol (the final concentration of ethanol was less than 0.1%) for 24 h in the absence or presence of 1 μM talarozole or 10 μM ketoconazole. Samples were harvested on ice. One milliliter of the medium was collected, the remaining medium was removed, cells were washed twice with ice-cold PBS, and harvested in 800 μL PBS. Cells and culture medium were then stored at −70 °C until retinoid extraction and determination of protein contents.

MTT Cell Viability Assay. PLHC-1 and ZFL cells in 96-well plates were exposed to pesticides (5−250 mg/L) diluted in the culture (100 μL/well) in triplicates for 1, 2, or 3 days. At the end of the incubation period, 10 μL of 5 g/L tetrazolium salt MTT was added to the wells, and the plates were incubated for 2−4 h to ensure the formation of purple formazan crystals. Subsequently, the medium was removed, the crystals were dissolved in 200 μL of DMSO, the plates were incubated for 15 min, shaken, and the optical density was measured at 575 nm using a Tecan SpectraFluor Plus microplate spectrophotometer. MTT data are expressed relative to control values measured in unexposed cells.

Cell Coexposure to at-RA and Pesticides with or without NAC. The same protocol as that described above was applied, with the following modifications. Cells were exposed to 250 mg/L of pesticides for 24 h. Concomitantly, cells were exposed to 50 nM at-RA dissolved in 95% ethanol (the final concentration of ethanol was less than 0.1%) for the last 6, 18, or 24 h of the pesticide incubation period. Cells and medium culture were harvested as described in the previous section. Zero-time cells and medium samples were obtained with nonexposed control cultures and with an unused culture medium containing 50 nM at-RA, respectively. Cells were harvested as usual. In some experiments, the effect of the antioxidant NAC on pesticide-induced modifications in at-RA contents was tested using coincubated cells with pesticides, at-RA, and 1 mM NAC. In these experiments, the duration of exposure was selected for the maximal effect of pesticides observed according to results obtained in kinetic studies (Figures 4 and 5).

Intracellular Thiol Content Determination. PLHC-1 and ZFL cell monolayers plated in 60 mm Petri dishes were exposed to 250 mg/L of pesticides for 3 and 1 days, respectively (ZFL cells were more sensitive to pesticide toxicity, especially to mixtures, hence the shorter exposure time). The next procedures were done on ice, in partial darkness, to prevent the oxidation of the thiols and because of the reagent's photosensitivity. At the end of the exposure period, the medium was removed, and cells were washed twice with ice-cold tris-phosphate EDTA buffer. PLHC-1 and ZFL cells were harvested in 800 μL and 500 μL buffer, respectively. Cells were then stored at −20 °C until further analysis. On the day of the assay, cells were thawed on ice and homogenized with a Kinematica Polytron PT 1600 E Homogenizer (Kinematica AG, Bohemia, NY) using a 5 mm PT-DA 05/2EC-E85 disperser for 45 s. Intracellular thiols were measured following reaction with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) in a 96-well plate: 200 μL of DTNB was added to 50 μL of cell homogenates in triplicate. The remaining homogenate was stored back at −20 °C until protein determination. A calibration curve was obtained with 200 μL of DTNB added to 50 μL of GSH at concentrations ranging from 0 to 1000 μM, diluted in tris-phosphate EDTA buffer. The optical density was measured at 410 nm using a Tecan SpectraFluor Plus microplate spectrophotometer. Cellular thiol contents are expressed relative to cellular protein contents (μmol/mg protein).

Lipid Peroxidation Determination: TBARS. Measurement of malonaldehyde (MDA) as a biomarker of lipid peroxidation was adapted from Ohkawa et al., Landry et al., and Paquet-Walsh et al.[21,23,26] PLHC-1 and ZFL cell monolayers plated in 100 mm Petri dishes were exposed to 250 mg/L of pesticides diluted in the culture medium. PLHC-1 cells were exposed for 1−3 days and ZFL cells for 1 day. The following procedures were done on ice. At the end of the
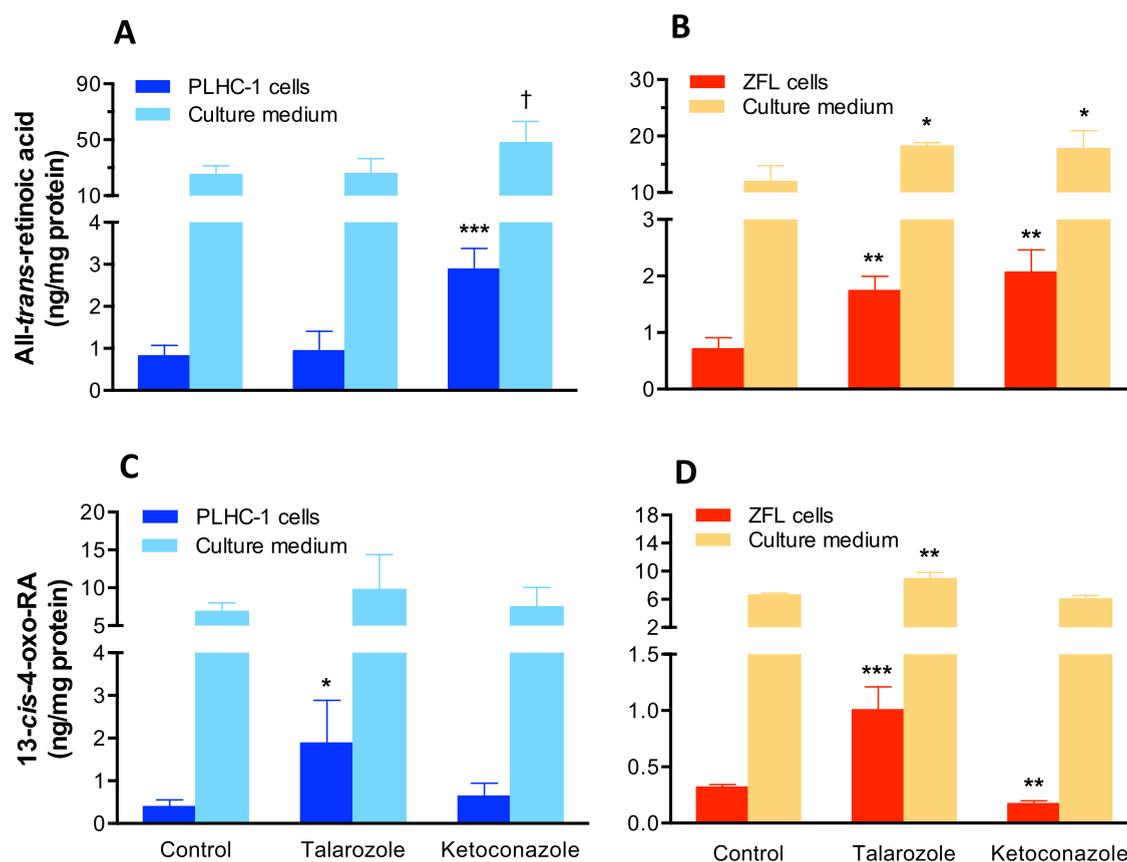
Figure 2. Inhibition of RA catabolism by CYP450 inhibitors. PLHC-1 (A, C) and ZFL (B, D) cells were incubated with 50 nM *at*-RA for 24 h in the absence or presence of 1 μM talarozole or 10 μM ketoconazole. All-*trans*-RA (A, B) and 13-*cis*-4-oxo-RA (C, D) were measured in cells and culture media. Results are expressed as mean ± SD estimated from three independent cell cultures. Data were analyzed by planned contrasts in one-factor linear models.

exposure period, the medium was removed, cells were washed twice with ice-cold phosphate-buffered saline (PBS), and harvested in 400 μL PBS. Cells were then stored at −70 °C until further analysis. On the day of the assay, cells were thawed on ice and homogenized with a Kinematica Polytron PT 1600 E Homogenizer using a 5 mm PT-DA 05/2EC-E85 disperser for 30 s. The homogenates were centrifuged at 4000*g*, for 10 min, at 4 °C. Then, 200 μL of the supernatant was collected, 100 μL of 0.15% sodium deoxycholate was added, and the samples were vortexed and allowed to rest on ice for 10 min. The remaining homogenates were stored back at −70 °C for protein and triglyceride determination. Then, 100 μL of 50% trichloroacetic acid was added to the samples, and the latter were centrifuged at 9000*g*, for 15 min, at 4 °C. Subsequently, 75 μL of the supernatant was transferred to a 96-well plate, in triplicate, with the addition of 50 μL of 50% trichloroacetic acid and 75 μL of 1.3% thiobarbituric acid (dissolved in 0.3% NaOH). The plate was wrapped in an aluminum sheet and heated on a dry heat block incubator at 80−90 °C for 1 h. This incubation allows the products of lipid peroxidation to react with thiobarbituric acid, generating MDA, which was subsequently measured. Following this incubation, the aluminum sheet was removed, and the plate was allowed to rest on ice for 15 min to stop the reaction. A calibration curve was obtained using 200 μL of 1,1,3,3-tetramethoxypropane diluted in PBS at concentrations ranging from 0 to 7.5 μM treated, as were the cell supernatant samples. The optical density was measured first at 530 nm to measure MDA and then at 650 nm to correct for turbidity, using a Varioskan Lux microplate spectrophotometer (Thermo Fisher Scientific, St. Laurent, QC, Canada). The optical density at 650 nm was subtracted from that at 530 nm. Levels of MDA are expressed relative to cellular protein contents (μmol/mg protein).

Triglyceride Content Determination. Cellular triglyceride contents were estimated using the EnzyChrom Triglyceride Assay Kit, on ice, as described by the manufacturer. Briefly, the working reagent was prepared by mixing, for each well, 100 μL of assay buffer, 2 μL of enzyme mix, 5 μL of lipase, 1 μL of ATP, and 1 μL of dye reagent. A calibration curve was prepared ranging from 0 to 1 mM and kept on ice using the standard provided. The samples and the standard dilutions were vortexed, 10 μL was transferred on a 96-well plate, in triplicate, and 100 μL of the working reagent was added. The plate was incubated at room temperature for 30 min, and the optical density was measured at 575 nm in a Tecan SpectraFluor Plus microplate spectrophotometer.

