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THE FUNCTIONAL INTEGRITY OF NORTHERN LEOPARD FROG (*RANA PIPIENS*) AND GREEN FROG (*RANA CLAMITANS*) POPULATIONS IN ORCHARD WETLANDS. I. GENETICS, PHYSIOLOGY, AND BIOCHEMISTRY OF BREEDING ADULTS AND YOUNG-OF-THE-YEAR

MEGAN L. HARRIS,† CHRISTINE A. BISHOP,*‡ JOHN STRUGER,§ MICHAEL R. VAN DEN HEUVEL,‖
GLEN J. VAN DER KRAAK,# D. GEORGE DIXON,‖ BRIAN RIPLEY,†† and JAMES P. BOGART#
†Centre for Toxicology, University of Guelph, Guelph, Ontario N1G 2W1, Canada
‡Canadian Wildlife Service, §Ecosystem Health Division, Environment Canada, Box 5050, Burlington, Ontario L7R 4A6, Canada
‖Department of Biology, University of Waterloo, Waterloo, Ontario N2L 3G1, Canada
#Department of Zoology, University of Guelph, Guelph, Ontario N1G 2W1, Canada
††Pesticide and Trace Contaminants Laboratory, Agriculture and Food Laboratory Services Centre,
Ontario Ministry of Agriculture, Box 3650, 95 Stone Road W., Guelph, Ontario N1H 8J7, Canada

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Abstract—Northern leopard frogs (*Rana pipiens*) and green frogs (*Rana clamitans*) were evaluated at eight wetland sites, four of which were within apple orchards, to determine if environmental changes associated with orchard management affected measured biological parameters. Size, age, genetic variation, condition indices, levels of circulating steroid hormones, 7-ethoxyresorufin-*O*-deethylase activity (EROD), and organochlorine and organophosphorus residues in breeding males sampled at pond sites in orchards were compared to the same parameters measured in breeding males from reference sites. Also, the size and physiological condition of young-of-the-year captured in orchard and reference ponds were compared. No evidence of a reduction in genetic variation was found in populations of either species at any sites, but unexpectedly high average heterozygosity values (0.191–0.282) in concert with low overall fixation indices (0.012–0.059) in adults of both species did suggest that pond populations were interacting with neighboring populations in nonorchard habitats. Few significant differences in levels of circulating steroid hormones or condition indices of breeding males were found among sites. Significant EROD induction in male green frogs collected from one orchard site during one sampling event was the only indication that a metabolic challenge due to presence of cytochrome P450-inducing toxicants may have existed, whereas elevated concentrations of organochlorines (dichlorodiphenyltrichloroethane [DDT]- or endosulfan-related) in green frog tissues suggested that frogs at three orchard sites were taking up pesticides. Significant differences in size of equivalent-age male and juvenile leopard frogs and green frogs occupying different study sites suggested that suboptimal habitat characteristics existed at one or two of the four orchard sites. However, site-specific habitat deficiencies could not be related to orchard study sites in general, and, thus, wetlands in apple orchards appeared to provide viable breeding habitat for both northern leopard frogs and green frogs.

Keywords—Amphibians Genetic variation Steroid hormones Ethoxyresorufin-*O*-deethylase Pesticides

INTRODUCTION

In southern Ontario, Canada, ponds and canals in farmland are important wetland habitat for amphibians and other wildlife. The amount of natural wetlands in the province is shrinking [1,2], and waterways within farmland are becoming more critical as wetland habitat. In such areas, spraying of fields with pesticides and fertilizers has the potential to affect the associated wildlife community. Several investigators have suggested that frogs may be particularly sensitive to agricultural-source chemical inputs to aquatic systems [3–6].

North American temperate anurans (frogs and toads) occupy spatially and temporally diverse habitats, but they are linked by their universal dependence on temporary or permanent water bodies for breeding and early life stage development. In Ontario, one toad species and at least seven frog species may use wetlands in agricultural landscapes [2,7]. During breeding, these species display energetically expensive behavior that includes ovulation by females and vocal advertisement by males [8]. Both sexes of many spring breeders proceed with such reproductive activities directly after their winter dormancy, before a rigorous feeding phase. It follows that any additional stressors presented to individuals at this time could affect their chances both for survival and immediate or future reproductive success.

The research described in this and a companion paper [9] investigated the population integrity of northern leopard frogs (*Rana pipiens*) and green frogs (*Rana clamitans*) using wetlands in apple orchards in southern Ontario, Canada. The northern leopard frog and green frog were chosen because they seem to represent a range in sensitivity to environmental change that exists within the class Amphibia. Studies of historical trends in size of green frog populations show that they often seem unaffected by human activities in rural areas [10]; conversely, localized leopard frog population extinctions in western North America have been linked to natural chaotic events or human-induced environmental change [10,11]. Both chosen species are common in the rural apple-growing region of Hamilton–Wentworth County [2], where the study sites were

* To whom correspondence may be addressed (cab.bishop@ec.gc.ca)

located, and neither species spends much time in wooded areas away from agricultural activity [12].

The research intent was to identify any effects of apple orchard management practices on adult frogs reproducing in proximity to the orchards. This was achieved by comparing traits of frog populations using orchard wetlands to other populations using wetlands in conservation areas. Gross, genetic, physiological, and biochemical characteristics of breeding males sampled at sites in apple orchards were evaluated relative to the same characteristics measured in breeding males from reference sites. Organochlorine and organophosphorus concentrations in fat of males were compared among sites. The size and condition of young-of-the-year captured at orchard sites were also evaluated relative to the size and condition of individuals of the same year class captured at reference sites. Genetic characterization was completed, because reduced variation might constrain the long-term adaptive potential of a population [13], or it could show selection at contaminant-resistant alleles [14]. Body size was assessed, because it is highly plastic in many amphibian species and strongly affected by surrounding habitat [15]. Other characteristics of males and juveniles were assessed in an effort to uncover any pattern of response to toxicant stress, similar to those described for fish by Gibbons and Munkittrick [16]. Age was estimated to check for skewedness toward abnormally old or young breeding stock. Condition indices and levels of circulating steroid hormones were measured to assess immediate nutritional and reproductive status. The hepatic activity of the P4501A1 detoxification enzyme, 7-ethoxyresorufin-*O*-deethylase (EROD), was measured in males to test for a direct response to toxicant exposure. Measurement of chemical residue stores in fat provided an estimate of toxicant body burdens that could be compared with residues detected in water and sediments. By assessing these variables, a comprehensive estimation of population integrity was performed.

MATERIALS AND METHODS

Description of study sites

Four wetland study sites were selected within apple orchards in rural areas between Hamilton, Brantford, and Guelph, Ontario, Canada (Fig. 1; OCD1, OCD2, OCD3, OCD4). The 1993 reference sites were selected within neighboring conservation areas (Fig. 1; REF1, REF2). Two additional reference sites were used in 1994 (Fig. 1; REF3, REF4), because only one of the original reference sites supported a breeding population of northern leopard frogs. The additional reference sites, at the University of Guelph Arboretum Nature Reserve, and on private land southeast of Guelph, Ontario, were thought to be further removed from agricultural inputs and were known to support large amphibian communities.

Wetlands associated with study orchards are used as a source of water for pesticide dilution before application. Three of the four orchard sites were dug-out ponds with associated natural wetlands; the fourth (OCD2) was a small canal with minimal water movement. The canal was widened in two areas within the orchard, dug out in one, and was used as a water source for spray-tank filling. Any influences of orchard management on breeding anuran species inhabiting that canal were thought to be similar to those experienced in the ponds. Reference sites were similar in structure to three of the orchard wetland sites in that they were permanent ponds with associated marsh. The two original reference sites were within 7 km of three of the orchards. The additional sites were further removed (~25 km), but were subject to roughly the same local weather and climate profiles over the period of study. Further study site characterization may be found in Harris [17].

