HEPATIC RETINOIDS OF BULLFROGS IN RELATION TO AGRICULTURAL PESTICIDES

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Abstract—Agricultural pesticides often have been cited as a factor affecting indigenous amphibian populations, but possible effects of pesticides and other factors associated with agricultural practices are understood poorly. Adult bullfrogs (Rana catesbeiana) were collected within the Yamaska River basin (Quebec, Canada) in subwatersheds representing low, medium, and high agricultural activities and 53 pesticides were analyzed in surface water. More pesticides were detected in subwatersheds associated with high agricultural activities like Rivière Noire and Rivière à la Barbe and pesticide concentrations were higher compared to the other study sites. Female and male body weights differed between sites. In the case of males, body weight was significantly less at Rivière à la Barbe. Liver retinol stores were decreased significantly in male bullfrogs from Rivière Noire, although total retinyl esters concentrations varied between sites having the highest concentration at Yamaska-Nord where the agricultural activity was considered low. The ratio of hepatic retinyl palmitate to retinol tended to be higher for male bullfrogs from Rivière Noire and Rivière à la Barbue. These results suggest that factors associated with intensive agricultural practices may affect the body weight and retinoid stores in male bullfrogs living in these agroecosystems.

Keywords—Pesticides Amphibians Rana catesbeiana Retinol Retinyl ester

INTRODUCTION

Over the last two decades, frogs worldwide have been subject to major impacts including population declines, decreased diversity, local extirpation, and, depending on the area, high incidences of malformations [1]. Multiple factors may contribute to these effects, not the least of which are habitat loss and water contamination [2–3]. In agricultural ecosystems, habitat loss typically is associated with the draining of marshes and ponds to maximize surface areas available for both cultivation and the spreading of manure. In addition, intensive agricultural practices involve hydrological changes due to surface and often subsurface drainage. Among the sources of water contamination are fertilizers, manure, soil erosion, veterinary products, and pesticides. Frogs are confined to less suitable habitats in these modified ecosystems and potentially are exposed to many pollutants, including pesticides.

Toxicology research has not identified adequately the effects of agricultural pesticides, per se, on resident amphibian populations. Most field experiments have compared agricultural to nonagricultural sites. Previous studies conducted in southern Quebec have associated agriculture with statistically significant increases in genomic variability and abnormal DNA profiles (aneuploidy or chromosomal fragmentation) of erythrocytes of green frogs (Rana clamitans) [4,5] in addition to apparent (nonstatistical) increases in hindlimb deformities of several species including bullfrogs [6]. However, from a remediation management perspective, variables potentially influencing population health need to be examined. For example, a geographically large-scale, multispecies and multihypothesis experiment identified atmospheric pesticide drift as a causal factor for amphibian declines in nonagricultural areas in California, USA [7]. The sampling design of the present investigation attempts to evaluate the influences of agricultural activities and potential pesticide exposure on bullfrogs.

Our general hypothesis is that chemical contaminants of the environment affect the metabolism or homeostasis of endogenous retinoids. Retinol and esterified retinol usually are stored in the liver. These stores compensate for inadequate nutritional intake and physiological demands (e.g., ovogenesis, reproduction, migration) and are converted to highly active forms such as retinoic acid. Retinoids were investigated because, when unbalanced, they are related to impairment of numerous processes including the immune response, reproduction, growth, and embryonic development (malformations); all these components may, alone or together, influence the status of frogs and contribute to their decline. Several researchers have proposed that the limb malformations in amphibians have a retinoid-based etiology [8,9]. More specifically, these investigators have focused on the possibility that compounds present in the environment, so-called environmental retinoids, may interact with retinoid receptors. However, such compounds have not been identified in aquatic sediments or surface water runoff in agricultural habitats at concentrations known to cause malformations. On the other hand, environmental organochlorine compounds like polychlorinated biphenyls are well known to alter stored retinoids in other vertebrates [10]. In this context, retinoid-based biomarkers have been validated successfully in-field for different species of mammals, fish, and birds [11–13]. Almost no information is published regarding other classes of contaminants. The only
paper dealing with pesticides, retinoids, and frogs is that of Keshavan and Deshmukh [14] reporting a decrease of hepatic retinoids in frogs exposed to dichlorodiphenyl trichloroethane (DDT) and carbaryl.

