Impact of Organochlorine Contamination on Levels of Sex Hormones and External Morphology of Common Snapping Turtles (Chelydra serpentina serpentina) in Ontario, Canada

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Aquatic ecosystems have been contaminated with pesticides and industrial chemicals on a global scale (1). Organochlorine compounds are of particular significance because of their persistence, tissue specificity, widespread use, and toxicity. Snapping turtles (Chelydra serpentina) bioaccumulate organochlorines because of their longevity (2) and their trophic status as omnivores. Because organochlorines are typically lipid soluble and are metabolized slowly, they tend to accumulate in lipid-rich tissues of snapping turtles, such as adipose tissue and reproductive organs (3). Significant levels of polychlorinated biphenyls (PCBs), organochlorine pesticides, and polychlorinated dibenzodioxins (PCDDs) occur in snapping turtle eggs from contaminated sites in Ontario (4,5).

Snapping turtles have temperature-dependent sex determination (TSD), such that males are produced when the eggs are incubated between 22 and 28°C and females are produced at incubation temperatures outside this range (6). TSD is mediated through differential hormone action or production (7), so hormones have an upstream or causal role in sex determination. Exposure of snapping turtle eggs to 17β-estradiol during incubation at male-producing temperatures produced all females (8), whereas exposure to estrogen antiserum caused some masculinization of embryos at female-producing temperatures (7). Female differentiation in snapping turtles appears to be primarily estrogen dependent, whereas male differentiation appears to be dependent on the absence of estrogen (9).

Many potential endocrine disrupting compounds are present in snapping turtle eggs and may influence sex differentiation (10). There is evidence that reptiles are sensitive to endocrine disruptors. Sluder eggs (Trachemys scripta) exposed to estrogenic PCBs produced females at male-producing temperatures (11). Furthermore, the presence of these organochlorines may disrupt normal endocrine function and morphology in sexually mature reptiles. Female alligators (Alligator mississippiensis), at a site contaminated from a spill of Dicofol and DDT, had abnormally high concentrations of 17β-estradiol in blood plasma, whereas males had depressed testosterone levels and increased levels of 17β-estradiol. Also, juvenile alligators born after the spill had poorly organized testes and small phallic (12,13). Environmental contaminants have also been implicated in demasculinization of Florida panthers (Felis concolor cory) (14), in lower sex steroid and reduced male secondary sex characteristics in white suckers (Catostomus commersonii) (15), and in feminization of California gulls (Larus californicus) (16).

Feminization in turtles may be expressed by changes in the circulating levels of hormones and changes in sexually dimorphic morphology. The precloacal length is longer in male turtles than in females (17) because this is where the penis is located. We examined the penis of a dissected adult male snapping turtle (carapace length = 33.9 cm); the tip of the penis in the relaxed position was within 1.5 cm from the cloacal opening. The precloacal length of male snapping turtles is usually longer than the two posterior plastron lobes, whereas it is shorter in females (17). In males, the precloacal area grows in length faster than the carapace, whereas in females, these two areas grow at the same rate (17). If exposure to exogenous endocrine-disrupting compounds during sexual development results in feminization, it may reduce the rate of growth of the precloacal length. Consequently, the ratio of the precloacal length to the posterior lobe (PPR) would be smaller in contaminated turtles.

We tested the hypothesis that snapping turtles from contaminated sites would be feminized when compared to snapping turtles from noncontaminated sites. We predicted that snapping turtles at contaminated sites would have depressed testosterone levels and elevated estrogen levels in their blood plasma and would have smaller PPRs. An alternative hypothesis is that variation in sexually dimorphic morphology and levels of sex steroids is dependent on abiotic factors such as temperature, length of active season, or other climatic factors. We tested this hypothesis by determining if latitude could explain the observed differences in testosterone and estrogen levels and sexually dimorphic morphology.