Genetic characterization of adult frog populations

Genetic variation in adult populations of northern leopard frogs and green frogs was estimated using allozyme variation, detected by horizontal starch gel electrophoresis. In 1993 and 1994, liver, spleen, heart, kidney, and muscle tissue were examined for the following enzyme systems and gene loci: aconitase hydratase (ACON-1), acid phosphatase (ACPH-2), creatine kinase (CK), aspartate aminotransferase (GOT-1, GOT-2), glycerol-3-phosphate dehydrogenase (αGPD-1, αGPD-2), isocitrate dehydrogenase (IDH-1, IDH-2), L-lactate dehydrogenase (LDH-1, LDH-2), malate dehydrogenase (MDH-1, MDH-2), malate dehydrogenase–nicotinamide adenine dinucleotide phosphate (oxidized form) ($NADP^+$) (ME-1, ME-2), mannose-6-phosphate isomerase (MPI), peptidase-A (PEP-A), phosphogluconate dehydrogenase (6PGD-1), glucose-6-phosphate isomerase (PGI), phosphoglucomutase (PGM-1, PGM-2, PGM-3), L-iditol dehydrogenase (SDH), and superoxide dismutase (SOD). In 1994, clipped toes were used, in addition to the aforementioned organs; toe extracts exhibited a distinct form of 6PGD, which was recorded as 6PGD-2. The specific tissue and buffer systems used to optimize resolution of each enzyme are similar to those outlined by Murphy et al. [18] and are described in detail in Harris [17].

Tissue samples were homogenized by hand with a glass rod in one or two drops of distilled water, spun for 2 min in a microcentrifuge, and supernatant-saturated filter paper wicks were loaded into 300-ml 12% starch gels (Connaught Laboratories, North York, ON, Canada). In a refrigerated room (4°C), current was supplied to ice-packed, J-cloth-wicked gels by Heathkit®-regulated high-voltage power supplies (Heath, Benton Harbor, MI, USA). Voltage varied according to the buffer system used: 250 volts for pH 6.5 amine citrate (morpholine) gels [19] and pH 8.0 Poulik gels [20]; and 150 volts for pH 6.7 and 8.0 tris citrate gels [20]. When a dye marker had migrated to the anodal edge of the gel, the gel was removed and horizontally sliced into four to five slices; each slice was histochemically stained [18] to reveal the allelic bands in the gel matrix.

When multiple loci were represented in one enzyme (e.g., MDH-1, MDH-2), the locus that migrated the farthest toward the anode was designated as locus 1, and subsequent loci were numbered in order of migration distance. Alleles at a given locus were also scored relative to migration rate. The allele that migrated the farthest toward the anode was designated as allele A for each species independently.

Gene frequencies and average heterozygosities (H) were calculated for each species at each site using the program GENEPAC developed by J.P. Bogart. Unbiased jackknife estimators [21] of Wright's F statistics (inbreeding coefficients) and their standard deviations were calculated using G-STAT 3.0 [22].

Age and size structure of frog populations

In an effort to eliminate sampling bias, breeding males were selected as the adult subgroup sampled for age, size, condition, and biochemical analyses. Adult female frogs are more cryptic than males; they neither call nor establish territories in more accessible shallow water as do males. Individuals were marked using the relatively noninvasive [23,24] and widely applied

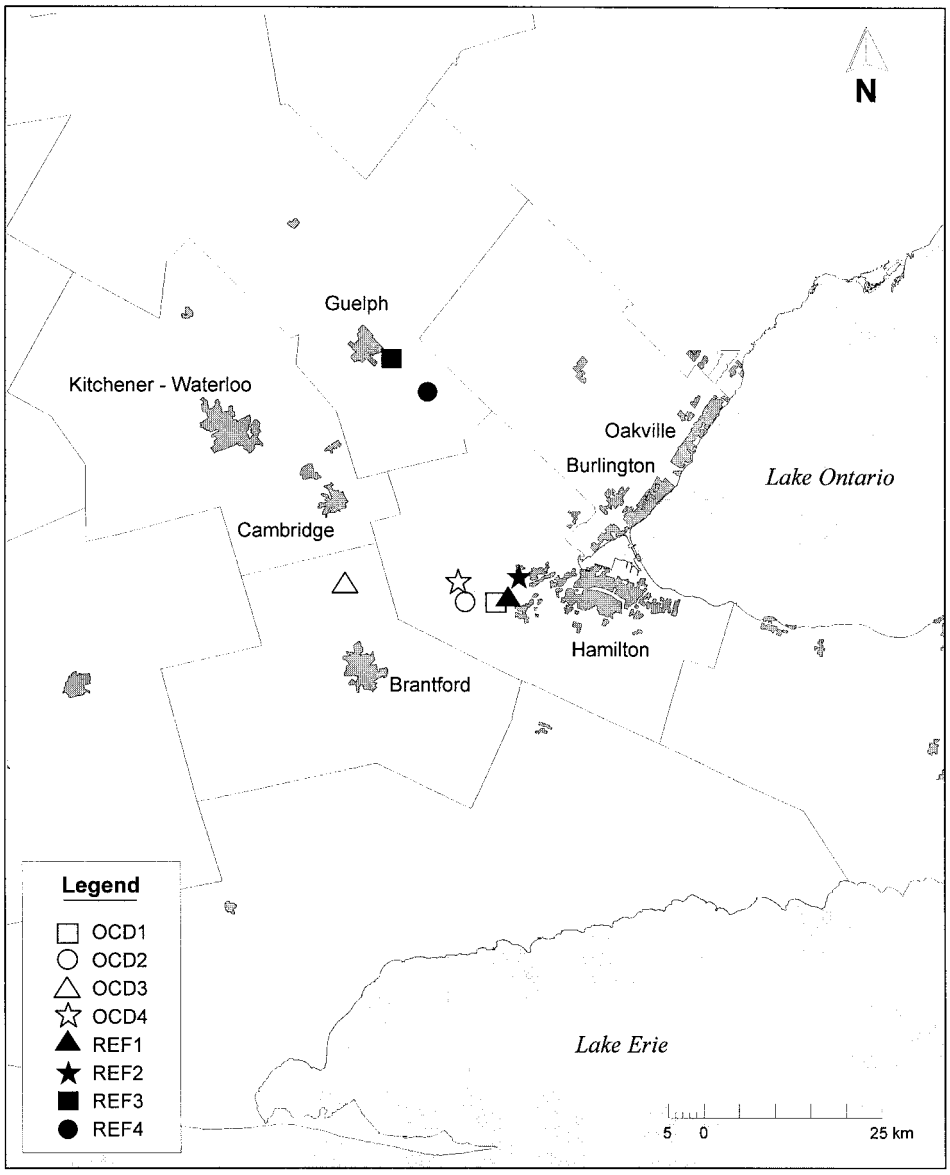

Fig. 1. The location of orchard (OCD1–4) and reference (REF1–4) study sites in Ontario, Canada.

[25–27] technique of toe-clipping. An alphanumeric coding system [28] was used to provide a unique mark for each individual at a given site. Most frog and toad species rarely move more than 1 km from their natal site [29]. Because the wetland sites used were all separated by at least 1 km, it was assumed that individuals were not traveling from one site to another. The sex of captured individuals was determined using external characteristics such as the presence of vocal sacs and swollen thumb pads in male leopard frogs, and the presence of yellow pigment on the throats of male green frogs. They were measured for snout–vent length (SVL) with a ruler, and weighed with a 30-, 50-, or 500-g Pesola spring scale (Avinet, Dryden, NY, USA).

Age of males was estimated using modifications of the skeletochronological technique of Leclair and Castanet [30]. The first two phalanges of a digit were clipped and immediately fixed in 10% buffered formalin. Fixed toes were skinned, decalcified for 4 to 7 h in 3% nitric acid, then rinsed overnight in cold, running tap water. The largest complete phalange was removed from the rest of the digit and processed for frozen sectioning [31]. The phalange was embedded in a frozen matrix of 1:4 OCT compound:distilled water. Sections were cut at 15 to 20 μm on a Perkin-Elmer Cryocut cryostat (Perkin-Elmer, Norwalk, CT, USA) maintained at −22°C. Sections were transferred to a distilled water bath, then stained with Ehrlich's hematoxylin. Cross sections from the mid-diaphyseal region of the phalange were identified under a dissecting microscope, transferred to slides, and mounted in glycerin.