Statistical Analysis. The viability (MTT) data were analyzed according to the following concentration−response curves

$$y = y_{min} + \frac{y_{max} - y_{min}}{1 + 10^{(\log LC_{50} - x)hillslope}}$$

where Y_{max} and Y_{min} are the maximal and minimal ratios of cell viability, respectively, and the LC_{50} is the concentration of pesticide for which a cell viability ratio (MTT activity ratio) of 0.5 is observed. These analyses were performed using Prism 6 software (GraphPad Software, San Diego, CA).

All of the data related to oxidative stress (thiols, proteins, MDA, triglycerides, and appropriate ratios) and retinoid measurements were analyzed using linear models, by setting the control group as intercept and comparing each exposed group to the control group by planned contrasts. For the measurements of MDA in PLHC-1 cells, the time of exposure and pesticides were not treated as separate factors but were combined, and the control group was considered as a 0 day exposure. The linear models for the analysis of retinoid contents after cells' exposure to pesticides considered the time of exposure as a second
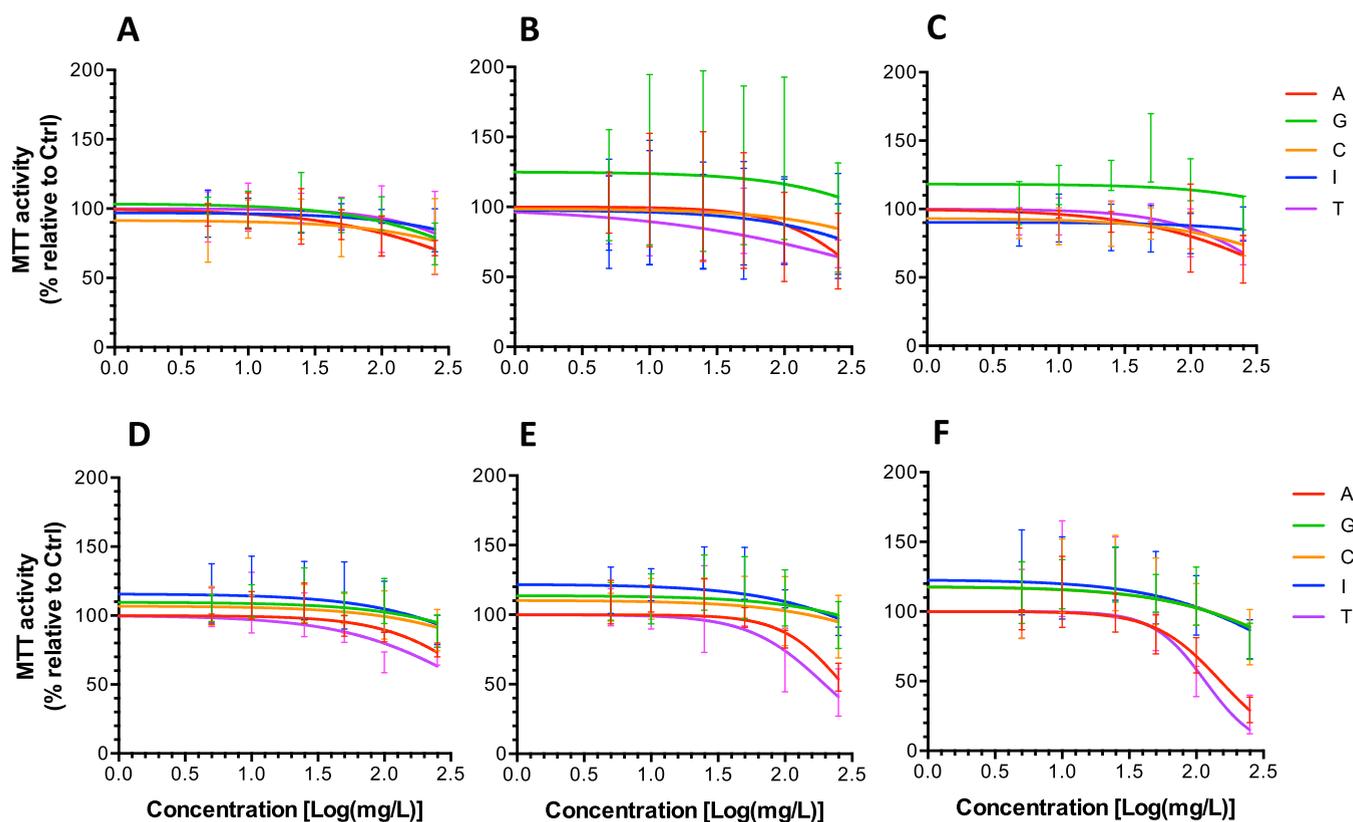
Figure 3. Concentration–response curves to pesticides. PLHC (A–C) and ZFL (D–F) cells were exposed to increasing concentrations of atrazine, glyphosate, clothianidin, imidacloprid, and thiamethoxam ranging from 0 to 250 mg/L for 1 (A, D), 2 (B, E) or 3 (C, F) days. MTT activity is expressed relative to control measured in the absence of pesticides. Data shown are mean ± SD estimated in three independent cell cultures. Data were analyzed by nonlinear regression, as described in the Materials and Methods section. A: atrazine; G: glyphosate; C: clothianidin; I: imidacloprid; and T: thiamethoxam.

factor, which was set as a discrete measure, without the interaction with the pesticide factor. The levels of all three isoforms of RA (9-*cis*-RA, 13-*cis*-RA, and *at*-RA) were summed to yield RA levels.

The data of the following models were log-transformed to correct for heteroscedasticity: extracellular 13-*cis*-4-oxo-RA levels in PLHC-1 cells and intracellular 13-*cis*-4-oxo-RA levels in ZFL cells after coexposure to *at*-RA and CYP450 inhibitors; intracellular RA levels in PLHC-1 and ZFL cells after coexposure to *at*-RA, NAC, and clothianidin or thiamethoxam, as well as after coexposure to *at*-RA, NAC, and glyphosate in ZFL cells; extracellular RA levels in ZFL cells after coexposure to *at*-RA, NAC, and clothianidin or thiamethoxam; and extracellular 13-*cis*-4-oxo-RA levels in ZFL cells after coexposure to *at*-RA, NAC, and atrazine. The data of the following models were square-rooted, rather than log-transformed, to correct for heteroscedasticity, because of the presence of zeros: intracellular RA levels and extracellular 13-*cis*-4-oxo-RA levels in PLHC-1 cells after coexposure to *at*-RA and pesticides; and intracellular and extracellular RA levels, as well as extracellular 13-*cis*-4-oxo-RA levels, in ZFL cells after coexposure to *at*-RA and pesticides. The heteroscedasticity of the thiol contents in PLHC-1 cells could not be corrected by transforming the data, so it was accounted for with a weighted linear model, each value being weighted by the inverse of its intragroup variance.[27,28] The proportion of isomers of RA of each sample was compared by the permutational multivariate analyses of variance (PERMANOVA) by setting the treatment and time as explanatory variables. Euclidean distance matrices were calculated, and 9999 permutations were computed. The use of Euclidean distances is often discouraged because they are affected by the presence of double zeros, but this distance measure was chosen precisely to account for double zeros, *i.e.*, cell samples devoid of the same isomer should be considered similar. Afterward, pairwise comparisons were accomplished by multivariate analyses of variance (MANOVA) to determine

which pesticide significantly modifies the ratios of isomers. Euclidean distance matrices were again calculated, but this time, 99 999 permutations were computed, and *p*-values were adjusted through the FDR method. The retinoid isomer contents (ng/mg protein) were plotted by principal component analyses (PCAs) for each cell line, regardless of the duration of exposure to *at*-RA, with ellipsoids corresponding to standard deviations. The PERMANOVA models were built using the *adonis2* function within the *vegan* package,[29] the PCAs were computed with the *rda* function in the *vegan* package,[29] and the pairwise comparisons MANOVA models were built using the *pairwise.perm.manova* function within the *RVAideMemoire* package.[30]

The statistical significance was assessed at $\alpha = 0.1$ († = tendency), 0.05 (*), 0.01 (**), or 0.001 (***). Statistical analyses were performed on R.[31]