Materials and Methods

Snapping turtles were caught at five different sites in Southern Ontario. Lake Sasajewun (45°35′N, 78°30′W), which is located at the wildlife research station

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(WRS) in Algonquin Provincial Park, is a 45-ha dystrophic lake with no history of industrial or agricultural discharge. Snapping turtle eggs from Lake Sasajewun contain very low to nondetectable organochlorine concentrations (5). Jack Lake (44°42'N, 78°02'W) is a dystrophic lake with numerous cottages along the shores but no industry, and it has only background levels of organochlorine contaminants in plankton (7). These levels are consistent with Ontario lakes that are contaminated primarily through atmospheric deposition (18). Cootes Paradise (43°17'N, 79°53'W) in Hamilton Harbour, is a 45-ha eutrophic wetland adjacent to heavy industry and sewage treatment plants on the shore of Lake Ontario. Lynde Creek Marsh (43°45'N, 78°57'W) in Whitby, Ontario, is a eutrophic marsh and creek that drain into Lake Ontario. Big Creek Marsh (42°31'N, 80°29'W) is a 615-ha shoreline marsh that drains a large, agriculturally based watershed into Lake Erie. Snapping turtle eggs collected in 1989 from Lake Sasajewun had significantly lower concentrations of total PCB congeners and p,p'-DDE [0.32 and 0.04 µg/g wet weight (ww), respectively] than Cootes Paradise (30.57 and 4.52 µg/g ww) or Lynde Creek Marsh (37.57 and 3.62 µg/g ww), whereas eggs from Big Creek turtles had intermediate concentrations (6.23 and 0.74 µg/g ww) (4,5). For this study, Lake Sasajewun and Jack Lake were considered control sites, Lynde Creek Marsh and Cootes Paradise were designated as contaminated sites, and Big Creek was considered to be a moderately contaminated site.

**Materials and Methods**

The morphology of snapping turtles was compared from data collected from Lake Sasajewun in 1987–1991, Jack Lake and Lynde Creek Marsh in 1995, Cootes Paradise in 1994–1995, and Big Creek Marsh in 1986–1987. Snapping turtles were caught with baited or unbaited hoop traps from early May to late August. The traps were left overnight and checked every morning. We trapped 15 female and 5 male snapping turtles at Lake Sasajewun from May 29 to July 22, 5 females and 4 males at Lynde Creek from June 25 to July 7, 14 males and 6 females at Jack Lake from July 25 to Aug 14, and 90 females and 80 males at Cootes Paradise from May 8 to Aug 22. Two observers measured turtles: one at Cootes Paradise, Jack Lake, and Lynde Creek and another at Big Creek and Lake Sasajewun. Because we had separate observers taking measurements at different sites, there was a risk that observer bias could affect our results. However, one of the observers from Lake Sasajewun had measured snapping turtles from Cootes Paradise in 1992, which allowed us to test for this potential bias. We compared measurements from Cootes Paradise between 1992 and 1994–1995. Precloacal length was measured from the posterior of the plastron to the cloaca with the tail stretched into a natural position (19). The posterior lobe was measured as the length of the two posterior plastron scutes (Fig. 1). Whenever possible, the males were positively sexed by eliciting eversion of the penis and females were identified by capturing nesting turtles.

In 1995, blood samples were obtained from adult snapping turtles (carapace length ≥20 cm) (18) from Lake Sasajewun, Jack Lake, Cootes Paradise, and Lynde Creek Marsh. Blood samples were taken within an hour from when the turtles were removed from the trap, usually between 0900 and 1100 hr. Approximately 4 ml blood was taken from the caudal vein using 5-ml lithium heparin vacutainers and 22 gauge double-sided needles. The blood samples were stored in a cooler in ice and water from 1 to 5 hr and then centrifuged in a clinical centrifuge for 10 min. The blood plasma was transferred to two cryovials, frozen at -70°C, and later transferred to a -20°C freezer. Plasma testosterone and estrogen levels were measured by radioimmunoassay (RIA) (20). The plasma samples were extracted (100 µl) by adding 5 ml diethyl ether to each sample and freezing the sample on dry ice and acetone. The ether phase was removed and evaporated and then reconstituted with assay buffer. Each sample was analyzed in duplicate. Validation of these assays for snapping turtles was based on the demonstration that serial dilutions of turtle plasma were parallel to the standardized curves (not shown).

**Chlorinated hydrocarbon analysis.** Blood plasma samples were frozen at -20°C until preparation for analyses by capillary gas-liquid chromatography. A total of 9 plasma samples from male snapping turtles from Cootes Paradise, 10 from Jack Lake, 4 from Lake Sasajewun, and 2 from Lynde Creek Marsh, was analyzed for PCBs and pesticides. Blood samples from females were pooled for each site and analyzed as single samples for non-ortho PCBs and PCDDs/polychlorinated dibenzofurans (PCDFs). Only samples from females were used because the entire samples from males were used for PCB and pesticide analyses. Six samples from Cootes