The number of lines of arrested growth (LAGs) in the bone cross section were counted under a Zeiss inverted microscope (Carl Zeiss Canada, Don Mills, ON, Canada; magnification 25×). In temperate climates, a line is created in dormant amphibians every winter [30]. Because all individuals were collected in the spring, it was assumed that the LAG deposited the previous winter would not be visible at the edge of the bone. Thus, the edge was counted as the last LAG. Because different toes were used for each individual, it was impossible to determine the extent of endosteal resorption by measuring bone diameter [30]. However, Leclair [32] showed that leopard and green frog populations in Quebec exhibited little to no

endosteal resorption, and it was assumed that no LAG were completely resorbed in individuals captured at the orchard and reference sites. Individuals with a partial LAG on the edge closest to the marrow had that LAG counted as a first-year, partially resorbed LAG.

On one occasion between July 18 and 27, 1994, emerging (newly metamorphosed) green frogs were surveyed to estimate the body size of young-of-the-year. Three researchers spent 1.5 h at each pond site catching as many individuals as possible. The same three people sampled every site to minimize sampler bias. At the end of the 1.5 h, all young-of-the-year were measured for SVL with a ruler, and weighed with a 10- or 30-g Pesola spring scale before being released. By mid-July, transforming leopard frogs had mostly dispersed, and therefore were not surveyed.

Measures of condition and biochemical indicators of stress and contaminant exposure

Tissues from male northern leopard frogs were collected for condition and biochemical analyses from April 24 to May 6, 1993, and April 18 to May 12, 1994. Tissues from male green frogs were collected from May 9 to June 25, 1993, and June 5 to July 10, 1994. The persistence of vocal advertisement was used as an indicator of breeding activity for each species.

Four condition indices were used to measure the physiological state of the males: total body mass adjusted for SVL as well as liver, gonad, and fat-body masses corrected for total body mass. Because Jørgensen [15] noted that fat-body size in amphibians is often reduced before and during breeding, assessment of the combined gonad–fat-body adjusted mass was also included (the sum of gonad and fat-body masses corrected for total body mass).

Hepatic mixed function oxygenase (MFO) enzyme induction was estimated on postmitochondrial supernatant by measuring catalytic activity against 7-ethoxyresorufin (7-ER). This induction, herein referred to as EROD activity, was determined in liver extracts from males using a modification of the procedure developed by Pohl and Fouts [33]. Animals were double-pithed and the liver, free of the gall bladder, was removed, weighed, and frozen in liquid nitrogen. Liver extracts were prepared according to Förlin and Andersson [34]. The EROD reaction mixture contained 0.1 M HEPES buffer pH 7.8 (Sigma, St. Louis, MO, USA), 5.3 mM Mg^{2+}, 0.5 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Boehringer Mannheim, Mannheim, Germany), 1.5 μM 7-ER (Sigma), and about 0.5 mg/ml of protein. The reaction was initiated by adding the 7-ER, then stopped after 10 min by adding 2.0 ml of ice-cold methanol. In 1993, fluorescence was determined on a Turner model 430 spectrofluorometer (Turner Designs, Sunnyvale, CA, USA) with excitation wavelength set at 550 nm and emission wavelength set at 585 nm. In 1994, fluorescence was determined on a Millipore Cytofluor 2350 plate-reading fluorometer (Milli-Q, Bedford, MA, USA) with a 530-nm excitation filter and a 590-nm emission filter. Protein was measured using the method of Bradford [35], with bovine serum albumin (Sigma) as the standard. The EROD specific activity was calculated as pmoles resorufin/mg protein/min.

Levels of circulating steroid hormones were measured in plasma of adult breeding males by radioimmunoassay [36]. Zerani et al. [37] determined that the stress of capture and holding significantly altered circulating levels of testosterone and estradiol in male European green frogs (*Rana esculenta*) after 6 h postcapture; therefore, blood was not collected if animals could not be sampled within 6 h. Collected blood was spun in hematocrit tubes in a Damon IEC MB microhaematocrit centrifuge (International Equipment, Needham Heights, MA, USA), and the serum portion (nonheparinized) was separated and stored at −80°C until further analysis. Plasma 17β-estradiol and testosterone were the specific steroids measured. Antiserum for testosterone was obtained from ICN Biomedicals (St. Laurent, PQ, Canada), whereas that for 17β-estradiol was obtained from Z. Yarron. Radiolabeled steroids were purchased from Amersham Canada (Oakville, ON, Canada), and unlabeled steroids were purchased from Sigma. Radioactivity was measured on a Micromedic Systems Taurus automatic liquid scintillation spectrophotometer (Titertek Instruments, Huntsville, AB, Canada).

Statistical evaluation of biological data

All analyses of size, age, physiological, and biochemical data were performed using SYSTAT® version 5.0 [38]. Size of adult males was compared among study sites by analyses of covariance (ANCOVAs) with age used as the covariate. In both years, the age–size relationship in green frogs was not statistically significant; therefore, age was dropped as a covariate, and one-way analyses of variance (ANOVAs) were performed separately on length and mass data. Snout–vent length and body mass data were log-transformed to meet assumptions of normality. Size of young-of-the-year, and average age, log-transformed hepatic EROD activity, and log-transformed plasma steroid concentrations of males were also compared among sites by one-way ANOVAs. Where significant differences were detected ($\alpha = 0.05$), an unplanned multiple comparison was conducted using the Tukey honestly significant difference (HSD) method (with a Tukey–Kramer adjustment when sample sizes were unequal). Average condition indices were compared among sites using ANCOVAs on log-transformed values. Analyses of covariance were performed both on complete data sets and on data sets with selected sites (represented by five or fewer data points) dropped. Because the final outcomes (detected significance) of the duplicate ANCOVAs were the same, values for all sites are listed.

Pesticide residue analysis of pond water, sediments, and frog tissues

Water samples were taken every 2 weeks in 1994 for pesticide residue analysis. Eight sample sets were collected between May 2 and August 8. Two or three 1-L grab samples of pond water from each study site were transported on ice and kept cold for 2 to 12 h before analysis. If it was necessary to hold the samples overnight (12 h), they were stored in the dark at 4°C. Samples were analyzed for 11 classes of pesticides. If a pesticide was detected with the general class scans, further analysis was completed to confirm and quantify the specific compound.

Water samples designated for organophosphorus, propargite, organonitrogen (triazoles), organochlorine, pyrethroid, and phthalimide analyses were extracted twice with 50 ml dichloromethane in the presence of 50 ml saturated aqueous sodium chloride. Extracts were dried with anhydrous sodium sulfate, evaporated, and redissolved in hexane to give a 500-fold concentration factor. A portion of the sample was further cleaned up for organochlorine analysis [39]. Both extracts were then evaluated by gas chromatography with electron capture and flame photometric detection [40–42]. Detectable residues of captan and the pyrethroids, permethrin and deltamethrin,

were also verified in extracts subjected to extraction, clean up, and fractionation for organochlorine analysis.

Samples for ethylenebisdithiocarbamate (EBDC) fungicide analysis were evaluated using a modified CS_2 evolution technique [43]. Results were expressed as concentrations of zineb ($C_4H_6ZnN_2S_4$, a type of EBDC) equivalents. Zineb or related residues in a 200-ml water sample were decomposed with hot mineral acid (100 ml 4 N hydrochloric acid) and 5 ml 40% (w/v) stannous chloride. During 35 min of reflux (decomposition time), evolved gases were drawn through two adsorption traps containing 10 ml 6.5% (w/w) sodium hydroxide and 5 ml benzene, and 12.5 ml Cullen's color reagent, respectively. Digested samples were allowed to sit for 15 min before absorbance was read with a Milton Roy Spectronic 601 UV-Visible spectrophotometer (Fisher Scientific, Toronto, ON, Canada) at a wavelength of 435 nm. Absorbance was compared with mancozeb standard absorbance, and concentrations of EBDCs were calculated from a standard curve.

Triazine and acetanilide herbicides were extracted from 1-L water samples (adjusted to pH 9.5) twice with 50 ml chloroform. Extracts were dried with anhydrous sodium sulfate, evaporated to dryness, and redissolved in 3.0 ml methanol. Residues were measured by packed column gas chromatography with an electrolytic conductivity detector (nitrogen-mode) [44,45]. *N*-Methylcarbamate insecticides and thiocarbamate herbicides were measured in acidified extractions using capillary column gas chromatography with nitrogen–phosphorous detection [46]. The extraction procedure was the same as that detailed for organophosphorus and organochlorine pesticides.