■ RESULTS

Contribution of CYP26A1 in the Catabolism of *at*-RA in Fish Hepatic Cell Lines. To characterize RA catabolism in fish hepatic cell lines and to estimate the contribution of CYP26A1 in this process, PLHC-1 and ZFL cells were exposed for 24 h to 50 nM *at*-RA in the presence or absence of two CYP450 inhibitors: talarozole (1 μM), a specific CYP26 inhibitor, and ketoconazole (10 μM), a general CYP450 inhibitor, both of which have been shown to successfully inhibit CYP450 in fish.[32,33] The RA isomers *at*-RA, 13-*cis*-RA, and 9-*cis*-RA and the metabolite 13-*cis*-4-oxo-RA were all detected in both cell lines. None of the cell treatments modified the total cellular protein content (ranging from 13 to 23 μg/cm²).
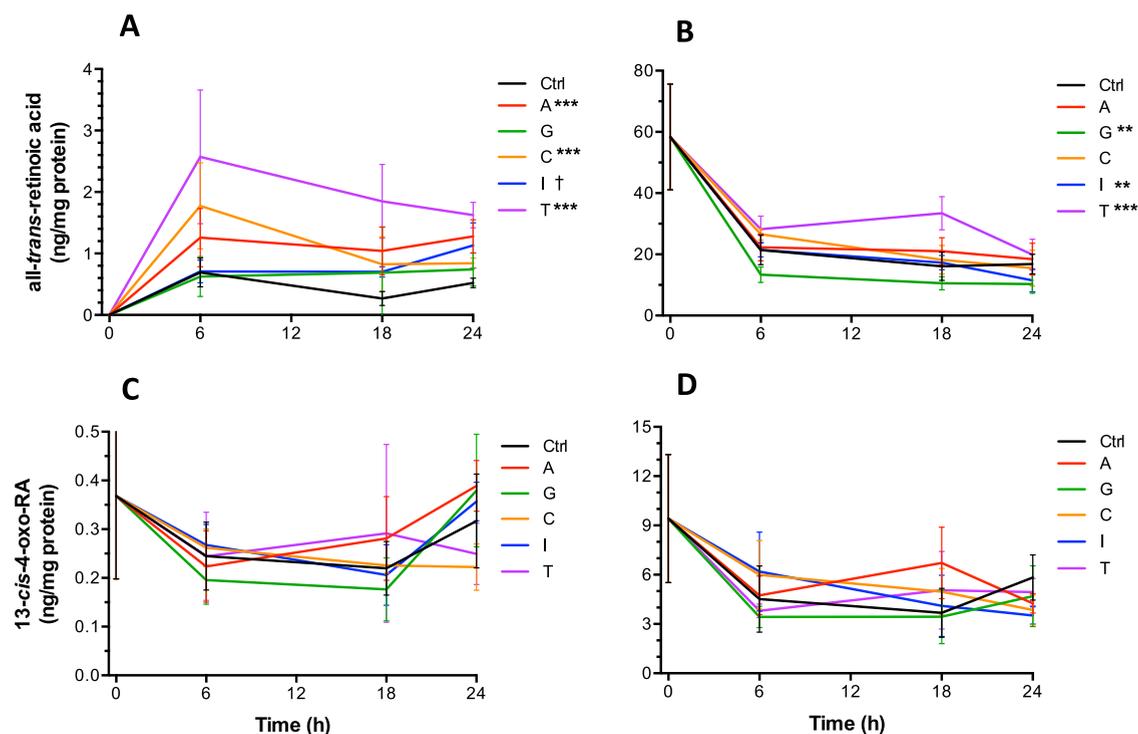
Figure 4. Levels of all-*trans*-RA and 13-*cis*-4-oxo-RA in PLHC-1 cell cultures as a function of time incubation with *at*-RA. PLHC-1 cells were exposed to 250 mg/L atrazine, glyphosate, clothianidin, imidacloprid, or thiamethoxam for 24 h and concomitantly incubated with 50 nM *at*-RA for the last 6, 18, or 24 h of treatment. Control cells were incubated with *at*-RA in the absence of pesticides. All-*trans*-RA (A, B) and 13-*cis*-4-oxo-RA (C, D) were measured in cells (A, C) and the culture medium (B, D). Data shown are mean ± standard deviation (SD) estimated from three independent cell cultures. Ctl: control; A: atrazine; G: glyphosate; C: clothianidin; I: imidacloprid; T: thiamethoxam. Data were analyzed with a two-factor linear model taking into account pesticide exposure (factor 1) and the time of exposure (factor 2). A significant result indicates a significant difference between RA levers in exposed cells as compared to those in control cells throughout the time points.

In PLHC-1 cells, talarozole had no effect on the intracellular and extracellular *at*-RA levels (Figure 2A). However, ketoconazole led to 3-fold higher intracellular contents of *at*-RA, as a result of a significant inhibition of catabolism. In contrast, in ZFL cells, both talarozole and ketoconazole led to a 2.5-fold increase in the intracellular contents of *at*-RA, showing similar levels of inhibition of RA catabolism by the specific and the generalist inhibitors (Figure 2B). Surprisingly, talarozole also caused a significant increase in the intracellular contents of 13-*cis*-4-oxo-RA in both cell lines, also in its levels in the culture medium of ZFL cells (Figure 2C,D). Significantly lower intracellular contents of 13-*cis*-4-oxo-RA were observed with ketoconazole only in ZFL cells (Figure 2D). These results suggest that some CYP450 isoforms of the CYP26 family are responsible for most of the *at*-RA catabolism in ZFL cells but not in PLHC-1 cells. Also, CYP26 would not be involved in the formation of the metabolite 13-*cis*-4-oxo-RA.

Effect of Pesticides on Cell Viability. Cells' sensitivity to pesticides was investigated with MTT assays. Concentration–response curves following 1, 2, or 3 day exposures were obtained with PLHC-1 (Figure 3A−C) and ZFL cells (Figure 3D−F). In both cell lines, atrazine and thiamethoxam were the most toxic pesticides. However, the sensitivity of the two cell lines was clearly different. While PLHC-1 cell mortality remained low (∼20%) even following a 3 day exposure to the highest level, up to 50 and 75% mortalities were observed in ZFL cell cultures following a 2 and 3 day exposure, respectively, to atrazine and thiamethoxam. The low PLHC-1 cell mortality prevented any estimation of LC$_{50}$ values, but nonlinear regression analysis of data obtained with ZFL cells

gave the following estimates at 48 h of exposure: LC$_{50}$ = 273 ± 42 and 201 ± 39 mg/L for atrazine and thiamethoxam, respectively. Estimates at 72 h of exposure were LC$_{50}$ = 153 ± 21 and 119 ± 25 mg/L for atrazine and thiamethoxam, respectively. According to these results, in some of the subsequent experiments, PLHC-1 cells were exposed to 250 mg/L of pesticides for 1−3 days, while ZFL cells were exposed to 250 mg/L of pesticides for 1 day only. This ensured a comparable and not too high level of cell mortality (∼20% max) in both cell lines. High concentration was prioritized over a long duration of exposure to manifest acute mechanisms of toxicity rather than chronic ones.

Perturbation of *at*-RA Catabolism by Pesticides. To test whether pesticides may affect *at*-RA catabolism in the two fish hepatic cell lines, *at*-RA and the metabolite 13-*cis*-4-oxo-RA were measured in the cells and the culture medium following a 6, 18, and 24 h exposure to *at*-RA in cells treated with pesticides for 24 h. Control data were obtained with cells exposed to *at*-RA exclusively. In unexposed PLHC-1 cells, *at*-RA could not be detected, whereas very low basal levels of intracellular *at*-RA of 0.06 ± 0.009 ng/mg protein were measured in ZFL. In both cell lines, increases in cellular *at*-RA plateaued at a 6 h exposure to *at*-RA with twice as high levels of accumulation in ZFL compared to PLHC-1 cells (2.02 ± 0.19 vs 0.81 ± 0.29 ng/mg protein) (Figures 4A and 5A). Also, in both cell lines, a concomitant decrease in the level of *at*-RA was observed at 6 h but it remained stable at 18 and 24 h (Figures 4B and 5B). Interestingly, decreases in extracellular *at*-RA were higher than increases in cellular contents, which is in accordance with the cellular catabolism activity. For
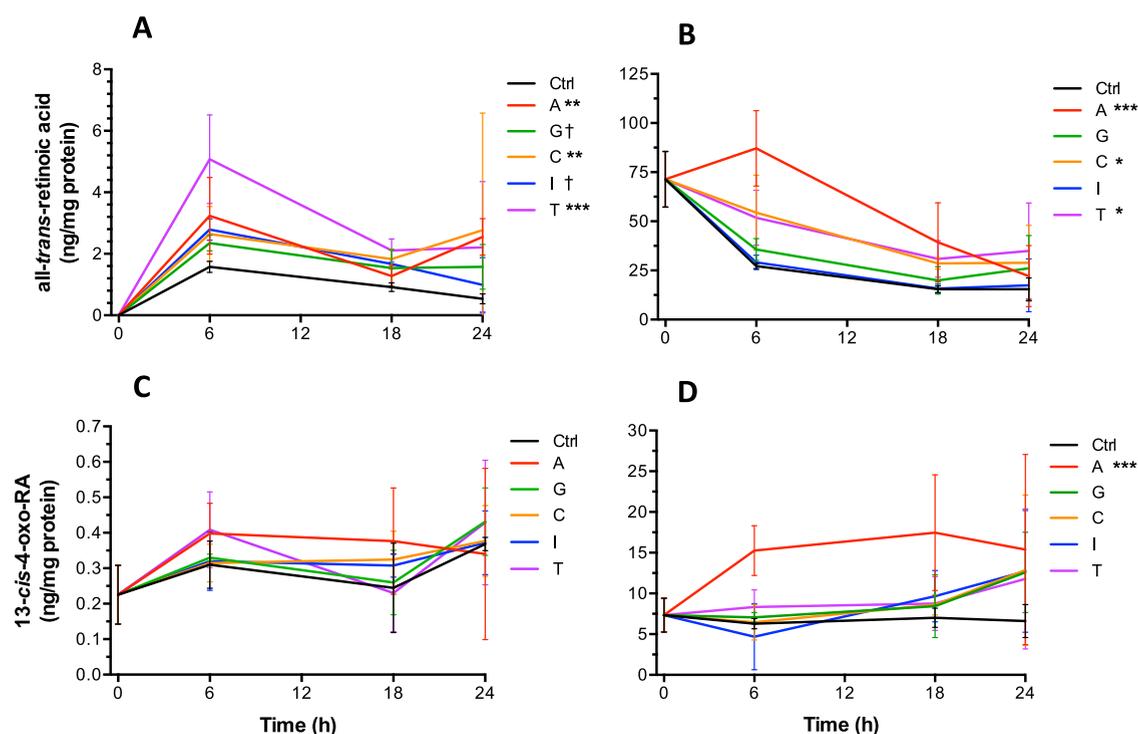
Figure 5. Levels of all-*trans*-RA and 13-*cis*-4-oxo-RA in ZFL cell cultures as a function of time incubation with *at*-RA. ZFL cells were exposed to 250 mg/L atrazine, glyphosate, clothianidin, imidacloprid, or thiamethoxam for 24 h and concomitantly incubated with 50 nM *at*-RA for the last 6, 18, or 24 h of treatment. Control cells were incubated with *at*-RA in the absence of pesticides. All-*trans*-RA (A, B) and 13-*cis*-4-oxo-RA (C, D) were measured in cells (A, C) and the culture medium (B, D). Data shown are mean ± SD estimated from three independent cell cultures. Ctl: control; A: atrazine; G: glyphosate; C: clothianidin; I: imidacloprid; T: thiamethoxam. Data were analyzed with a two-factor linear model taking into account pesticide exposure (factor 1) and the time of exposure (factor 2). A significant result indicates a significant difference between RA levers in exposed cells as compared to those in control cells throughout the time points.

example, in the presence of thiamethoxam, the cellular content of *at*-RA in PLHC-1 increased from an undetectable level to 2.6 ± 1.1 ng/mg protein after a 6 h exposure to *at*-RA, whereas the extracellular content dropped from 58 ± 17 to 28 ± 4 ng/mg protein (Figure 4A,B).

For both cell lines, exposure to pesticides leads to higher cellular contents of *at*-RA (1.5−5-fold), except for glyphosate in PLHC-1 cells, and imidacloprid for which only a tendency was observed. However, the extracellular levels of *at*-RA were not as much affected by the pesticides, especially in the PLHC-1 cell cultures where increases were noted with thiamethoxam, exclusively. In ZFL cell cultures, higher levels of extracellular *at*- were measured following exposure to atrazine, clothianidin, or thiamethoxam.