The bullfrog, *Rana catesbeiana*, was investigated because it is considered to be an indigenous, nonthreatened species in Quebec; it is a voracious predator (high potential exposure to contaminated prey); its life cycle mostly is aquatic (chronic exposure to waterborne pollutants); and it has a relatively long life expectancy of eight to nine years (long lifetime exposure).

The study design involves a series of independent sampling sites (tributaries and small watersheds) within the Yamaska River drainage basin. The sampling sites were selected to encompass habitats ranging from wildlife conservation areas to areas of intensive agriculture (combined corn, soya, pork, and poultry). Potential exposure to pesticides in the different tributaries was evaluated by the analysis of surface water for the concentrations of 53 pesticides in the triazine, organophosphate, and aryloxyacid categories. Water samples were collected a month after the peak spraying period, in order to estimate chronic, low-level exposure. For the first time, retinoids are investigated in wild populations of amphibians and their potential use as biomarkers is discussed.

### MATERIALS AND METHODS

#### Study areas

During the month of July 2003, 17 to 28 bullfrogs were captured in each of the six subwatersheds within the Yamaska River drainage basin (Fig. 1, sites 1–6). The sampling sites, thus, were independent and represented local agricultural activities and pesticide use patterns. The selection of subwatersheds was based on the percentage of surface area under intensive cultivation [15] classed as low (0–19%), moderate (20–59%) or high (>60%). According to this classification, the two sites associated with low agricultural activity were Deborah Stairs (site 1), a 2.5-ha pond located in a private wildlife reserve, and Rivière Yamaska-Nord (site 2), in a protected wetlands area created in 1992 as an enlargement of this tributary to the Yamaska River. Sites associated with high agricultural activity were Rivière Noire (site 5), Rivière à la Barbue (site 6), and Rivière Chibouet (site 7), these three subwatersheds being characterized by intensive corn and soya cultivation as well as high-density pork and poultry production. The Rivière Pot-au-Beurre subwatershed (site 4) was classed as a medium-intensity site having between 20 and 39% of its surface area cultivated intensively. The sampling site was located downstream from the farms in a 1,400-ha wetland reserve called the Baie Lavallière. The medium-intensity Yamaska River site (site 3) was located upstream from the intensive agricultural regions and to some extent was influenced by the confluence of three tributaries draining low-intensity farmland. The mouth of the Yamaska River (site 8) was located on the St. Lawrence River shore. Sites 4 and 8 had between 40 and 59% of the land under intensive cultivation.

#### Bullfrogs

The field collections and handling of animals conformed to animal care permits issued by the Université du Québec à Montréal and the Société de la faune et des parcs du Québec. No bullfrogs were collected at sites 7 and 8. Further investigation will be needed to confirm the presence and the number of bullfrogs before sampling at these two sites. A maximum of 30 adult bullfrogs was allowed for capture at sites 1 to 6, and 12 per site were euthanized for liver retinoid analysis. The field collections took place between 21 h and 2 h. Bullfrogs were localized along the water’s edge by their calls and were fixed individually in the beam of a spotlight before being captured by hand or dip net. This method tends to select for males rather than females. The frogs were kept in heavy plastic containers (40 × 75 × 55 cm) filled to a depth of 8 cm, approximately, with pond or river water at a maximum density of six individuals per container. Air holes provided aeration, secured lids prevented predation, and the containers were hidden by vegetation. A field laboratory was set up early the next day and all animals were processed within 16 h of capture. Water temperature was maintained within normal limits (15–25°C) by keeping the containers in the shade and exchanging water as necessary.

Adult bullfrogs were anesthetized individually in water containing 0.1% tricane methanesulphonate (MS222) buffered with 0.2% NaHCO₃. Animals were weighed, measured (total length and snout vent length), sexed, and examined for external anomalies. The adults were distinguished from juveniles by rougher skin and the absence of black spots. Sexually mature male frogs were identified by yellow coloration of the throat and the larger development of the thumb (digit 1 of forelimb palms) [16]. For retinoid analysis, the anesthetized animals were euthanized by cervical severance and maturity was confirmed by the development of sexual organs, presence of testis,
and fat bodies for males, and for females, the well-developed ovaries and the presence of mature eggs. The liver was removed immediately and frozen in liquid nitrogen. Samples were stored at −80°C until analysis for retinoids.