Sediment samples were collected from study ponds with a mini-Ponar sediment sampler (MSE Engineering, Downsview, ON, Canada), once in the spring of 1994, and again in the fall of 1994. Samples were frozen at −20°C until analysis. Two 20-g samples of wet sediment were ultrasonically extracted in preparation for organophosphorus and organochlorine analysis [47]. The sample intended for organophosphorus analysis was extracted using a 1:4 acetone:hexane mixture; the other sample was extracted using a 1:1 (v:v) acetone:hexane solution. Extracts were partitioned with water, back-extracted with dichloromethane, concentrated, cleaned up, and fractionated, either on a 3% (w/w) (for chlorinated compounds) or on a 10% (w/w) (for organophosphorus compounds) deactivated silica gel column. Organophosphorus and organochlorine pesticide residues were detected on a dual column capillary HP5890 gas liquid chromatograph (Hewlett Packard, Mississauga, ON, Canada) with electron capture and nitrogen–phosphorus detection. Polychlorinated biphenyls (PCBs) were identified using a packed column HP-5700 gas liquid chromatograph with electron capture detection. Extracts were assayed for 13 organophosphorus compounds (naled, phorate, dimethoate, terbufos, fonofos, diazinon, disulfoton, malathion, chlorpyrifos, parathion, ethion, phosmet, and azinphos-methyl), 17 organochlorine pesticides (α-hexachlorocyclohexane [BHC], lindane, heptachlor, aldrin, heptachlor epoxide, α- and γ-chlordane, α- and β-endosulfan, dieldrin, endrin, *o,p′*-dichlorodiphenyl trichloroethane [DDT], *p,p′*-dichlorodiphenyl dichloroethylene [DDE], *p,p′*-dichlorodiphenyl dichloroethane [DDD], *p,p′*-DDT, mirex, methoxychlor), and total PCBs.

In 1993 and 1994, frog fat bodies were collected from breeding males. Leopard frog fat bodies were too small to provide sufficient tissue for residue analysis. Green frog fat bodies were pooled and residues extracted in an acetonitrile:water solution (2:1, v:v) according to Braun and Lobb [48]. A portion of the extract was cleaned up for organochlorine analysis using column chromatography [39]; the remainder was concentrated and analyzed for organophosphorus pesticides. Organochlorines were assayed with an HP5890 gas chromatograph with electron capture detection and either a DB-5 (pesticides) or an HP-5 (PCBs) fused silica column. The PCBs were identified and quantified by comparison with Arochlor 1254 standards. Organophosphorus pesticides were assayed using a HP5890 gas chromatograph with flame photometric detection (phosphorus filter, 525 nm), and a DB-608 fused silica column (Chromatographic Specialties, Brockville, ON, Canada).

RESULTS

Genetic characterization of adult frog populations

Twenty-four loci, representing 16 enzymes, were successfully scored in green frog tissues (Table 1). Nineteen of these 24 loci exhibited polymorphism in at least one of the populations sampled. Average heterozygosity (*H*) values ranged between 0.219 and 0.282. Using the qualitative guidelines suggested by Wright [49], most of the fixation index (F_{ST}) values ranged between 0 and 0.05, indicating little genetic differentiation. Moderate differentiation was expressed at the ACPH-2 (0.060), ME-1 (0.135), and SDH (0.055) loci, whereas very great differentiation was expressed at the 6PGD-2 (0.450) locus. The F_{ST} value for all loci combined was 0.012, indicating little genetic differentiation between the subpopulations sampled.

Northern leopard frogs had 23 scorable loci, representing 15 enzyme systems (Table 1). Eighteen loci were polymorphic in at least one of the populations sampled. Average heterozygosities (*H*) ranged between 0.191 and 0.273. Moderate genetic differentiation [49] was exhibited at the ACON-1 (0.055), CK (0.124), GOT-1 (0.131), MPI (0.066), αGPD-1 (0.07), and 6PGD-2 (0.124) loci, whereas great differentiation was exhibited at the 6PGD-1 (0.251) and PGM-3 (0.218) loci. The overall F_{ST} for all loci was 0.059, suggesting moderate differentiation of the subpopulations sampled.

Age and size of breeding male frogs

During 1993 and 1994, 130 adult green frogs and 83 adult leopard frogs were sampled for size (Figs. 2 and 3) and age (Table 2) analysis. No significant differences in average body mass and SVL of male green frogs within a pond site between years were detected at OCD1, OCD2, OCD4, or REF1. However, significant differences were found between years of capture in both average mass ($p = 0.023$) and in average SVL ($p = 0.036$) of individuals captured at REF2. No males and one female marked in the summer of 1993 were recaptured in 1994. In 1993, sampling for northern leopard frogs was poor, partly because of a short (~1-week) breeding season. Because all sites could not be sampled adequately within that period, the 1993 leopard frog data (age, size, condition, biochemistry) were dropped from the analyses.

Among pond sites, male green frogs exhibited significant differences in average SVL, and average body mass, but not in average age. Significant differences in average mass were detected in both 1993 and 1994, whereas significant differences in average SVL were detected only in 1994 (Fig. 2). The average size of males from reference sites tended to be greater than that of individuals from orchard sites, but most

Table 1. Number of alleles expressed at each scorable locus and overall average heterozygosity for northern leopard frogs and green frogs at each study site. Sample sizes are shown in brackets after site codes[a]

	No. of alleles—green frogs						No. of alleles—leopard frogs				
Locus[b]	OCD1 (n = 14)	OCD2 (n = 15)	OCD3 (n = 5)	OCD4 (n = 15)	REF1 (n = 31)	REF2 (n = 20)	OCD2 (n = 9)	OCD3 (n = 20)	OCD4 (n = 4)	REF3 (n = 11)	REF4 (n = 26)
ACON-1	3	3	2	2	3	3	2	2	2	2	2
ACPH-2	3	3	1	3	3	3	—	—	—	—	—
CK	2	2	1	2	2	2	2	2	2	2	2
GOT-1	3	3	3	4	4	3	3	3	1	3	3
GOT-2	1	1	1	1	1	1	1	1	1	1	1
αGPD-1	1	1	1	1	1	1	2	2	2	2	2
αGPD-2	1	2	1	2	2	2	2	2	2	2	2
IDH-1	1	1	1	1	1	1	1	1	1	1	1
IDH-2	2	1	2	2	3	3	1	2	1	2	2
LDH-1	2	2	2	2	2	2	2	1	2	1	3
LDH-2	2	1	2	2	2	1	1	1	1	1	1
MDH-1	2	2	2	2	2	2	3	2	2	3	2
MDH-2	1	1	1	1	1	1	1	1	1	1	1
ME-1	1	1	2	1	1	1	1	1	1	2	2
ME-2	2	2	2	2	2	2	—	—	—	—	—
MPI	4	3	4	4	3	4	5	5	4	5	5
PEP-A	1	1	1	1	1	1	1	1	1	1	1
6PGD-1	3	1	2	2	1	2	3	2	2	3	2
6PGD-2	1	1	2	2	1	1	2	3	1	2	2
PGI	2	2	2	2	2	2	2	2	2	2	2
PGM-1	2	2	2	2	2	2	3	3	1	3	3
PGM-2	2	2	1	3	2	3	1	2	2	2	3
PGM-3	—	—	—	—	—	—	3	2	1	1	1
SDH	3	3	2	3	3	3	2	2	3	3	3
SOD	2	2	2	2	2	2	2	2	2	2	3
Average heterozygosity	0.281	0.219	0.282	0.261	0.237	0.248	0.273	0.191	0.236	0.251	0.212

[a] OCD1–4 = orchard study sites; REF1–4 = reference study sites.
[b] Loci are spelled out in the Materials and Methods.