Intracellular contents of 13-*cis*-4-oxo-RA were similar in both cell lines and did not vary significantly over the time studied or in the presence of pesticides (Figures 4C and 5C). The influence of pesticides on culture media was observed only in ZFL cells, where atrazine doubled extracellular 13-*cis*-4-oxo-RA (Figure 5D). These results suggest that all studied pesticides may inhibit *at*-RA catabolism in fish hepatic cell lines and underline some differences between the two *in vitro* models.

Implication of Oxidative Stress in the Perturbation of *at*-RA Catabolism by Pesticides. The possible involvement of oxidative stress in the perturbation of *at*-RA catabolism was investigated using cells' coexposure with pesticides and NAC used as an antioxidant. These experiments were conducted using the optimal period of incubation with *at*-RA as selected following analyses of data on pesticide effects on *at*-RA

catabolism using linear models (Figures 4 and 5). In PLHC-1 cells, we selected 24 h for atrazine, clothianidin, imidacloprid, and thiamethoxam. Glyphosate was not considered because it did not modify intracellular *at*-AR contents in these cells. The ZFL cells were exposed to 24 h for atrazine and glyphosate and 6 h for clothianidin, imidacloprid, and thiamethoxam.

In PLHC-1 cells, NAC nullified the increase in cellular *at*-RA following exposure to atrazine or imidacloprid but not thiamethoxam (Figure 6A). In these experiments, clothianidin did not significantly affect cellular contents of *at*-RA, regardless of the presence or absence of NAC, and the data do not suggest any effect of the antioxidant (Figure 6A). In ZFL cells, NAC abolished the increase in cellular *at*-RA induced by atrazine but not by glyphosate (Figure 6B). In studies conducted over a 6 h exposure to *at*-RA, significant higher cellular levels of *at*-RA were obtained in cells treated with NAC alone (Figure 6C). Therefore, the intracellular *at*-RA contents following exposure to clothianidin, imidacloprid, and thiamethoxam were also compared to cells treated with NAC alone and used as control. According to these analyses, only thiamethoxam significantly increased the cellular *at*-RA contents. These results suggest that atrazine impairs *at*-RA catabolism through oxidative stress in both cell lines. This would also be the case for imidacloprid in PLHC-1 cells and for thiamethoxam in ZFL cells. However, clothianidin- and glyphosate-induced modifications in *at*-RA catabolism would not involve oxidative stress.

Effects of Pesticides on Lipid Peroxidation and Cellular Thiol Contents. The effect of pesticides on the cells' redox state was investigated by monitoring the variation
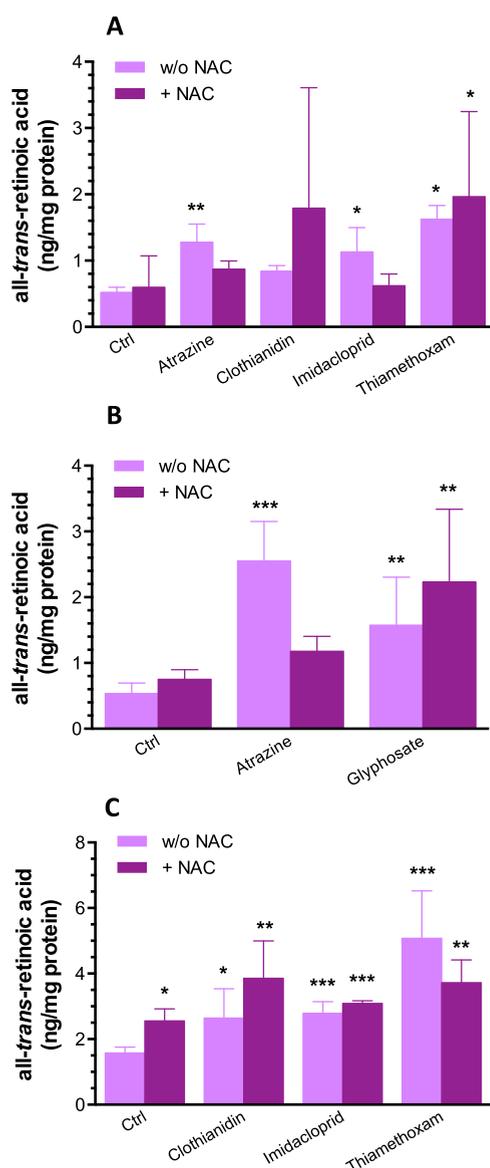
Figure 6. Effect of the antioxidant NAC on pesticide-induced modification in *at*-RA catabolism. PLHC-1 (A) and ZFL (B, C) cells were exposed to 250 mg/L atrazine, glyphosate, clothianidin, imidacloprid, or thiamethoxam in the absence or presence of 1 mM NAC for 24 h, concomitantly with a 24 h (A, B) or a 6 h incubation (C) with 50 nM *at*-RA. Results shown are mean ± SD estimated from three independent cell cultures. Data were analyzed by planned contrasts in one-factor linear models.

Figure 7. Effect of pesticides on lipid peroxidation and cellular thiol contents. PLHC-1 cells were exposed to 250 mg/L atrazine, glyphosate, clothianidin, imidacloprid, and thiamethoxam alone or in mixtures. Cellular malonaldehyde and thiol contents were measured following a 3 day exposure to pesticides. Results are expressed as mean ± SD estimated from three independent cell cultures. Data were analyzed by planned contrasts in one-factor linear models.

in MDA formation, a biomarker of lipid peroxidation, and intracellular thiols, used as a biomarker of antioxidative defense. None of the pesticide treatment produced a significant increase in MDA before 3 days of exposure at which time atrazine and glyphosate doubled the MDA formation in PLHC-1 cells without the effect in ZFL cells (Figure 7A). In PLHC-1 cells exposed to pesticides for 3 days, atrazine, alone or in a mixture with glyphosate, clothianidin, imidacloprid, or thiamethoxam, induced a 1.7-fold significant increase in intracellular thiol contents (Figure 7B). Clothianidin, thiamethoxam, and a mixture of glyphosate and imidacloprid also significantly increased the level of intracellular thiols albeit less impressively. In ZFL cells, cellular thiols did not vary. Although NAC modified the atrazine- and thiamethoxam-
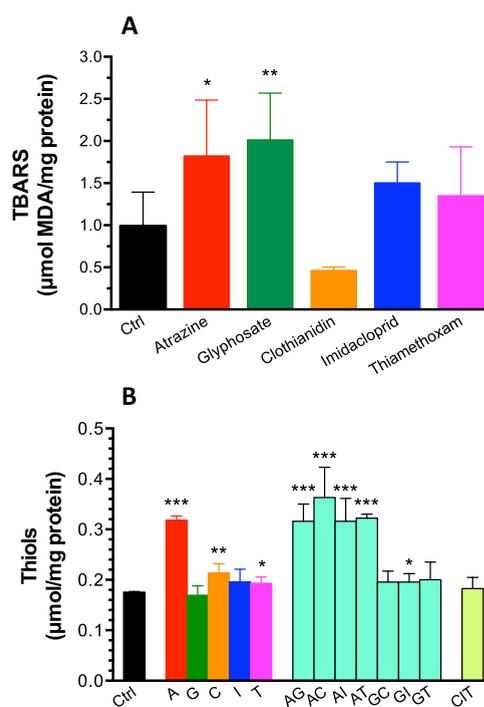
induced variation in *at*-RA catabolism in ZFL cells, the investigated biomarkers did not provide evidence of oxidative stress in these cells under our experimental conditions. None of the cell treatments modified cellular triglyceride contents ranging from 40 to 100 μg triglyceride/mg protein in both cell lines (data not shown).

Retinoic Acid Isomerization after Pesticide Exposure. The effect of pesticides on the fate of *at*-RA in cells was also studied by monitoring variations in the respective levels of RA isomers. Figure 8 shows the relative proportions of *at*-RA, 13-*cis*-RA, and 9-*cis*-RA in PLHC-1 and ZFL cells. In both cell lines, *at*-RA was consistently the major isomer of RA, which was expected since cells were exposed to *at*-RA. PERMANO-VAs revealed significant variation in *at*-RA isomerization with the time of exposure to *at*-RA. Interestingly, 9-*cis*-RA was absent in PLHC-1 control cells but appeared following exposure to pesticides with a more pronounced effect of the pesticide when cells were incubated with *at*-RA for 24 h compared to those for 6 or 18 h (Figure 8A−C). This variation in cellular 9-*cis*-RA with the time of incubation with *at*-RA cannot be related to higher intracellular RA since maximal accumulation was reached at 6 h (Figure 4). Contrary to PLHC-1 cells, 9-*cis*-RA was detected in control ZFL cells. However, similarly to what was observed in PLHC-1 cells, the contribution of 9-*cis*-RA to RA increased with longer exposure time to *at*-RA in the presence of pesticides (Figure 8D−F).