**Chemicals**

The MS222 was purchased from ICN Biomedicals (Irvine, CA, USA). Solvents used for analysis were obtained from Fisher Scientific (Montreal, QC, Canada). All-trans-retinol, all-trans-retinyl palmitate, pyridine, and acyl chloride were purchased from Sigma Chemicals (St. Louis, MO, USA). Retinyl esters were synthesized according to the method of Huang and Goodman [17] with some modifications. Briefly, 0.2 μmol all-trans-retinol was diluted in 100 μl benzene containing 1% pyridine and 0.2 μmol of acyl chloride (lauroyl, myristoyl, linoleoyl, or stearoyl) was added. The test tube was sealed under nitrogen and placed in a water bath at 50 to 60°C for 1 min. The reaction was completed at room temperature, the tube being vortexed at 10-min intervals over a 1-h period. Retinoid esters were extracted with 0.5 ml hexane and partially purified on a Florisil column (Sep-Pak, 1 ml, Waters, Milford, MA, USA).

**Retinoid analysis**

All retinoid analyses were conducted under yellow incandescent lights to prevent isomerization. The extraction procedure was modified from that of Spear and Moon [18]. Briefly, 0.2 g of bullfrog liver was dehydrated by grinding with 2 g of anhydrous sodium sulphate (Na₂SO₄). Extraction was achieved by adding 5 ml hexane and mixing for 10 min. After centrifugation (2 min at 2,000 g), a 500-μl aliquot was evaporated to dryness at 45°C under a gentle stream of nitrogen gas. The extract was redissolved in 200 μl acetonitrile and 50 μl was injected into the high-performance liquid chromatography.

The high-performance liquid chromatography conditions were as described in Doyon et al. [19] with the exception that a Nova-Pak C18 (4-μm particle size, 3.9 × 150 mm, Waters) was used as the analytical column. The peaks for retinol and retinyl palmitate were identified by comparing with authentic commercial standards. Oleate and palmitate esters coeluted under these high-performance liquid chromatography conditions and were quantified together as the palmitate ester. The peaks for retinyl laurate, myristate, linoleate, and stearate were identified by standards synthesized in our laboratory and quantified using the standard curve for retinyl palmitate corrected for molecular weight. The sum of concentrations of the individual retinyl esters, expressed in μmoles per gram (μmol/g), is referred to as total ester concentration. This value was taken to represent overall retinoid storage.

**Water analysis**

Bulk samples of surface water (10 L) were collected from a depth of 20 cm at all sites on August 4, 2003. Besides the six study sites, an additional sample was collected from the Rivière Chibouet subwatershed characterized by high agricultural intensity. A water sample also was taken near the mouth of the Yamaska River representing the combined influence of all subwatersheds to the Yamaska. The unfiltered samples were analyzed for organophosphorous, aryloxyacides, and triazines pesticides by Centre d’expertise en analyse environnementale du Québec [20]. For the analysis of aryloxyacid pesticides, unfiltered surface water was preserved by acidification (pH < 2) with sulphuric acid and stored at 4°C up to 21 d. Surrogate deuterated standards (2,4-D-d₃ and dicamba-d₃) were added and pesticides extracted from a 250-ml aliquot by solid phase extraction (C18: 1 g; J.T. Baker, Phillipsburg, NJ, USA). Analytes were eluted with methylene chloride: methanol (90:10), evaporated near dryness and the analytes were esterified with diazomethane. Following purification on a silica gel column (500 mg; J. T. Baker), an internal standard was added and samples were analyzed by gas chromatography-mass spectrometry (GC-MS) in the scanning mode. The analyses were performed using a model HP-6890 GC coupled to a model HP-5973 MS (both components Hewlett-Packard, Palo Alto, CA, USA). The chromatographic column was an HP-5MS (30-m length; 0.22-mm i.d.; 0.2-μm phase thickness; Agilent Technologies, Palo Alto, CA, USA). The method analyzed the following 16 pesticides: 2,4,5-T (2,4,5-trichlorophenoxyacetic acid), 2,4-DB (2,4-dichlorophenoxybuteric acid), 2,4-D (2,4-dichlorophenoxyacetic acid), bentazone, clopyralid, dicamba, dichlorprop, dinoseb, dromoxynil, fenoprop, 4-chloro-2-methylphenoxyacetic acid, 4-(4-chloro-2-methylphenoxy)butanoic acid, mecoprop, methylidiclofop, piclorame, and triclopyr. Overall, the quantitative limits of detection ranged from 0.01 to 0.04 μg/L in water with recoveries of 73 to 120%. Method performance specific to the pesticides detected in surface waters of present study are given in Table 1.