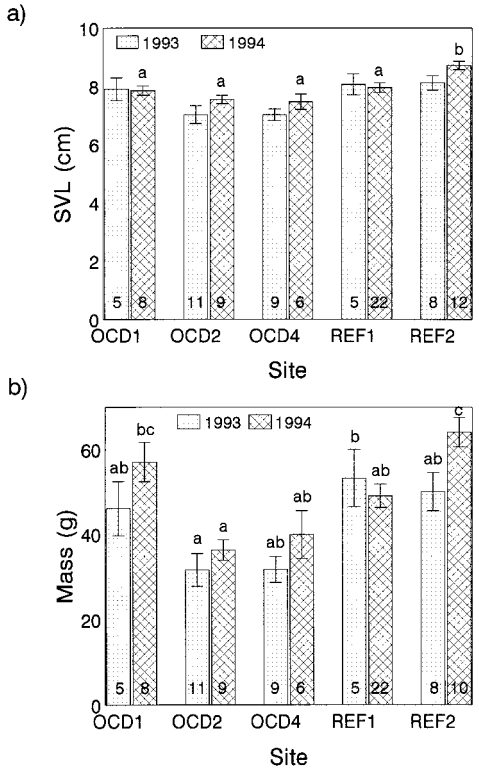

Fig. 2. Size of green frog males captured at study sites in 1993 and 1994. (**a**) Snout–vent length (SVL, cm). (**b**) Total body mass (mass, g). Values at the base of each bar are sample sizes. Sites with at least one similar letter are not significantly different from each other at an α level of 0.05 (years considered independently). Error bars represent standard error.

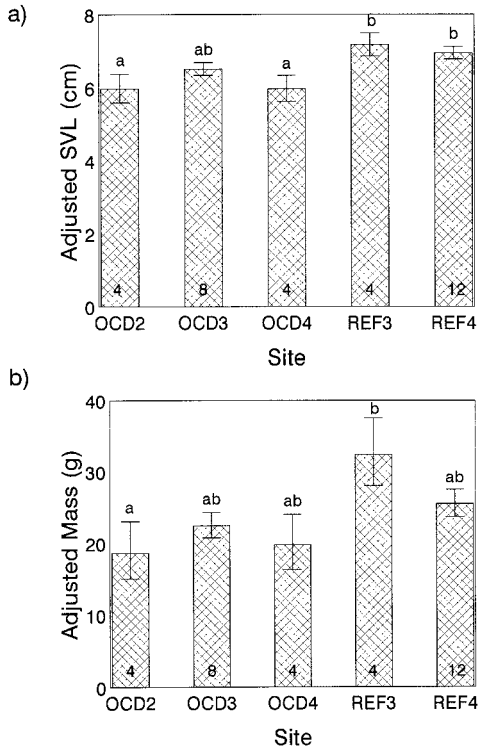

Fig. 3. Size of northern leopard frog males captured at study sites in 1994. (**a**) Snout–vent length (SVL, cm) adjusted for age. (**b**) Total body mass (mass, g) adjusted for age. Values at the base of each bar are sample sizes. Sites with at least one similar letter are not significantly different from each other at an α level of 0.05. Error bars represent standard error.

Table 2. A description of the age structure of male green frogs and northern leopard frogs captured at orchard (OCD) and reference (REF) sites. Mean age of leopard frog males at reference site 4 was significantly greater than mean age of males from other sites[a]

	Age of males in 1993				Age of males in 1994			
Site	*N*	Average (±SE)	Youngest	Oldest	*N*	Average (±SE)	Youngest	Oldest
Green frogs								
OCD1	3	2.7 (0.7)	2	4	5	3.6 (0.4)	3	5
OCD2	9	2.7 (0.4)	1	5	8	3.1 (0.2)	2	4
OCD3	1	5.0	5	5	0	NA	NA	NA
OCD4	8	2.6 (0.3)	2	4	5	3.2 (0.2)	3	4
REF1	4	3.3 (0.6)	2	5	23	2.9 (0.2)	2	6
REF2	8	3.1 (0.4)	2	5	12	3.0 (0.3)	2	5
Leopard frogs								
OCD2	—	—	—	—	4	2.3 (0.3)**	2	3
OCD3	—	—	—	—	8	2.6 (0.3)**	2	4
OCD4	—	—	—	—	4	2.5 (0.5)*	2	4
REF3	—	—	—	—	6	2.7 (0.3)*	2	4
REF4	—	—	—	—	19	4.0 (0.2)	2	6

[a] *N* = sample size; SE = standard error; NA = not applicable; * = $p < 0.05$; ** = $p < 0.01$.

of the size differences were not significant. In 1993, individuals from REF1 had significantly greater mass than individuals from OCD2 ($p = 0.050$), but did not differ significantly from individuals at any other site. In 1994, individuals from REF2 had significantly greater mass than individuals from OCD2 ($p < 0.001$), OCD4 ($p = 0.003$), and REF1 ($p = 0.016$), but did not differ significantly from individuals at OCD1. Individuals from REF2 exhibited significantly greater SVLs than individuals from all other sites: OCD1, $p = 0.026$; OCD2, $p = 0.001$; OCD4, $p = 0.002$; and REF1, $p = 0.009$. In 1993, only two breeding green frogs were collected from OCD3, and no evidence of green frog breeding activity was found at that site in 1994. Therefore, OCD3 was not included in green frog size, age, and condition analyses.

The average age of male green frogs did not differ significantly among breeding pond sites. However, differences were found in the age ranges represented at each site by breeding males (Table 2). In 1993, 5-year-old males were not found at OCD1 and OCD4, and 6 year olds were not found at any pond sites. In 1994, 5- or 6-year-old males were not found at OCD2 and OCD4, and 6 year olds were also not found at OCD1 and REF2. The age at maturity for males seemed to be 2 years posttransformation, with the exception of one 1-year-old captured while calling at OCD2 in 1993. In 1994, no 2-year-old calling males could be found at OCD1 and OCD4.

Breeding male leopard frog age had a highly significant effect on both SVL ($p = 0.001$) and body mass ($p = 0.002$). However, analyses of covariance still showed significant differences in average SVL ($p = 0.003$) and average mass ($p = 0.026$) of males among study sites (Fig. 3). Males from OCD2 were significantly smaller (in length and mass) than equivalent-age males from REF3 ($p = 0.02$), and males from OCD4 were significantly shorter than males at both reference sites (REF3, $p = 0.019$; REF4, $p = 0.04$). Individuals from OCD3 were intermediate in size, and not significantly different from individuals at any other site.

Average age of male leopard frogs from REF4 was significantly greater than average age of males from all orchard sites and from REF3 (Table 2). The age at maturity for male leopard frogs appeared to be 2 years at all breeding ponds. Site REF4 was the only pond in which 5- and 6-year-old frogs were found.

Reproductive and physiological condition of breeding male frogs

Male green frogs marked or collected from study sites in 1993 exhibited no significant differences in adjusted mean body mass among sites. In 1994, males from OCD1 were significantly heavier (when standardized for length) than males from OCD2 ($p = 0.001$) and REF1 ($p = 0.047$; Fig. 4). Males from OCD4 and REF2 were not significantly different in overall body condition from individuals from any other sites. Male leopard frogs sampled in 1994 exhibited no significant differences in adjusted mean body mass among sites.

No differences were demonstrated in organ condition indices of male green frogs from orchard sites versus reference sites (Table 3). Analyses of covariance detected no significant differences in average adjusted gonad mass, fat-body mass, or liver mass among green frogs from different pond sites in either year. A combined gonad–fat-body index also produced no significant differences among orchard and reference individuals.

Although some significant differences were detected in organ condition indices of male leopard frogs collected in 1994, there was still no obvious distinction between individuals from orchard and reference sites (Table 3). Analyses of covariance detected no significant difference in average adjusted gonad mass, but average adjusted liver mass of males was signifi-

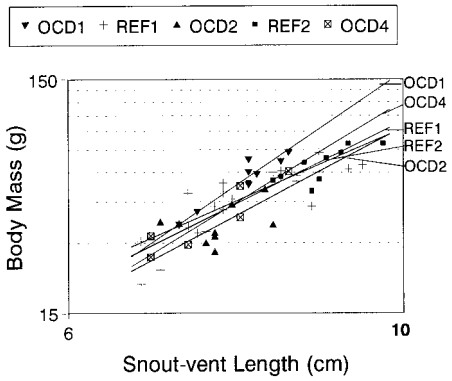

Fig. 4. Weight versus snout–vent length (condition index) of green frog males captured at study sites in 1994. Both axes are logarithmic. Slope and intercept values for lines were calculated using model II regressions.