Tables 1 and 2 show pairwise comparisons between the proportions of RA isomers in each cell line obtained following permutational MANOVAs. In both cell lines, atrazine and thiamethoxam significantly modified *at*-RA isomerization, and
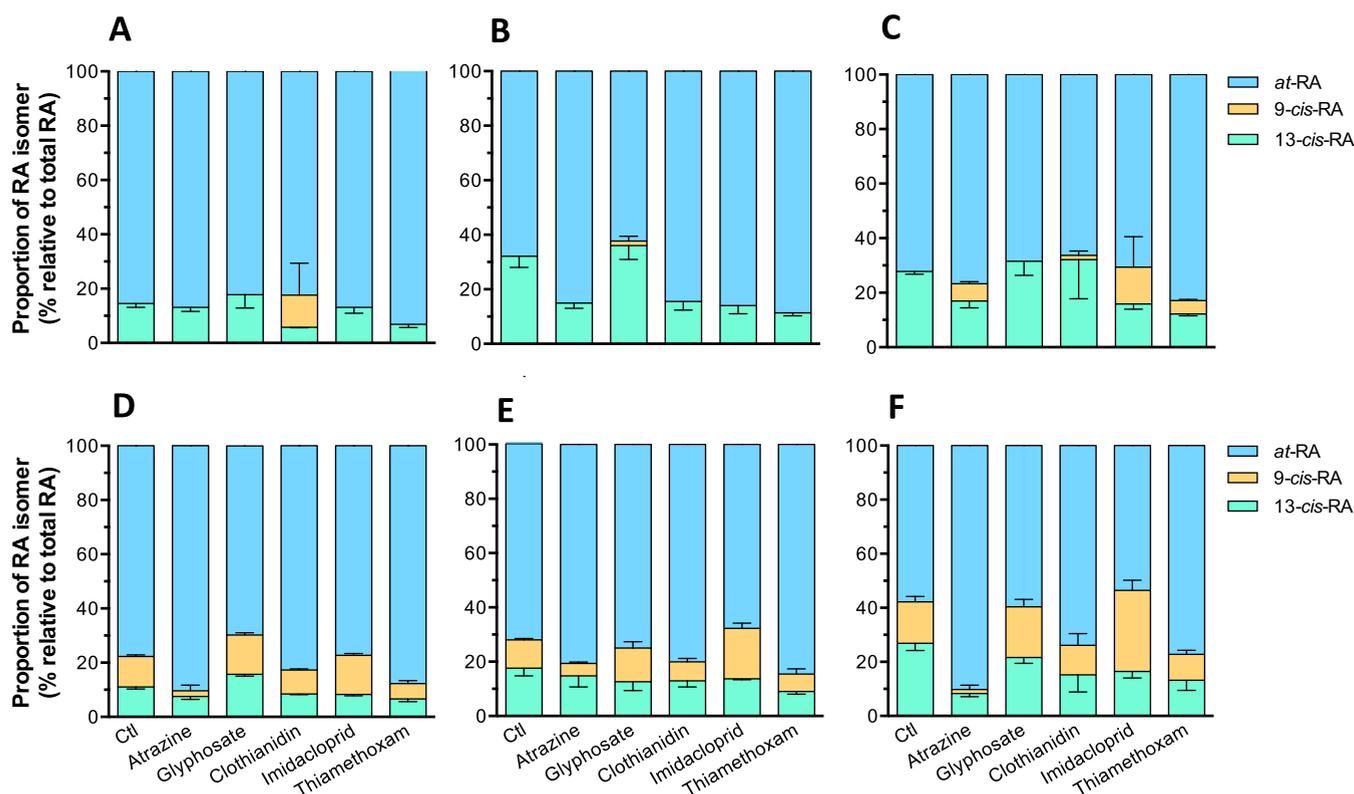
Figure 8. Effects of pesticides on RA isomerization. PLHC-1 (A−C) and ZFL (D−F) cells were exposed to 250 mg/L atrazine, glyphosate, clothianidin, imidacloprid, or thiamethoxam for 24 h and concomitantly incubated with 50 nM *at*-RA for the last 6 h (A, D), 18 h (B, E), or 24 h (C, F) of treatment. The proportions of all-*trans*-RA, 9-*cis*-RA, and 13-*cis*-RA relative to the total cellular RA are expressed as mean percentage ± SD estimated from three independent cell cultures.

Table 1. Pairwise Comparisons in PLHC-1 Cells Using Permutation MANOVAs on a Distance Matrix (Number of Permutations: 99 999, *p*-Values Lower Than 0.05 Shown in Bold)

p-values	CTL	A	G	C	I
A	**0.0438**				
G	0.5689	**0.0349**			
C	0.5773	0.9261	0.3249		
I	0.0729	0.9261	**0.0438**	0.9261	
T	**0.0045**	0.0589	**0.0045**	0.1666	0.1666

Table 2. Pairwise Comparisons in ZFL Cells Using Permutation MANOVAs on a Distance Matrix (Number of Permutations: 99 999, *p*-Values Lower Than 0.05 Shown in Bold)

p-values	CTL	A	G	C	I
A	**0.0020**				
G	0.5624	**0.0009**			
C	0.0931	0.0834	0.0567		
I	0.1246	**0.0009**	0.2963	**0.0244**	
T	**0.0081**	0.1939	**0.0056**	0.3573	**0.0024**

a trend was observed with imidacloprid in PLHC-1 cells. Also, in both cell lines, glyphosate modified the proportions of RA isomers observed in atrazine-treated cells, whereas thiamethoxam modified those measured in glyphosate-treated cells.

The PCAs show the Euclidean distances between the samples throughout all durations of exposure; the greater the distance between two samples on the biplot, the greater the

dissimilarity between the samples (Figure 9). The arrows illustrate the isomers; thus, for samples going in the direction of an arrow (relative to the control group), contents of the isomer are higher. The angles between the arrows illustrate the direction of the correlations between the three isomers; arrows going in the same direction imply a correlation, and arrows separated by a square angle are not correlated. In both cell lines, thiamethoxam increased the levels of *at*-RA, and glyphosate enhanced 13-*cis*-RA contents. Additionally, glyphosate and imidacloprid increased the levels of 9-*cis*-RA in ZFL cells, exclusively. Interestingly, in PLHC-1 cells, there is no correlation between the contents of 13-*cis*-RA and 9-*cis*-RA, and each of these isomers is weakly correlated with *at*-RA. In contrast, in ZFL cells, the levels of 13-*cis*-RA and 9-*cis*-RA are strongly correlated, and none is correlated with *at*-RA (Figure 9B). The isomerization of *at*-RA in PLHC-1 and ZFL cells varied over time and was affected by some pesticides, notwithstanding important differences between cell lines.

■ DISCUSSION

The aim of the study was to investigate whether herbicides and neonicotinoid insecticides modify RA catabolism in two fish hepatic cell lines widely used in *in vitro* toxicology studies. We first characterized RA catabolism in PLHC-1 and ZFL cells by evaluating the contribution of CYP26. In both cell lines, the metabolite 13-*cis*-4-oxo-RA was detected, and the three RA isomers eluted similarly to what was described in human intestinal cells and mouse liver.[34,35] The isoform CYP26A1, which has been cloned first in zebrafish,[36] is believed to be mainly responsible for *at*-RA clearance in the liver, in addition
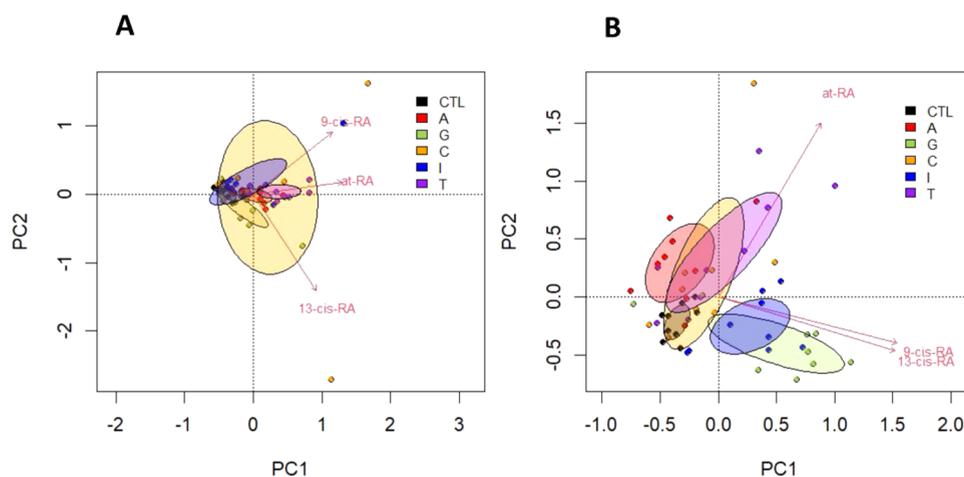
Figure 9. Principal component analysis of RA isomerization profiles following pesticide exposure in PLHC-1 (A) and ZFL (B) cells. Retinoid isomer contents (ng/mg protein) are plotted according to their Euclidean distances and regardless of the duration of exposure to *at*-RA. Ellipsoids correspond to standard deviations.

to CYP26B1, whereas other CYP450 isoforms would have a significant role in other tissues.[8,20,37] Our results suggest that *at*-RA catabolism is mediated mostly by CYP26 in ZFL cells, a result that agrees with the upregulation of the *cyp26b1* gene reported by others in the same cells exposed for 24 h to 1 μM *at*-RA.[38] However, CYP26 would not be responsible for *at*-RA catabolism in PLHC-1 cells, in which isoforms of other CYP450 families, including CYP1A and CYP3A, would play a major role (Figure 2). Indeed, these CYP450 can catalyze the 4-hydroxylation of *at*-RA and they are inhibited by ketoconazole in the fish liver.[14,39] Also, our results show that the formation of 13-*cis*-4-oxo-RA is not mediated by CYP26 in both cell lines. These observations agree with those of Topletz et al. (2015),[20] who investigated the induction of CYP26A1 by *at*-RA and its metabolites in HepG2 cells and in human hepatic microsomes. In the latter study, 4-oxo-*at*-RA, a substrate for CYP26A1, induced CYP26A1, but the formation of 4-oxo-*at*-RA from 4-OH-*at*-RA was not inhibited by ketoconazole (10 μM). However, the formation of 4-oxo-*at*-RA from *at*-RA was inhibited by talarozole (1 μM), which led the authors to conclude that CYP26A1 was responsible for the initial hydroxylation of *at*-RA into 4-OH-*at*-RA but not for the subsequent dehydrogenation of 4-OH-*at*-RA into 4-oxo-*at*-RA.[20]

As a result of the inhibition of 4-OH-*at*-RA formation, Topletz et al.[20] found decreased contents of 4-oxo-*at*-RA in HepG2 cells treated with talarozole. In our study, talarozole increased cellular levels of 13-*cis*-4-oxo-RA in PLHC-1 and ZFL cells, with a concomitant higher cellular content of *at*-RA in ZFL cells exclusively (Figure 2). Although the metabolic steps leading to 13-*cis*-4-oxo-RA from *at*-RA were not investigated, and considering the possible isomerization before or after 4-OH-*at*-RA formation, our data suggest the following: (1) CYP26 would not be responsible for the initial formation of 4-OH-*at*-RA and its subsequent dehydrogenation into 13-*cis*-4-oxo-RA; (2) a 4-OH-*at*-RA-independent metabolic pathway might exist for 13-*cis*-4-oxo-RA production from *at*-RA. In both cases, CYP26 would be responsible for the degradation of 13-*cis*-4-oxo-RA in fish hepatic cell lines but not for its formation. Involvement of CYP450 isoforms of families other than CYP26, namely, CYP2B and CYP3A, in the formation of