In the case of organophosphate and triazine pesticides, surrogate standards (atrazine-d₅ and propoxur) were added to samples of bulk surface water before being stored at 4°C up to 7 d. Pesticides were extracted from 150-ml aliquots by solid phase extraction (C18: 1 g; J.T. Baker) with elution in ethyl acetate:water (90:10). The solvent was evaporated and analytes recovered in ethyl acetate containing an internal standard. Analyses were performed with a Trace GC-MS system in the scan mode (Thermo-Quest, Waltham, MA, USA). The GC column used was a model HP-5MS (30-m length; 0.25-mm i.d.; 0.25-μm phase thickness; Agilent Technologies). A total of 37 pesticides and metabolites were analyzed with quantitative limits of detection ranging from 0.01 to 0.25 μg/L and recoveries varying between 63 and 128% depending upon the compound. Compounds potentially detected are the following: Atrazine, bendiocarbe, butilate, carbaryl, carbofuran, chlorothalonil, chlorfenvinphos, chloroxuron, chlorpyriphos, cyazinazine, deethylatrazine, desipropylatrazine, diazinon, dichlorvos, dimethanamide, dimethoate, diuron, E-dipyropylcarbamothioate, fenitrothion, fonofos, linuron, malathion, methidathion, methyldiclofop, metolachlor, metribuzine, methidathion, methyldiclofop, metolachlor, metribuzine, mevinphos, myclobutanil, 1-napthhol, parathion, methylparathion, propoxur, pyralid, dicamba, dichlorprop, dinoseb, dromoxynil, fenoprop, 4-chloro-2-methylphenoxyacetic acid, 4-(4-chloro-2-methylphenoxy) butanoic acid, mecoprop, methylidiclofop, piclorame, and triclopyr. Detection limits, recoveries, and sample-to-sample reproducibility of the method as it pertains to pesticides detected in surface waters of the present study are included in Table 1.

**Statistical analysis**

Differences in body weight and retinoid concentrations between sites and sexes were tested using two-way analysis of variance general linear model procedure. For the male data, retinol, total esters, and ratio of retinyl palmitate/retinol values were compared between sites using a one-way analysis of variance (general linear model procedure). When the general variance model was significant, multiple comparisons were conducted using the Tukey test. A Pearson correlation matrix between all variables was computed while a Kendall correlation was performed for body weight, liver weight, total esters, and...
retinol within each site. Statistical analyses were performed using Systat® 10.0 software (Systat Software, Richmond, CA, USA).

RESULTS

Water analysis

Of 53 possible measured pesticides, only 11 were present in the surface waters at levels above the limits of detection (Table 1). All the pesticides detected were herbicides. Among the six study sites, Deborah Stairs and Rivière Yamaska-Nord were less contaminated than Rivière Noire and Rivière à la Barbue. No pesticides were detected at the Deborah Stairs site. Atrazine and metolachlor were detected in all other samples, their concentrations being greatest at Rivière Noire and Rivière à la Barbue. The number of pesticides detected also was greater at Rivière Noire and Rivière à la Barbue. These two sites have a similar contamination profile except for metolachlor and dimethinamide, which, respectively, are two and 20 times higher for Rivière à la Barbue. Pesticide contamination associated with site 3 (Yamaska River) was lower than agricultural intensity [15]. However, pesticide contamination associated with site 1 (Yamaska River) was higher for Rivière a la Barbue. Pesticide contamination at sites 4, 5, and 6 was computed separately. The number of females for Rivière Noire was similar for all sites. Body weight data for females and males, therefore, were computed separately. The number of females for Rivière Pot-au-Beurre and Rivière à la Barbue was not high enough to conduct statistical analysis. Consequently, female body weight data were compared between the four remaining sites. The one-way analysis of variance showed a slightly significant difference between sites ($F_{4,112} = 3.167, p = 0.046$); the females from the Yamaska River were smaller than females from the Rivière Noire ($p = 0.039$; Fig. 2A). When considering male bullfrogs, the weight also differed between sites ($F_{5,89} = 18.8, p < 0.001$) showing a marked decrease at Rivière à la Barbue. Males from this site were significantly smaller than males from all other sites except Yamaska River ($p = 0.008$; Fig. 2B). A high significant correlation was observed between body weight and liver weight ($r = 0.879, p < 0.001$).