Table 3. Adjusted mean gonad, fat-body, and liver masses of male green and leopard frogs sampled at orchard (OCD) and reference (REF) sites. Upper and lower 95% confidence limits are included after each mean[a]

	1993 Samples										1994 Samples									
		Gonad			Fat body			Liver				Gonad			Fat body			Liver		
Site	N	$\bar{x}$	Upper CL	Lower CL	$\bar{x}$	Upper CL	Lower CL	$\bar{x}$	Upper CL	Lower CL	N	$\bar{x}$	Upper CL	Lower CL	$\bar{x}$	Upper CL	Lower CL	$\bar{x}$	Upper CL	Lower CL
Green frogs																				
OCD1	5	0.18	0.24	0.13	0.03	0.09	0.01	0.56	0.69	0.45	5	0.22	0.29	0.16	0.05	0.17	0.02	1.05	1.38	0.80
OCD2	5	0.16	0.27	0.09	0.06	0.27	0.02	0.92	1.96	0.43	5	0.23	0.31	0.16	0.02	0.07	0.003	0.90	1.21	0.67
OCD4	9	0.17	0.21	0.14	0.07	0.16	0.03	0.98	1.26	0.76	5	0.22	0.35	0.14	0.11	0.30	0.04	1.13	1.51	0.84
REF1	3	0.14	0.21	0.09	0.10	0.31	0.03	0.92	1.63	0.52	5	0.20	0.24	0.16	0.10	0.42	0.02	1.07	1.58	0.72
REF2	3	0.15	0.23	0.10	0.10	0.63	0.02	0.87	1.76	0.43	5	0.19	0.22	0.16	0.04	0.15	0.01	1.05	1.26	0.87
Leopard frogs																				
OCD2	—	—			—			—			4	0.03	0.05	0.02	0.04	0.11	0.01	0.65	0.97	0.44
OCD3	—	—			—			—			4	0.03	0.04	0.02	0.004	0.006	0.003	0.34	0.46	0.25
OCD4	—	—			—			—			4	0.04	0.06	0.02	0.03	0.04	0.02	0.51	0.88	0.29
REF3	—	—			—			—			4	0.03	0.04	0.02	0.01	0.02	0.004	0.36	0.61	0.22
REF4	—	—			—			—			6	0.03	0.04	0.02	0.01	0.04	0.003	0.31	0.41	0.24

[a] N = sample size; $\bar{x}$ = mean; CL = confidence limit.

Fig. 5. Size of young-of-the-year green frogs captured at study sites in 1994. (**a**) Snout–vent length (SVL, cm). (**b**) Total body mass (mass, g). Values at the base of each bar are sample sizes. Sites with the same letter are not significantly different from each other at an α level of 0.05. Error bars represent standard error.

cantly different among sites. Leopard frogs from OCD2 had significantly smaller livers than individuals collected from OCD3 ($p = 0.016$) and REF4 ($p = 0.013$). Fat-body size could not be evaluated, because there was a significant interaction between study site and the covariate, body mass ($p = 0.013$). A combined gonad–fat-body index produced no significant differences between sampled populations.

Size and physiological condition of young-of-the-year green frogs

A 1994 sample of 129 young-of-the-year green frogs revealed significant differences in average body mass and SVL (Fig. 5), as well as condition among study sites. However, no obvious distinctions were found between individuals from orchard sites versus reference sites. Young from OCD3 had significantly greater body mass than young from OCD2 ($p = 0.006$) and OCD4 ($p = 0.040$), and greater SVL than young from OCD2 ($p < 0.001$), OCD4 ($p < 0.001$), REF1 ($p = 0.028$), and REF2 ($p = 0.040$). Young from OCD4 were significantly smaller than individuals from all other sites. The adjusted mean body mass of individuals from OCD2 was significantly greater than that of counterparts from OCD4 ($p = 0.006$), but not different than that of young from other sites. Individuals still in the process of transforming were captured at all sites, indicating that no one particular pond site was distinct from other sites in timing of transformation events. Those still in the process of transformation were not included in the ANOVA. They comprised the following percentages at each site: OCD1, 17%; OCD2, 20%; OCD3, 17%; OCD4, 25%; REF1, 51%; and REF2, 11%.

Table 4. Average concentrations (±standard error [SE]), expressed in ng/ml, of estradiol (E) and testosterone (T) in plasma of breeding male green and leopard frogs[a]

	Green frogs			Leopard frogs		
Site	*n*	E (±SE)	T (±SE)	*n*	E (±SE)	T (±SE)
OCD1	5	0.443 (0.146)	15.79 (2.00)	NS	NS	NS
OCD2	4	0.621 (0.163)	15.36 (1.71)	4	0.532 (0.318)	11.25 (3.60)
OCD3	NS	NS	NS	3	0.188 (0.162)	4.25 (0.81)
OCD4	4	0.653 (0.298)	15.43 (1.55)	NS	NS	NS
REF1	5	0.408 (0.120)	19.83 (4.09)	NS	NS	NS
REF2	5	0.308 (0.076)	13.36 (2.05)	NS	NS	NS
REF3	NS	NS	NS	4	0.359 (0.161)	7.36 (5.35)
REF4	NS	NS	NS	6	0.357 (0.113)	4.63 (1.07)

[a] *n* = sample size; NS = none sampled; OCD1–4 = orchard study sites; REF1–4 = reference study sites.

Biochemical indicators of reproductive stress and exposure to P4501AI-inducers

In 1994, individuals removed from breeding populations for tissue analysis were also sampled for circulating plasma levels of estradiol and testosterone. Analyses of variance revealed no significant differences in average (log-transformed) concentrations of estradiol or testosterone in the plasma of green frogs or northern leopard frogs from orchard and reference sites (Table 4).

7-Ethoxyresorufin-*O*-deethylase activity showed few significant differences among sites. Green frogs sampled in 1993 at OCD4 showed significant EROD induction ($p = 0.009$) relative to individuals sampled from OCD1. Because no significant difference in EROD activity was found in individuals from both reference sites, reference samples were pooled, and used as control samples for a Dunnett's comparison; a significant ($p = 0.038$) EROD induction in green frogs captured at OCD4 ($\bar{x} = 36.1$ pmoles/mg protein/min), relative to the pooled sample of reference green frogs, was apparent (Fig. 6). No significant differences were found in EROD values in either northern leopard frogs ($p = 0.599$) or green frogs ($p = 0.212$) collected from study sites in 1994.

Pesticide residue analysis of pond water, sediments, and frog tissues

Differences were found among study sites in the number and magnitude of pesticide residues detected in pond water in 1994 (Table 5). Several chemicals, both those sprayed in orchards and those not normally used, were consistently detected at OCD1 and OCD2. Water samples from OCD3, OCD4, REF1, and REF2 were relatively clean, although atrazine or orchard-use pesticides were occasionally detected. Pyrethroids, EBDCs, thiocarbamates, captan, dinocap, and propargite were not detected in any of the water samples.

Water samples collected from July 26 to August 3, 1994, at OCD1 followed a known spray event in that orchard. A mixture of the insecticides Guthion® 50WP (50% azinphos-methyl; UniRoyal, Elmira, ON, Canada), Omite® (propargite; Bayer/Mobay, Etobicoke, ON, Canada), and Dikar® (dinocap and mancozeb; Rohm and Haas Canada, West Hill, ON, Canada) was sprayed throughout the orchard on July 26. Results indicated that azinphos-methyl, probably from that event, persisted in pond water for several days. All other water samples were taken without prior knowledge of spray schedules.

Although a few pesticides were detected in water from reference ponds, no organophosphorus or organochlorine compounds (including PCBs) were detected in reference pond sediments. Further, no organophosphorus pesticides or PCBs were detected in orchard pond sediments. Sediments from OCD1 and OCD2 contained endosulfan, whereas sediment from all four orchard ponds contained breakdown products of DDT (Table 6). Organochlorine concentrations in spring and fall sediment samples were essentially the same. Concentrations of DDE and DDD were highest in sediments from OCD1 and OCD3, ranging between 24.4 and 71.4 ng/g. Dichlorodiphenyldichloroethylene was present at OCD2 and OCD4 during one sampling event each at concentrations near the detection limit of 5.6 ng/g. β-Endosulfan was only detectable at OCD1 (39.9–64.5 ng/g).