4-OH-*at*-RA and 4-oxo-*at*-RA, has been reported by others in mice liver microsomes.[35]

Before investigating the effects of pesticides on *at*-RA metabolism, the cells' sensitivity to pesticides was estimated using MTT assays. The ZFL cells were found more sensitive than PLHC-1 cells (Figure 3). This difference between the two cell lines deserves to be investigated in future studies. However, in both cell lines, atrazine and thiamethoxam were the most toxic. Higher lethality with thiamethoxam compared to that with imidacloprid was also observed for yellow perch larvae.[23] The herbicide atrazine was strikingly more toxic than glyphosate. As discussed below, our data suggest atrazine-induced redox signal responsible for the impaired *at*-RA catabolism in both cell lines, but all our results do not support that oxidative stress is responsible for the high level of mortality in ZFL cells. *In vitro*, fish cells' sensitivity to pesticides varies considerably from one study to another. A 20% cell mortality was reported in ZFL cells exposed to 271 μg/L Roundup Transorb in PBS buffer, which shows much higher toxicity compared to our data, but the study did not provide an indication about the duration of exposure.[40] In flounder *P. olivaceus* gill FG cells, an IC_{50} of 38.5 μg/mL was estimated for MTT activity following a 48 h exposure to pure imidacloprid substance, closer to our findings.[41] In contrast, rainbow trout *Oncorhynchus mykiss* adrenocortical cells would be more resistant, at least to atrazine, as the LC_{50} would be higher than 18 g/L following a 60 min exposure to pure atrazine substance.[42] However, the LC_{50} was 93 mg/L for diazinon, an organophosphorus pesticide, showing that sensitivity to pesticides also varies considerably between pesticides for the same cell model. Compared to these *in vitro* data, the LC_{50} of 13 and 38 mg/L were estimated *in vivo* for rainbow trout *O. mykiss* following a 96 h exposure to the pure atrazine substance and the commercial formulation atrazine 500, respectively.[43]

Of note is the increase in MTT activity observed with some pesticides, especially glyphosate. MTT activity measurement was originally used as an indicator of cell survival and the chemosensitivity of drug-resistant tumor cells.[44] It has been widely used in cytotoxicity studies to monitor cell viability and to measure cell proliferation. However, none of the pesticides studied, including glyphosate, modified the total cell protein

contents (data not shown). Dose-dependent increases in MTT activity not related to variation in cell proliferation had also been reported by others.[45−48] Our results suggest that mitochondrial dehydrogenase activity might be stimulated under specific exposure to some pesticides, which deserves to be investigated in future studies.

It has been shown that pesticides may affect *at*-RA metabolism in various cell models or species. In the honeybee (*Apis mellifera*) exposed to atrazine and glyphosate, decreases in ROH contents, related to stimulated *β*-carotene 15-15′-oxygenase and ROLDH activities, could have modified *at*-RA levels with resulting detrimental effects on the bees.[49] In human hepatic cells HepG2, several organochlorine insecticides induced the overexpression of *cyp26* through binding and stimulation of RAR*α* and RAR*β*, two nuclear receptors of *at*-RA, leading to excessive catabolism of *at*-RA with the expected decrease in cell contents.[50] In contrast, the decreased CYP26 expression with a concomitant accumulation of ROH, possibly related to a decreased *at*-RA catabolism, was also reported in the livers of European common frogs *Rana temporaria* exposed to *p,p′*-DDE, a metabolite of the organochlorine insecticide DDT.[51] Conversely, Chen et al.[35] provided evidence for a stimulated catabolism of *at*-RA in mice exposed to conazole fungicides, as revealed by lower levels of hepatic *at*-RA with higher levels of 4-oxo-*at*-RA and 4-OH-*at*-RA, resulting from the overexpression of CYP2B and CYP3A but not of CYP26A1. Closer to our study model, Landry et al.[21] reported lower hepatic contents of DPAL in yellow perch from lake Saint-Pierre, compared to other populations located upstream and less exposed to pesticides. However, liver DROH contents were unchanged, as well as hepatic contents of the retinoid precursors *α*-carotene and *β*-carotene. Therefore, the authors suggested that DPAL depletion might result from an overmobilization of hepatic stocks to maintain basal concentrations of DROH. These results obtained by Landry et al.[21] prompted our hypothesis of an increased *at*-RA catabolism in fish exposed to pesticides, leading to lower levels of *at*-RA and higher levels of 13-*cis*-4-oxo-RA.

Contrary to the initial hypothesis that pesticides would stimulate *at*-RA catabolism, our results suggest that pesticides inhibit *at*-RA catabolism, as evidenced by higher *at*-RA contents in cells following pesticide exposure (Figures 4 and 5). The higher extracellular levels measured in these same cell cultures may be the result of the limited *at*-RA uptake in response to high intracellular contents, perhaps by regulating the uptake of *at*-RA mediated by RBP.[11,12] Hence, the overmobilization of DPAL stocks previously observed by Landry et al.[21] might be caused by an excessive conversion of DPAL into DROH by REH, rather than by an excessive catabolism of RA by CYP26A1. In other words, pesticides might stimulate the expression or the activity of REH, rather than that of CYP26A1. In this regard, Landry et al.[52] did observe a lower DPAL/DROH ratio in yellow perch living in the southern waters of lake Saint-Pierre. This lower DPAL/DROH ratio suggests a perturbation of the LRAT-ARAT/REH enzymatic equilibrium. If the effect of pesticides observed in our study with the PLHC-1 and ZFL cell lines translates *in vivo* in yellow perch, the inhibition of *at*-RA catabolism and its subsequent cellular accumulation could have detrimental consequences as either a deficiency or an excess of *at*-RA may affect the immune system, the ocular development, the formation of the cardiac system, the reproductive system, and limb formation during embryonic development.[7] Our data do

not provide evidence for any pesticide's effects on the intracellular contents of 13-*cis*-4-oxo-RA, and the higher extracellular levels noted after exposure to atrazine in ZFL cell cultures remain to be clarified. As a consequence of *at*-RA catabolism inhibition, lower intracellular contents of 13-*cis*-4-oxo-RA could be expected. However, 13-*cis*-4-oxo-RA is but one of many metabolites of *at*-RA,[7,10,14] and it might itself be further catabolized, possibly involving CYP26, as suggested by results obtained with talarozole in both cell lines (Figure 2) and by other investigators.[20]

We hypothesized that pesticide-induced perturbations of *at*-RA catabolism would be related to oxidative stress. This was studied by investigating the putative rescue effect of NAC on the pesticide-induced effects. *N*-acetylcysteine prevented the inhibition of *at*-RA catabolism by atrazine in both cell lines, by imidacloprid in PLHC-1 cells, and by thiamethoxam in ZFL cells (Figure 6). These data suggest the involvement of redox signals in the disruption of *at*-RA catabolism induced by these pesticides. However, data obtained with TBARS and thiol contents reveal that the relationship between perturbation in *at*-RA catabolism and oxidative damage is not straightforward. First, NAC prevented the effects of some pesticides in ZFL cells for which we have no evidence of oxidative stress. A more subtle redox imbalance in these cells could be revealed by measurements of biomarkers other than thiols and lipid peroxidation. Second, NAC did not rescue *at*-RA catabolism in cells exposed to glyphosate, while lipid peroxidation was measured with glyphosate in PLHC-1 cells (Figure 7). It is therefore possible that glyphosate induces oxidative stress but that it also inhibits *at*-RA catabolism independently of this stress. Third, higher levels of thiols were measured with atrazine but not with glyphosate. Different kinetics in increased cellular thiols in response to pesticides are conceivable. Alternatively, too fast an oxidation of thiols by glyphosate, but insufficient to manage the redox state of the cell and prevent lipid peroxidation, is also possible. Lipid peroxidation has also been reported in the liver of the fish *Channa punctatus* following exposure to atrazine as well as in the gills and in blood cells of the fish *C. punctatus* exposed to glyphosate.[53,54]

Data obtained with NAC suggest the involvement of redox signals in the disruption of *at*-RA catabolism induced by atrazine, imidacloprid, and thiamethoxam. Clearly, atrazine can induce oxidative damage, but redox imbalance would be sufficient, possibly by altering the expression or activity of the CYP450 isoform(s) responsible for the catabolism of *at*-RA. Some studies have shown that various CYP450 may be upregulated or in contrast downregulated under pro-oxidant conditions. In SVGA astrocytes, ethanol increased the expression of CYP2E1, but this effect disappeared with cells' cotreatment with the antioxidant vitamin A, suggesting that the increased CYP2E1 expression is mediated by oxidative stress.[55] In human HepG2 and rat H4 cells, pretreatment with H_2O_2 greatly reduced CYP1A1 overexpression normally induced by exposure to TCDD.[56] In these same cell lines, a dose-dependent downregulation of the CYP1A1 promoter activity and glutathione depletion was observed with increasing concentrations of H_2O_2.[56] In the rat liver and HepG2 cells, CCl_4 reduced the expression of CYP1A2, CYP2B1/2, CYP2C6, CYP2E1, and CYP3A2, with a concomitant lipid peroxidation, whereas pretreatment with antioxidant lignans moderated CYP450 downregulation and rescued the liver cells from lipid peroxidation.[57] Our data suggest that unlike atrazine, imidacloprid, and thiamethoxam, the effect of

glyphosate and clothianidin on *at*-RA catabolism would not involve oxidative stress (Figure 7B,C). Clothianidin is metabolized by various CYP450 isoforms including CYP3A4, CYP2C19, and CYP2A6,[58] and CYP26 is not the sole enzyme responsible for *at*-RA catabolism,[8] especially in PLHC-1 cells where ketoconazole, but not talarozole, inhibited catabolism (Figure 2). Therefore, it is conceivable that clothianidin might impair *at*-RA catabolism by competition for the CYP450. Glyphosate, however, is not metabolized by CYP450 and, in rat, seems to be mostly excreted without being metabolized at all.[59,60] Nonetheless, glyphosate has been shown to both lower expression and inhibit the activity of some CYP450, which could be responsible for the impaired *at*-RA catabolism observed in ZFL cells (Figure 5).[59,61]

All-*trans*-RA may be converted to 9-*cis*-RA and 13-*cis*-RA by isomerization and reciprocally.[14] In control PLHC-1 cells, 9-*cis*-RA was not detected, but atrazine and thiamethoxam induced the apparition of this isomer (Figure 8A–C). Similarly, conazole fungicides increased *at*-RA isomerization to 9-*cis*-RA in mouse hepatic microsomes.[33] In the present study, whether modifications in 9-*cis*-RA in PLHC-1 cells come from *at*-RA or 13-*cis*-RA isomerization or both remains to be verified, but our data suggest an important contribution of 13-*cis*-RA. On the contrary, the three isomers were all detected in control ZFL cells, and atrazine affected isomerization activity, leading to a lower proportion of 9-*cis*-RA, especially after a 24 h exposure to *at*-RA (Figure 8F). Why the isomerization profiles in control and atrazine-exposed PLHC-1 and ZFL cells differ deserves to be investigated. These differences between fish cell lines and, perhaps, between fish species are important to consider in future studies on *at*-RA metabolism in fish.