Retinoids

The livers of 67 frogs (51 males and 16 females) were analyzed for retinoids: Retinol and total esters. Both variables were sex-related as revealed by a two-way analysis of variance including site, retinol ($F_{1,53} = 10.27, p = 0.002$), and total esters ($F_{1,53} = 7.79, p = 0.007$). The interaction term (site × sex) for both retinoids was not significant (retinol $p = 0.24$; total esters $p = 0.78$), indicating a similar effect on retinoids for males and females within each site. These results suggest that liver retinoid data should be treated separately for males and females.

When performed on female data, the one-way analysis of variance did not demonstrate a significant difference between

### Table 1. Analysis of pesticide concentrations (μg/L) in surface water from subwatersheds sampled in the Yamaska River Basin, Quebec, Canada, 2003

<table>
<thead>
<tr>
<th>Analytical performance</th>
<th>Sampling sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Deborah Stairs</td>
</tr>
<tr>
<td>Clopyralid</td>
<td>0.03</td>
</tr>
<tr>
<td>Dicamba</td>
<td>0.03</td>
</tr>
<tr>
<td>Mecoprop</td>
<td>0.01</td>
</tr>
<tr>
<td>Dichlophenoxy-acetic acid (2,4-D)</td>
<td>0.01</td>
</tr>
<tr>
<td>Bentazone</td>
<td>0.04</td>
</tr>
<tr>
<td>Deethyl-atrazine</td>
<td>0.05</td>
</tr>
<tr>
<td>Simazine</td>
<td>0.04</td>
</tr>
<tr>
<td>Atrazine</td>
<td>0.01</td>
</tr>
<tr>
<td>Metolachlor</td>
<td>0.02</td>
</tr>
<tr>
<td>Dimethenamide</td>
<td>0.02</td>
</tr>
</tbody>
</table>

* — = Under the limit of detection.
sites for retinol ($F_{5,10} = 1.30, \ p = 0.316$) or for total esters ($F_{5,10} = 0.414, \ p = 0.829$). Moreover, the number of females in each group (5–11) was not sufficient to sustain a thorough statistical analysis; the data set was characterized by considerable individual variation. Therefore, the male data set was examined more closely.

The retinol concentration in males differed significantly between groups ($F_{5,45} = 3.313, \ p = 0.012$; Fig. 3A). When compared to the Yamaska-Nord site, bullfrogs from Rivière Noire subwatershed had significantly lower liver retinol stores ($p = 0.036$) and those associated with Rivière à la Barbue showed a similar trend, although not significant ($p = 0.06$). With regard to the total concentration of retinyl esters, differences were obtained between sites ($F_{5,45} = 4.89, \ p = 0.001$; Fig. 3B), bullfrogs from the Yamaska-Nord site having the highest mean concentration of total esters and being significantly different from Deborah Stairs ($p = 0.026$), Rivière Pot-au-Beurre ($p = 0.002$), and Rivière à la Barbue ($p = 0.001$) sites. The ratio of retinyl palmitate over retinol showed a slight increase for Rivière Noire and Rivière à la Barbue sites but no significant difference between sites was found ($F_{5,46} = 0.977; \ p = 0.442$, Fig. 4). Neither body weight versus liver retinol ($r = 0.270, \ p = 0.066$) nor body weight versus total esters ($r = 0.244, \ p = 0.098$) was correlated. Within sites, a significant correlation was found between total esters and retinol for the Deborah Stairs site ($r = -0.643, \ p = 0.05$).