Dichlorodiphenyltrichloroethane, its breakdown products, and endosulfan were also the only organochlorines detected

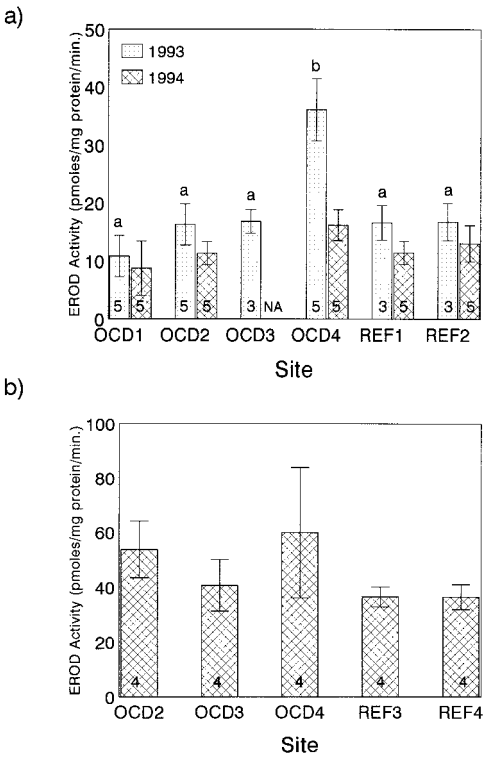

Fig. 6. Hepatic 7-ethoxyresorufin-*O*-deethylase (EROD) activity (in pmoles/mg protein/min) detected in (**a**) male green frogs, and (**b**) male northern leopard frogs in 1993 and 1994. Values at the base of each bar are sample sizes. Sites with the same letter are not significantly different from a pooled sample of reference individuals (REF1 + REF2) at an α level of 0.05 (years considered independently). Error bars represent standard error. NA = not applicable; none sampled.

Table 5. Pesticide residues (in μg/L) detected in water samples collected at orchard (OCD) and reference (REF) pond sites in 1994. Compounds are categorized as sprayed in orchards (S) or not sprayed in orchards (NS)

		Site						
Residue (detection limit)	Sample date	OCD1	OCD2	OCD3	OCD4	REF1	REF2	REF3
Atrazine[NS] (0.04 μg/L)	May 16	ND[a]	ND	ND	ND	0.37	ND	ND
	May 30	0.07	0.37	0.08	0.07	ND	ND	—
	June 13	0.051	0.063	0.063	0.055	0.2	0.055	ND
	June 27	ND	15.0	0.094	0.072	0.053	0.039	ND
	July 11	0.081	10.0	0.13	0.11	0.078	0.055	—
	July 25	ND	6.7	ND	ND	ND	ND	—
	August 8	ND	5.9	0.4	0.11	0.073	0.59	—
Azinphos-methyl[S] (0.01 μg/L)	June 27	1.0	ND	ND	ND	ND	ND	
	July 27	0.059	—	—	—	—	—	—
	July 28	0.06	—	—	—	—	—	—
	July 29	0.066	—	—	—	—	—	—
	July 31	0.21	—	—	—	—	—	—
	August 3	0.09	—	—	—	—	—	—
	August 8	0.097	0.3	ND	ND	ND	ND	—
Diazinon[S] (0.01 μg/L)	June 13	0.78	ND	ND	ND	ND	ND	—
	June 27	0.42	ND	ND	ND	ND	ND	ND
	July 11	0.22	ND	ND	0.18	ND	ND	—
	July 25	ND	ND	ND	0.09	ND	ND	—
	July 27	0.04	—	—	—	—	—	—
	July 28	0.03	—	—	—	—	—	—
	July 29	0.04	—	—	—	—	—	—
	July 31	0.04	—	—	—	—	—	—
	August 3	0.03	—	—	—	—	—	—
	August 8	0.057	ND	ND	0.068	ND	ND	—
Endosulfan[S] (0.01 μg/L)	July 25	0.53	ND	ND	ND	0.051	ND	—
	August 8	0.163	ND	ND	ND	ND	ND	—
Metolachlor[NS] (0.04 μg/L)	May 30	ND	0.29	ND	ND	ND	ND	—
	June 27	ND	8.8	ND	ND	ND	ND	ND
	July 11	ND	5.1	ND	ND	ND	ND	—
	August 8	ND	1.5	ND	ND	ND	ND	—
Pirimicarb[NS] (0.04 μg/L)	August 8	0.48	ND	ND	ND	ND	ND	—
Simazine[S] (0.04 μg/L)	July 11	0.28	ND	ND	ND	ND	ND	—

[a] ND = not detected.

in green frog fat bodies (Table 7). No organophosphorus pesticides or PCBs were detectable in tissue extracts. As in the sediment samples, DDE levels were highest in individuals from OCD1 and OCD3 (0.99 and 2.81 μg/g, respectively). Negligible levels of DDD or DDT were found in frogs from OCD2, OCD3, and OCD4. Some residues of DDE and endosulfan sulfate were detected in tissue samples from all sites, although frogs from reference sites had concentrations consistently near the detection limit. Endosulfan sulfate concentrations in frog fat tissues collected from OCD2, OCD3, and OCD4 were well above that background level, averaging 1.3, 10.0, and 1.4 μg/g, respectively. These values do not exhibit the same relative trend among orchard sites as the sediment β-endosulfan concentrations, but, together, the residue data indicate that endosulfan contamination existed at all four orchard sites.

DISCUSSION

No consistent differences were detected in the genetic, physiological, or biochemical makeup of northern leopard frogs or green frogs that distinguished populations occupying orchard ponds or canals from populations occupying reference ponds. Genetic variation was not reduced in populations of either species inhabiting orchard ponds relative to populations from reference ponds. Age differences detected in adult male leopard frog populations were associated with one particular reference site that supported individuals that were as much as 2 years older than males from other sites. Differences in size of equivalent-age male leopard frogs and green frogs as well as juvenile green frogs occupying different study sites suggest that suboptimal habitat factors existed at one or two of the four orchard sites. Induction of EROD in male green frogs collected from one orchard site during one sampling event was the only indication that a contaminant-related metabolic challenge may have existed. Multiple detections of pesticides in pond water, and elevated concentrations of organochlorines (either DDT- or endosulfan-related) in sediments and green frog tissues confirmed that exposure to pesticides existed at all orchard sites.

Size differences in equivalent-age male leopard frogs and green frogs inhabiting orchard and reference ponds were an indication that conditions at some orchard sites supported lower potential adult growth. Breeding male green frogs from OCD2 and OCD4 were consistently smaller than males breeding at other sites. Young-of-the-year green frogs captured in 1994 also exhibited reduced size at these two sites, which suggests that the factor or factors responsible initially acted at a premetamorphic stage. Although size differences were not always as distinct in the male leopard frogs (i.e., sometimes significant differences were only detected in length and not in mass), males from OCD2 and OCD4 were also generally smaller than equivalent-age males from reference sites. A few young-of-the-year leopard frogs were also captured at OCD4, and were significantly smaller than young from other sites (M.L. Harris, unpublished data). Because leopard frog and green frog individuals that transform in the same year represent eggs laid in two different years, further support would be given

Table 6. Organochlorines detected in pond sediments collected in the spring and fall of 1994[a]

Site	Sample date	α-Endosulfan (ng/g)	β-Endosulfan (ng/g)	p,p′-DDE (ng/g)	p,p′-DDD (ng/g)
OCD1	May 2	3.1	39.9	71.4	44.4
	September 23	4.9	64.5	64.5	24.4
OCD2	May 2	1.5	ND[b]	10.9	ND
	September 28	ND	ND	ND	ND
OCD3	May 2	ND	ND	43.6	31.9
	September 7	ND	ND	58.3	24.6
OCD4	May 2	ND	ND	ND	ND
	September 8	ND	ND	8.9	ND
REF1	May 2	ND	ND	ND	ND
	September 7	ND	ND	ND	ND
REF2	May 2	ND	ND	ND	ND
	September 9	ND	ND	ND	ND
REF3	May 2	ND	ND	ND	ND
	September	NS[c]	NS	NS	NS

[a] DDE = dichlorodiphenyldichloroethylene; DDD = dichlorodiphenyldichloroethane; OCD1–4 = orchard study sites; REF1–3 = reference study sites.
[b] ND = nondetection as follows: α-endosulfan = 1.4 ng/g, β-endosulfan = 2.9 ng/g, p,p′-DDE = 5.6 ng/g, p,p′-DDD = 6 ng/g.
[c] NS = not sampled.

to the theory that the unknown growth-limiting variable(s) had persisted over more than one generation at least at OCD4.