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**Table 1. Comparison of CL, carapace length-adjusted PPR, and precloacal length of male snapping turtles among sites**

| Site                  | CL (cm)* | PPR**  | Precloacal (cm)*** |
|-----------------------|----------|--------|-------------------|
| Lake Sasajewun (R)    | 32.67 ± 3.67 AB | 1.324 ± 0.173 A | 13.80 ± 2.49 AB  |
| (n = 86)              |          |        |                   |
| Jack Lake (R)         | 32.87 ± 3.43 AB | 1.328 ± 0.121 A | 14.74 ± 1.98 A  |
| (n = 14)              |          |        |                   |
| Cootes Paradise (Con) | 32.90 ± 4.51 B  | 1.123 ± 0.141 B | 13.30 ± 2.76 B  |
| (n = 75)              |          |        |                   |
| Big Creek (Con)       | 29.86 ± 4.94 A | 1.136 ± 0.116 B | 12.75 ± 2.46 C  |
| (n = 37)              |          |        |                   |
| Lynde Creek (Con)     | 31.83 ± 2.48 AB | 1.165 ± 0.146 B | 13.83 ± 2.26 ABC|
| (n = 3)               |          |        |                   |

Abbreviations: CL, carapace length; PPR, precloacal/posterior lobe ratio; R, reference site; Con, contaminated site; SD, standard deviation. Values of CL are mean ± SD, and PPR and precloacal length are least square mean ± SD. Similar letters (A,B,C) indicate no significant differences among sites (p>0.05).

*ANOVA, F= 3.94; p= 0.0049.
**ANOVA, F= 30.90; p=0.0001.
***ANOVA, F= 8.225; p<0.0001.
Paradise, four from Lynde Creek, three from Jack Lake, and seven from Lake Sasajewun were pooled for each site. The organochlorine pesticides dichlorodiphenyltrichloroethane (p,p'-DDE), 1,2,4,5- and 1,2,3,4-chlorobenzene, hexachlorobenzene (HCB), octachlorostyrene (OCS), photo-mirex (p-mirex) and mirex, dieldrin, cis- and oxychlordane, cis- and trans-nonachlor, heptachlor epoxide, α- and β-hexachlorocyclohexane (HCH), and 37 PCB congeners were measured in the turtle blood plasma using analytical procedures described by Peakall et al. (21) with modifications detailed by Turle et al. (22) and Bishop et al. (5).

The plasma samples were thawed to room temperature and extracted on a column with dichloromethane (DCM):hexane (1:1 v/v). The extract was evaporated to 1–2 ml and extracted on an 8-g 1.2% water-deactivated Florisil column (Floridin Corp., Berkeley Spring, WV). This was eluted into three fractions and analyzed separately on a fused silica capillary column (60 m × 0.25 mm; 0.1 µm DB-5 film thickness; J&W Scientific, Inc., Folsom, CA). The limit of detection for the organochlorine varied between 0.0003 µg/g ww to less than 0.0001 µg/g ww. The percent lipid content was determined for each sample analyzed; however, due to the small sample size and low lipid content, most of the results for lipid contents were 0%. Only four blood plasma samples from Jack Lake had detectable concentrations of lipids. Although there is a strong relationship between the percent lipid content and lipophelic contaminant concentrations (22), we were unable to use lipid content as a covariate. The value of total PCBs reported here is the sum concentration of the following congeners: 44, 49, 52, 60, 66, 74, 87, 99, 101, 105, 110, 118, 128, 137, 138, 141, 145, 149, 151, 153, 158, 170, 171, 172, 174, 180, 182, 183, 185, 194, 195, 200, 201, 203, 206, 1260 [International Union of Pure and Applied Chemistry (IUPAC) number] (24). The contaminant analysis of one blood plasma sample was accidently measured twice, so the mean of the two estimates was used for subsequent statistical analyses.

Concentrations of 20 PCDDs, 26 PCDFs, and 6 non-ortho PCBs were measured in blood plasma of female snapping turtles collected from all four sites using capillary gas chromatography and mass spectrometry (25). The blood samples were pooled for each site. Initially, samples were extracted by solid-phase extraction using a C18 bonded silica cartridge, but this extraction method was unsuccessful. Thus, liquid/liquid extraction, using Florisil column chromatography, was used instead. Samples were prepared with anhydrous sodium sulfate and extracted with DCM:hexane (1:1 v/v). The sample was spiked with a primary internal standard mixture (13C-12-labeled PCDDs/PCDFs and non-ortho PCBs at a level of 50–100 ng/kg on top of the column prior to extraction). Samples were analyzed with a double focusing VG Autospec GC/MS (Hewlett-Packard, Wilmington, DE) at 10,000 resolution. The minimum detectable concentration for congeners varied from 0.01 to 0.16 ng/kg ww. Minimum detection limits for non-ortho PCBs ranged from 0.01 to 1.05 ng/kg ww. Problems with extraction resulted in sample loss and less than 50% recovery in some cases.