**DISCUSSION**

Compared with other water analyses within the Yamaska River drainage basin [21], the pesticide concentrations reported here are relatively low. This difference probably is due to the fact that the water had been sampled late in the growing season. The relative lack of pesticides is opposed to the findings of Meili et al. [24] for the nearby St. Maurice River. Bullfrogs from the two study sites differ in their biochemical response to sublethal concentrations of 2,4-D. This may be due to the difference in pesticide concentrations between the two watersheds. The Yamaska River drains a mixed-use landscape and contains a larger proportion of forest cover than the St. Maurice River. This may suggest that the concentrations of estrogenic contaminants are higher in the St. Maurice River and that the effects are more pronounced in this population of bullfrogs.

**Fig. 3.** Liver retinoids of male bullfrogs sampled at study sites, Quebec, Canada. Bars represent mean and standard deviation. Values at the base of bars are sample sizes. Sites sharing at least one similar letter are not significantly different from each other ($p \leq 0.05$). (A) Retinol and (B) total esters.

**Fig. 4.** Ratio of retinyl palmitate over retinol. Bars represent mean and standard deviation. All sites in Quebec, Canada.
season whereas most herbicides are applied in the spring. Pesticides detected in water samples, therefore, represent residual concentrations consistent with low level, chronic exposures. Dicamba exceeded water quality criteria for irrigation. According to the Conseil canadien des ministres des ressources et de l’environnement [22] the concentration should be less than 0.006 µg/L, which is 10 times lower than the value detected in the Rivière Noire and Rivière à la Barbue and 20 times lower than the concentration at the mouth of the Yamaska River. None of the pesticide concentrations in surface waters exceeded criteria for aquatic life established by the Quebec and Canadian governments [22,23].

Among the six study sites, Rivière Noire and Rivière à la Barbue had the highest overall concentrations and the greatest number of pesticides, including the new generation pesticide clopyralide. Most traditional pesticides are applied at rates of approximately kg/ha, but clopyralide and other new generation products are used in the g/ha range. The fact that this product nonetheless is detected in surface water with values similar to other herbicides suggests a high mobility and low degradation in the environment and should raise concern. The chemical analysis for pesticides at sites 1, 2, 4, 5, and 6 conformed with agricultural activity of their respective subwatersheds as defined by Primeau et al. [15]; however, pesticide levels at site 3 (Yamaska River) were lower than expected. This discrepancy may be explained by the influence of three subwatersheds draining land associated with low agricultural intensity that flowed into the Yamaska River immediately upstream from site 3. One should consider that the Yamaska River, being a much larger river than other sampled sites, has a greater dilution potential, which would influence the contaminant concentration.

Body size is an indicator of good health or fitness of frogs [24] and, for males, it is related to territorial defense and, therefore, mating success. The results for body weight in our study varied for both sexes. Despite a significant statistical result, female body weight differences between sites are not convincing due to the small number of females collected and high individual variation. In male frogs, body weight varied among sites and smaller males were observed at Rivière à la Barbue, where many pesticides were detected (Fig. 1). Assuming an effect of pesticides, the male bullfrogs from Rivière Noire also should have been smaller as the contamination profile is very close to that of Rivière à la Barbue (Table I). It is interesting to note the large difference in dimethenamide between these two sites. Decreased body weight and snout vent length were reported for tadpoles or adult frogs exposed to atrazine [25], metolachlor [26], and dichlorvos [27] and dimethenamide has been proven to interfere with body weight gains in rats [28] and newts [29]. Other chemicals are known to affect the growth of bullfrogs. In a laboratory experiment, adult bullfrogs (30 per group) exposed to atrazine, nonylphenol, chlorothalonil, and carbaryl demonstrated growth retardation based on weight (M. Boily, Université du Québec à Montréal, Quebec, Canada, unpublished data). A chronic exposure to metolachlor and dimethenamide could explain partially the small body size observed in males from Rivière à la Barbue. In addition to possible effects of chemical contaminants, ecological parameters such as food availability and competition for food also may influence body size in frogs. Among the contaminants found in water samples of the present study, adverse effects also have been noted for tadpole gonad development [30–32] and malformations related to atrazine [33]. Also, DNA damage has been observed for bullfrog tadpoles exposed to atrazine, metolachlor, and 2,4-D amine [34]. Few studies have been conducted with clopyralide, but this herbicide caused weight loss in rats [35].