Body size at metamorphosis in amphibians reflects the relative importance of risks associated with predation versus inter- and intraspecific competition in a given aquatic system [50]. Nutrient data collected in 1994 was not indicative of conditions that might cause lack of food availability in water from OCD2 or OCD4 [9], nor was there an obvious difference in size of predator populations in either orchard system relative to the other study sites (M.L. Harris, unpublished data). Despite the documented existence of pesticide influxes to the pond and canal environments, no indication was found that chemical exposure was responsible for size differences. In short, no glaring habitat characteristic explains the smaller size of individuals from OCD2 and OCD4. That size may even be within the realm of variability associated with normal differences in natural systems. It is more likely that several factors contributed to observed size differences, and some of them may have been related to orchard management.

No 5- or 6-year-old leopard frogs were found at any sites other than REF4, which might suggest that this site had an uniquely favorable environment for that species. A survey of 43 mature leopard frogs captured in southwestern Quebec also did not reveal any 5- or 6 year olds [30]. Average SVLs published in the same paper ranged from 6.38 to 6.76 cm for adult males captured in northern Michigan, USA, Wisconsin, USA, and Quebec, Canada. Therefore, size of males at our reference sites (6.85–7.46 cm) was also greater than published values. That implies that size and age of leopard frogs at orchard sites were not unusually low, but, rather, size and age of leopard frogs at REF4 were unusually elevated.

Differences in relative concentrations of organochlorines in sediments and tissues supports a sediment sink for DDT and its derivatives, but not for endosulfan. Dichlorodiphenyltrichloroethane and its breakdown products were not detectable in reference pond sediments, suggesting that those present in orchard site sediments reflected persistence from historical use. Russell et al. [51] found similar concentrations of DDE in whole spring peepers from Point Pelee National Park, Ontario, Canada, where routine spraying of DDT for mosquito control ceased in 1967. Where endosulfan concentrations were high in tissues, sediment concentrations were generally undetectable and vice versa. Endosulfan is still used as an insecticide in the orchards (Thiodan® 50WP, Agrevo Hoescht Noram Canada, Cambridge, ON, Canada), and it is likely that tissue burdens were the result of aqueous or diet contamination. Because both species of frogs are important prey species for many reptiles, birds, and small mammals, the potential for biomagnification of these organochlorines up the food chain is substantial.

Although relatively elevated concentrations of organochlorines were detected in frogs and sediment in orchard wetlands when compared to those in reference wetlands, no gross, physiological, or biochemical repercussions of those contaminant body burdens could be detected. Reduced size of adults and juveniles did not coincide with higher tissue organochlorine concentrations. When EROD induction was found in green frogs from OCD4, no obvious elevated presence of organo-

Table 7. Organochlorines detected in the fat bodies of breeding male green frogs collected in 1993 and 1994. Values listed are an average of *N* number of pooled samples[a]

Site	*N*	Total weight of sample (g)	*n*	Endosulfan sulfate (μg/g)	DDE (μg/g)	DDD (μg/g)	DDT (μg/g)
OCD1	1	0.69	11	0.12	0.99	ND	ND
OCD2	1	0.35	10	1.30	0.12	0.21	0.04
OCD3	1	0.99	3	10.0	2.81	0.05	0.46
OCD4	1	1.08	14	1.40	0.15	0.02	ND
REF1	3	2.22	8	0.086	0.17	ND	ND
REF2	2	1.91	12	0.074	0.05	ND	ND

[a] *n* = number of individuals represented in each pooled sample; DDE = dichlorodiphenyldichloroethylene; DDD = dichlorodiphenyldichloroethane; DDT = dichlorodiphenyltrichloroethane; OCD1–4 = orchard study sites; REF1–2 = reference study sites; ND = not detected.

chlorines was found in sampled tissues. Induction of EROD in those individuals indicates that they were being exposed to some other P4501A1-inducing organic compound, either through the aquatic medium or through ingestion of contaminated food. Because breeding behavior typically precludes active foraging in vocalizing males, it is probable that the exposure was aqueous in nature. The induced individuals were all captured on June 23, 1993. The spray schedule for OCD4 indicated that whole orchard sprays of Dithane® DG (Rohm and Haas Canada, West Hill, ON, Canada) and Guthion® 50WP were conducted on June 6 and June 20. Neither active ingredient is recognized as a P4501A1-inducer. Vindimian et al. [52] found a correlation between fish hepatic EROD induction and river water loads of herbicide, fungicide, and insecticide mixtures originating from French vineyards. Although they could not determine what compound present in the mixture was causing the induction, they did suggest that trace amounts of inducers in herbicide formulations might be partly responsible. Similarly, an obvious inducing compound could not be identified in this study, either from data on recently sprayed compounds or from tissue residues.

Genetic variation, estimated by allele frequencies, was high in northern leopard frogs and green frogs from all sites, suggesting that there was no selection for pollutant-resistant alleles or genetic constraints on adaptive potential prevalent in any of the pond populations. However, the high H values do have implications for the genetic connectivity of the studied populations. Wahlund's principle states that the frequency of homozygous genotypes is reduced in a pooled population of subpopulations relative to the unmixed subpopulations [53]. High H values in both species relative to published values for the northern leopard frog of 0.027 [54] and for the typical amphibian of 0.079 [55] imply that the studied populations experienced exchanges with a larger genetic pool. The low overall F_{ST} values derived for both species corroborate the heterozygosity values, in that they do not indicate partitioning of subpopulations or obstructed gene flow. Together, these data indicate that pond populations are interacting with neighboring subpopulations via immigration and emigration. This suggests that the discontinuous landscape occupied by semiaquatic frogs in the rural region studied is not sufficiently fragmented to cause genetic isolation. It also implies that some populations might be present, not because the wetland habitat is suitable, but because individuals are dispersing there from superior neighboring habitat.

Although amphibians are traditionally monitored when they congregate to breed [56], this study highlights some of the limitations of such analyses when applied to investigations of populations in modified or polluted environments. The genetic analyses in this study indicate that breeding populations do interact with neighboring populations to some degree. In addition, residue analyses indicated that even frog populations occupying reference ponds in conservation areas were exposed to pesticides. Therefore, comparisons to frogs that were completely uninfluenced by agricultural activities were never accomplished. Amphibians do not migrate in the same spatial sense as birds, anadromous fish, and small mammals, but they do occupy varying, seasonally specific spatial units [29]. Breeding congregations may be easiest to census, but, in terms of physiological status, they may better reflect the environmental quality of a small area (a few square kilometers), rather than of a specific pond or canal. When the status of a particular wetland is in question, the premetamorphic amphibian stages may provide a more accurate depiction of biotic responses to environmental conditions [9].

The broad objective outlined upon initiation of this study was to determine if, given an increasingly fragmented network of wetlands, canals and ponds in farmland can serve as suitable habitat for semiaquatic amphibians. Inhabiting some waterways seemed to have repercussions on the growth of young and adult frogs, and contaminant burdens in one pond redirected the short-term allocation of energy in male green frogs to a metabolic detoxification response; however, indications of detrimental effects on adult frogs were infrequent, most often could not be related to chemical exposure, and probably resulted from several habitat conditions that were not universally present in orchards. Based on the information obtained on wild adults and young-of-the-year at study sites over the short term, wetlands in apple orchards can provide viable breeding habitat for both northern leopard frogs and green frogs.

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