The particular patterns of *at*-RA isomerization observed following exposure to pesticides could be detrimental to the cell. Higher levels of *at*-RA were measured in both cell lines exposed to atrazine or thiamethoxam (Figures 4A and 5A). Hence, as a protective defense against too high transcriptional activity, one would expect higher isomerization toward the inactive isomer 13-*cis*-RA, which exhibits very low binding affinity for the nuclear retinoic acid receptor RAR compared to *at*-RA.[8,62] However, in both cell lines, the proportion of 13-*cis*-RA to *at*-RA was rather lower following exposure to atrazine or thiamethoxam. Thus, isomerization toward the inactive form of RA in response to high levels of cellular *at*-RA does not take place. Conversely, in mouse hepatic microsomes, isomerization to 13-*cis*-RA was observed although conazole fungicides decrease cellular RA.[35] In the presence of pesticides or fungicides, isomerization may not be able to maintain an adequate level of active RA. Our results also show higher proportions of total active isomers (*at*-RA + 9-*cis*-RA) in PLHC-1 and ZFL cells exposed to either atrazine or thiamethoxam (Figure 8C,F), which may lead to the overexpression of target genes with critical impacts for the cell. Of note, the proportion of inactive 13-*cis*-RA increased with the time of exposure to *at*-RA in the absence of pesticides, suggesting that isomerization, in addition to catabolism, would normally prevent the cells from overstimulated transcriptional activity. However, pesticides, in addition to inhibiting *at*-RA catabolism, would also affect the protection provided by isomerization. This dual action of pesticides on cellular mechanisms that control levels of active RA deserves to be investigated in future studies. The putative link between redox imbalance, modification in cellular thiols, and RA isomerization should also be studied as nonenzymatic isomerization

of RA isomers has been reported to involve molecules containing sulfhydryl groups, *i.e.*, thiols.[16,17,63]

CONCLUSIONS

This study shows that CYP26A1 is responsible for RA catabolism in ZFL cells but not in PLHC-1 cells. However, pesticides inhibit RA catabolism in the two fish hepatic cell lines. Atrazine, imidacloprid, and thiamethoxam, but not glyphosate and clothianidin, impair RA catabolism through oxidative stress. Additionally, pesticides modify the isomerization of *at*-RA and decrease the cell's capability to cope with too high levels of active isomers. The data allow for a better understanding of how pesticides may affect growth and development in fish through retinoid perturbation. This study also provides valuable data showing that ZFL and PLHC-1 cells represent good *in vitro* models to investigate retinoid metabolism in hepatic fish cells in relation to environmental contamination.

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Notes

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REFERENCES

(1) de la Chenelière, V.; Brodeur, P.; Mingelbier, M. Restauration des habitats du lac Saint-Pierre: un prérequis au rétablissement de la perchaude. *Le naturaliste canadien* **2014**, *138*, 50−61.

(2) Magnan, P.; Brodeur, P.; Paquin, É.; Vachon, N.; Paradis, Y.; Dumont, P.; Mailhot, Y. État du stock de perchaudes du lac Saint-Pierre en 2016. Comité scientifique sur la gestion de la perchaude du lac Saint-Pierre. 2017. Chaire de recherche du Canada en écologie des eaux douces; Université du Québec à Trois-Rivières et ministère des Forêts: de la Faune et des Parcs.

(3) Mailhot, Y. Plan de gestion de la pêche à la perchaude et suivi scientifique de la population de la perchaude. In*Compte rendu du deuxième atelier sur les pêches commerciales*, Bernard et, M.; Groleau, C., Eds.; Ministère de l'Environnement et de la Faune du Québec, 1997; pp 141−146.

(4) Giroux, I.; Hébert, S.; Berryman, D. Qualité de l'eau du Saint-Laurent de 2000 à 2014: paramètres classiques, pesticides et contaminants émergents. *Le Naturaliste Canadien* **2016**, *140*, 26−34.

(5) Ministère du Développement durable. de l'Environnement et des Parcs, (MDDEP). In *Portrait de la qualité des eaux de surface au Québec 1999 − 2008*, Direction du suivi de l'état de l'environnement: Québec, 2012; p 97, ISBN 978-2-550-63649-6 (PDF).

(6) Bernatchez, L.; Giroux, M. *Les poissons d'eau douce du Québec et leur répartition dans l'Est du Canada*, Broquet: Ottawa, 2000.

(7) Ross, S. A.; McCaffery, P. J.; Drager, U. C.; De Luca, L. M. Retinoids in embryonal development. *Physiol. Rev.* **2000**, *80*, 1021−1054.

(8) Novák, J.; Beníšek, M.; Hilscherová, K. Disruption of retinoid transport, metabolism and signaling by environmental pollutants. *Environ. Int.* **2008**, *34*, 898−913.

(9) Napoli, J. L. Biochemical pathways of retinoid transport, metabolism, and signal transduction. *Clin. Immunol. Immunopathol.* **1996**, *80*, S52−S62.

(10) Napoli, J. L. Interactions of retinoid binding proteins and enzymes in retinoid metabolism. *Biochim. Biophys. Acta, Mol. Cell Biol. Lipids* **1999**, *1440*, 139−162.

(11) Blomhoff, R.; Green, M. H.; Berg, T.; Norum, K. R. Transport and storage of vitamin A. *Science* **1990**, *250*, 399−404.

(12) Goodman, D. S.; Raz, A. Extraction and recombination studies of the interaction of retinol with human plasma retinol-binding protein. *J. Lipid Res.* **1972**, *13*, 338−347.

(13) Smith, J. E.; Milch, P. O.; Muto, Y.; Goodman, D. S. The plasma transport and metabolism of retinoic acid in the rat. *Biochem. J.* **1973**, *132*, 821−827.

(14) Marill, J.; Idres, N.; Capron, C. C.; Nguyen, E.; Chabot, G. G. Retinoic acid metabolism and mechanism of action: a review. *Curr. Drug Metab.* **2003**, *4*, 1−10.

(15) Bastien, J.; Rochette-Egly, C. Nuclear retinoid receptors and the transcription of retinoid-target genes. *Gene* **2004**, *328*, 1−16.

(16) Shih, T. W.; Shealy, Y. F.; Strother, D. L.; Hill, D. L. Nonenzymatic isomerization of all-*trans*-and 13-*cis*-retinoids catalyzed by sulfhydryl groups. *Drug Metab. Dispos.* **1986**, *14*, 698−702.

(17) Shih, T. W.; Lin, T. H.; Shealy, Y. F.; Hill, D. L. Nonenzymatic isomerization of 9-*cis*-retinoic acid catalyzed by sulfhydryl compounds. *Drug Metab. Dispos.* **1997**, *25*, 27−32.

(18) Reijntjes, S.; Blentic, A.; Gale, E.; Maden, M. The control of morphogen signaling: Regulation of the synthesis and catabolism of retinoic acid in the developing embryo. *Dev. Biol.* **2005**, *285*, 224−237.

(19) Idres, N.; Marill, J.; Flexor, M. A.; Chabot, G. C. Activation of retinoic acid receptor dependent transcription by all-*trans*-retinoic acid metabolites and isomers. *J. Biol. Chem.* **2002**, *277*, 31491−31498.

(20) Topletz, A. R.; Tripathy, S.; Foti, R. S.; Shimshoni, J. A.; Nelson, W. L.; Isoherranen, N. Induction of CYP26A1 by metabolites of retinoic acid: evidence that CYP26A1 is an important enzyme in the elimination of active retinoids. *Mol. Pharmacol.* **2015**, *87*, 430−441.

(21) Landry, C.; Houde, M.; Brodeur, P.; Spear, P.; Boily, M. Lipophilic antioxidants and lipid peroxidation in yellow perch subjected to various anthropogenic influences along the St. Lawrence River (QC, Canada). *Ecotoxicol. Environ. Saf.* **2017**, *139*, 316−325.

(22) Bruneau, A.; Landry, C.; Giraudo, M.; Douville, M.; Brodeur, P.; Boily, M.; Gagnon, P.; Houde, M. Integrated spatial health assessment of yellow perch (*Perca flavescens*) populations from the St. Lawrence River (QC, Canada), part B: cellular and transcriptomic effects. *Environ. Sci. Pollut. Res.* **2016**, *23*, 18211−18221.

(23) Paquet-Walsh, A.; Bertolo, A.; Landry, C.; Deschamps, L.; Boily, M. Interactive effects of neonicotinoids and natural ultraviolet radiation on yellow perch (*Perca flavescens*) larvae. *Sci. Total Environ.* **2019**, *685*, 690−701.

(24) Solari, M.; Paquin, J.; Ducharme, P.; Boily, M. P19 neuronal differentiation and retinoic acid metabolism as criteria to investigate atrazine, nitrite, and nitrate developmental toxicity. *Toxicol. Sci.* **2010**, *113*, 116−126.

(25) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(26) Ohkawa, H.; Ohishi, N.; Yagi, K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* **1979**, *95*, 351−358.

(27) Johansen, S. The Welch-James approximation to the distribution of the residual sum of squares in a weighted linear regression. *Biometrika* **1980**, *67*, 85−92.

(28) Lee, C. H.; Cook, S.; Lee, J. S.; Han, B. Comparison of two meta-analysis methods: inverse-variance weighted-average and weighted sum of Z-scores. *Genomics Inform.* **2016**, *14*, 173−180.