The profile of liver retinoids in wild bullfrogs revealed a similar pattern to that of other vertebrates; retinol as the major nonesterified form and, among esterified forms, palmitate/oleate present at the highest concentrations. Because bullfrogs spend most of their time in water, we expected to find all-trans-dehydroretinol and dehydroretinyl esters, which frequently are detected in fish [36]. A few individuals showed a small concentration of dehydroretinol, but this compound typically was below the detection limit.

The data pertaining to retinoids in females were not treated statistically due to the low number of females captured. The females typically are attracted to the males’ calls and only enter the male territory to breed. When disturbed, they hide in deeper water. Therefore, the sampling method favored the capture of male frogs and the lower number of females does not necessarily represent an unusual sex ratio.

For male bullfrogs, the three sites having the lowest burden of pesticide contamination (Yamaska-Nord, Deborah Stairs, and Yamaska River) showed the highest hepatic retinol concentrations (Fig. 2A). The significantly lower hepatic retinol level at the Rivière Noire site may be associated with intensive agricultural practices including herbicide use. According to what is known of retinoid regulation in mammals, hepatic retinol is the form available to enter systemic circulation from which it is delivered to extra hepatic tissues and cells. Generally it is accepted that hepatic retinol primarily is derived from the hydrolysis of esterified retinoids stored in the liver. Such a link could not be confirmed in our study because total esters were correlated poorly with retinol ($r = 0.339$, $p = 0.02$). It is interesting to note that, within sites, a relationship between hepatic esters and retinol was found to be significant for only one location: Deborah Stairs. Being the most pristine site of our study, it is possible that male bullfrogs from this location were at biochemical equilibrium between retinol and its esters compared to other sites but further investigation will be needed to confirm this assumption. The ratio of retinyl palmitate/oleate to retinol often is used to get a better idea of the metabolism of stored retinoids. In laboratory and environmental studies, a higher ratio has been associated with adverse (contaminated) conditions. In our study, higher ratios for Rivière Noire and Rivière à la Barbue were observed, although not significant (Fig. 4). These results may be attributed to a lack of retinyl ester mobilization (hydrolysis) or enhanced ester formation (esterification). Both esterification and hydrolysis steps have been shown to be affected by organochlorine exposure in the liver of rats and fish as well as in the yolk sac of Japanese quail embryos [37–40]. Perhaps hydrolysis or esterification enzymes were affected in the bullfrogs due to water contaminants associated with intensive agriculture. As a top predator, the bullfrog may accumulate high levels of such compounds as DDT, dichlorodiphenyldichloroethylene (DDE), and polychlorinated biphenyls.

To examine whether the decrease in liver retinol concentrations might affect mobilization from liver to blood, retinol concentrations in plasma were analyzed in the same bullfrogs. A full account of the plasma retinol concentrations will be published separately. A highly significant positive correlation was obtained between liver and plasma retinol ($r = 0.560$, $p$
Hepatic retinoids in bullfrogs

< 0.001). These results are consistent with a partial inhibition of retinol release into the blood stream.

Many water contaminants associated with intensive agricultural practices may affect retinoid storage. The results of the present study suggest that herbicides may be involved and may decrease retinyl ester mobilization (hydrolysis) or enhance ester formation (esterification). However, because most herbicides cannot be measured adequately in tissues, biomarkers associated to these commonly used compounds are needed. The results of this study indicate that retinoid-based biomarkers could be developed for male bullfrogs. A more complete interpretation must await detailed analyses for other waterborne contaminants and a more complete investigation into temporal variation in the concentrations of such contaminants including pesticides.

The present study further demonstrates that intensive agriculture may be related to decreased body size in male bullfrogs as well as altered retinoids in the liver. The apparent absence of adult bullfrogs from the most contaminated site, Rivière Chibouket, merits further investigation.

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REFERENCES


Taj RF, Jehanesan A, Selvanayagam M, Manohar GJ. 1988. Effect of organophosphorous (Nuvan) and carbamate (Baygon) compounds on Rana hexadactyla (Lesson) with a note on body protein and liver glycogen. Gazette 15:25–32.


Hayes T, Haston K, Tsui M, Hoang A, Haefele C, Vonk A. 2003. Atrazine-induced hermaphroditism at 0.1 ppb in American leopar