(29) Oksanen, J.; Blanchet, F. G.; Friendly, M.; Kindt, R.; Legendre, P.; McGlinn, D.; Minchin, P. R.; O'Hara, R. B.; Simpson, G. L.; Solymos, P.; Stevens, M. H. H.; Szoecs, E.; Wagner, E. *vegan: Community Ecology Package. R package version 2.5-7*. https://CRAN.R-project.org/package=vegan, 2020 (accessed Nov 30, 2021).

(30) Hervé, H. *RVAideMemoire: Testing and Plotting Procedures for Biostatistics. R package version 0.9-80*. https://CRAN.R-project.org/package=RVAideMemoire, 2021 (accessed Nov 30, 2021).

(31) R Core Team. *R: A language and environment for statistical computing*; R Foundation for Statistical Computing: Vienna, Austria https://www.R-project.org/2018.

(32) Hegelund, T.; Ottosson, K.; Râdinger, M.; Tomberg, P.; Celander, M. C. Effects of the antifungal imidazole ketoconazole on CYP1A and CYP3A in rainbow trout and killifish. *Environ. Toxicol. Chem.* **2004**, *23*, 1326−1334.

(33) Quintanilla, C. A.; Ho, R. K. The Cdx transcription factors and retinoic acid play parallel roles in antero-posterior position of the pectoral fin field during gastrulation. *Mech. Dev.* **2020**, *164*, No. 103644.

(34) Lampen, A.; Meyer, S.; Arnhold, T.; Nau, H. Metabolism of vitamin A and its active metabolite all-*trans*-retinoic acid in small intestinal enterocytes. *J. Pharmacol. Exp. Ther.* **2000**, *295*, 979−985.

(35) Chen, P. J.; Padgett, W. T.; Moore, T.; Winnik, W.; Lambert, G. R.; Thai, S. F.; Hester, S. D.; Nesnow, S. Three conazoles increase hepatic microsomal retinoic acid metabolism and decrease mouse hepatic retinoic acid levels in vivo. *Toxicol. Appl. Pharmacol.* **2009**, *234*, 143−155.

(36) White, J. A.; Guo, Y. D.; Baetz, K.; Beckett-Jones, B.; Bonasoro, J.; Hsu, K. E.; Dilworth, F. J.; Jones, G.; Petkovich, M. Identification of the retinoic acid-inducible all-trans-retinoic acid 4-hydroxylase. *J. Biol. Chem.* **1996**, *271*, 29922−29927.

(37) Thatcher, J. E.; Zelter, A.; Isoherranen, N. The relative importance of CYP26A1 in hepatic clearance of all-*trans* retinoic acid. *Biochem. Pharmacol.* **2010**, *80*, 903−912.

(38) Marqueno, A.; Flores, C.; Casado, M.; Porte, C. Dusregulation of lipid metabolism in PLHC-1 and ZFL cells exposed to tributyltin and all-trans retinoic acid. *Aquat. Toxicol.* **2021**, *231*, No. 105733.

(39) Miranda, C. L.; Henderson, M. C.; Buhler, D. R. Evaluation of chemicals as inhibitors of trout cythochrome P450s. *Toxicol. Appl. Pharmacol.* **1998**, *148*, 237−244.

(40) Goulart, T. L. S.; Boyle, R. T.; Souza, M. M. Cytotoxicity of the association of pesticides Roundup Transorb and Furadan 350 SC on the zebrafish cell line, ZF-L. *Toxicol. In Vitro* **2015**, *29*, 1377−1384.

(41) Su, F.; Zhang, S.; Li, H.; Guo, H. *In vitro* acute cytotoxicity of neonicotinoid insecticide imidacloprid to gill cell line of flounder *Paralichthy olivaceus*. *Chin. J. Oceanol. Limnol.* **2007**, *25*, 209−214.

(42) Bisson, M.; Hontela, A. Cytotoxic and endocrine-disrupting potential of atrazine, diazinon, endosulfan and mancozeb in adrenocortical steroidogenic cells of rainbow trout exposed in vitro. *Toxicol. Appl. Pharmacol.* **2002**, *180*, 110−117.

(43) Wan, M. T.; Buday, C.; Schroeder, G.; Kuo, J.; Pasternak, J. Toxicity to Daphnia magna, Hyalella azteca, Oncorhynchus kisutch, Oncorhynchus mykiss, Oncorhynchus tshawytscha, and Rana catesbeiana of atrazine, metolachlor, simazine, and their formulated products. *Bull. Environ. Contam. Toxicol.* **2006**, *76*, 52−58.

(44) Carmichael, J.; Degraff, W. G.; Gazdar, A. F.; Minna, J. D.; Mitchell, J. B. Evaluation of a tetrazolium-based semiautomated

colorimetric assay: assessment of chemosensitivity testing. *Am. Assoc. Cancer Res.* **1987**, *47*, 936−942.

(45) Gieni, R. S.; Li, Y.; HayGlass, K. T. Comparison of [³H] thymidine incorporation with MTT- and MTS-based bioassays for human and murine IL-2 and IL-4 analysis. Tetrazolium assays provide markedly enhanced sensitivity. *J. Immunol. Methods* **1995**, *187*, 85−93.

(46) Bernhard, D.; Schwaiger, W.; Crazzolara, R.; Tinhofer, I.; Kofler, R.; Csordas, A. Enhanced MTT-reducing activity under growth inhibition by resveratrol in CEM-C7H2 lymphocytic leukemia cells. *Cancer Lett.* **2003**, *195*, 193−199.

(47) Hamel, P.; Abed, E.; Brissette, L.; Moreau, R. Characterization of oxidized low-density lipoprotein-induced hormesis-like effects in osteoblastic cells. *Am. J. Physiol.: Cell Physiol.* **2008**, *294*, C1021−C1033.

(48) Mantha, M.; Jumarie, C. Cadmium-induced hormetic effect in differentiated Caco-2 Cells: ERK and p38 activation without cell proliferation stimulation. *J. Cell. Physiol.* **2010**, *224*, 250−261.

(49) Hedrei Helmer, S.; Kerbaol, A.; Aras, P.; Jumarie, C.; Boily, M. (2015). Effects of realistic doses of atrazine, metolachlor, and glyphosate on lipid peroxidation and diet-derived antioxidants in caged honey bees (*Apis mellifera*). *Environ. Sci. Pollut. Res.* **2015**, *22*, 8010−8021.

(50) Lemaire, G.; Balaguer, P.; Michel, S.; Rahmani, R. Activation of retinoic acid receptor-dependent transcription by organochlorine pesticides. *Toxicol. Appl. Pharmacol.* **2005**, *202*, 38−49.

(51) Leiva-Presa, A.; Mortensen, A. S.; Arukwe, A.; Jenssen, B. M. Altered hepatic retinol and CYP26 levels in adult European common frogs (*Rana temporaria*) exposed to p, p′-DDE. *Mar. Environ. Res.* **2006**, *62*, S10−S15.

(52) Landry, C.; Houde, M.; Brodeur, P.; Boily, M. Biological markers to establish a relationship between the health status of the St. Lawrence River yellow perch (*Perca flavescens*) with a gradient of anthropogenic disturbances. *Sci. Total Environ.* **2020**, *726*, No. 138515.

(53) Nwani, C. D.; Lakra, W. S.; Nagpure, N. S.; Kumar, R.; Kushwaha, B.; Srivastava, S. K. Toxicity of the herbicide atrazine: effects on lipid peroxidation and activities of antioxidant enzymes in the freshwater fish *Channa punctatus* (Bloch). *Inter. J. Environ. Res. Public Health* **2010**, *7*, 3298−3312.

(54) Nwani, C. D.; Nagpure, N. S.; Kumar, R.; Kushwaha, B.; Lakra, W. S. DNA damage and oxidative stress modulatory effects of glyphosate-based herbicide in freshwater fish, *Channa punctatus*. *Environ. Toxicol. Pharmacol.* **2013**, *36*, 539−547.

(55) Jin, M.; Ande, A.; Kumar, A.; Kumar, S. Regulation of cytochrome P450 2e1 expression by ethanol: role of oxidative stress-mediated pkc/jnk/sp1 pathway. *Cell Death Dis.* **2013**, *4*, No. e554.

(56) Morel, Y.; Barouki, R. Down-regulation of Cytochrome P450 1A1 Gene Promoter by Oxidative Stress, Critical Contribution of Nuclear Factor 1. *J. Biol. Chem.* **1998**, *273*, 26969−26976.

(57) Xie, Y.; Hao, H.; Wang, H.; Guo, C.; Kang, A.; Wang, G. Reversing effects of lignans on CCl4-induced hepatic CYP450 down regulation by attenuating oxidative stress. *J. Ethnopharmacol.* **2014**, *155*, 213−221.

(58) Shi, X.; Dick, R. A.; Ford, K. A.; Casida, J. E. Enzymes and inhibitors in neonicotinoid insecticide metabolism. *J. Agric Food Chem.* **2009**, *57*, 4861−4866.

(59) Hietanen, E.; Linnainmaa, K.; Vainio, H. Effects of phenoxyherbicides and glyphosate on the hepatic and intestinal biotransformation activities in the rat. *Acta Pharmacol. Toxicol.* **2009**, *53*, 103−112.

(60) Brewster, D. W.; Warren, J.; Hopkins, W. E., II Metabolism of glyphosate in Sprague-Dawley rats: tissue distribution, identification, and quantitation of glyphosate-derived materials following a single oral dose. *Fundamen. Appl. Toxicol.* **1991**, *17*, 43−51.

(61) Abass, K.; Pelkonen, O. The inhibition of major human hepatic cytochrome P450 enzymes by 18 pesticides: comparison of the N-in-one and single substrate approaches. *Toxicol. In Vitro* **2013**, *27*, 1584−1588.

(62) Allenby, G.; Bocquel, M. T.; Saunders, M.; Kazmer, S.; Speck, J.; Rosenberger, M.; Lovery, A.; Kastner, P.; Grippo, J. F.; Chambon, P.; Levin, A. A. Retinoic acid receptors and retinoid X receptors: interactions with endogenous retinoic acids. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 30−34.

(63) Urbach, J.; Rando, R. R. Thiol dependent isomerization of all-*trans*-retinoic acid to 9-cis-retinoic acid. *FEBS Lett.* **1994**, *351*, 429−432.