Statistical analyses were performed using Statistica v 5.0 (26). The precloacal/posterior lobe ratios (PPR) and precloacal lengths were compared among sites using analysis of covariance (ANCOVA), with carapace length as a covariate to adjust for body size. We used χ2 analysis to determine if there was a significant overlap of PPR between males and females at Cootes Paradise and at Lake Sasajewun. Regression analysis was used to determine relationships between sex steroid levels and morphology and between sex steroid levels and morphology with latitude. Analysis of variance (ANOVA) was used to determine if there were any differences in mean hormone levels or morphology among sites. Because the blood sampling periods among sites did not completely overlap and body size varied among sites, we used carapace length and Julian date as covariates to eliminate variation in hormone levels due to these confounding factors. The arithmetic means and standard deviations (SD) of PCB and pesticide concentrations were not lipid-normalized because the lipid content was so low as to be unmeasurable for most samples. ANOVA was used to find any differences in PCB and pesticide concentrations among sites. Partial correlations were used to measure relationships between testosterone levels and concentration of organochlorine compounds, both within sites and between sites. Because the estrogen levels were highly variable and sample sizes were so low, the partial correlations between estrogen and organochlorine were spurious; thus, we did not present the results here. We used Spearman rank correlations to test for relationships between the pooled PCDDs/PCDFs and non-ortho PCBs from each site and between the mean testosterone and estrogen levels of both male and females for each site. Linear regressions were used to determine if PCB and pesticide concentrations varied with body size. A modified Tukey test for unequal sample sizes was used for post hoc multiple comparisons (27) for all ANOVAs and ANCOVAs.

This study complied with the University of Guelph animal care protocol.

Results

Morphology. The PPRs of males from the three Great Lakes' sites (contaminated sites) were significantly smaller than those from Jack Lake and Lake Sasajewun (reference sites) (Table 1). There were no significant within-site differences among the reference sites or contaminated sites. Although there were no significant differences in the PPRs or precloacal lengths between females from Jack Lake and the three contaminated sites, the PPRs and precloacal lengths of females from two of the contaminated sites were significantly smaller than those from Lake Sasajewun (Table 2). Similarly, the precloacal lengths of both sexes were longer at control sites than at most contaminated sites. Only the male PPR was significantly different between Lynde Creek Marsh and the control sites, but since the sample from Lynde Creek Marsh was small, it was difficult to detect a difference.

There were clear differences in the modal distribution of PPR in the turtles between males and females from Cootes Paradise and Lake Sasajewun (Fig. 2). Males had larger

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Table 2. Comparison of CL, carapace length-adjusted PPR, and precloacal length of female snapping turtles among sites

| Site                  | CL (cm)* | PPR**  | Precloacal (cm)** |
|-----------------------|----------|--------|-------------------|
| Lake Sasajewun (R)    | 28.52 ± 0.07 BC | 0.626 ± 0.081 AB | 6.33 ± 0.085 BC   |
| (n = 25)              |          |        |                   |
| Jack Lake (R)         | 23.76 ± 0.67 ABC | 0.752 ± 0.076 ABC | 8.16 ± 0.84 ABC   |
| (n = 5)               |          |        |                   |
| Cootes Paradise (Con) | 27.00 ± 2.33 AB | 0.647 ± 0.108 B  | 6.74 ± 1.37 B     |
| (n = 70)              |          |        |                   |
| Big Creek (Con)       | 25.99 ± 2.74 AB | 0.633 ± 0.084 C  | 6.26 ± 0.86 C     |
| (n = 156)             |          |        |                   |
| Lynde Creek (Con)     | 28.52 ± 0.107 BC | 0.626 ± 0.081 AB | 6.33 ± 0.085 BC   |
| (n = 5)               |          |        |                   |

Abbreviations: CL, carapace length; PPR, precloacal/posterior lobe ratio; R, reference site; Con, contaminated site; SD, standard deviation. Values of carapace length are mean ± SD, and PPR and precloacal length are least square mean ± SD. Similar letters (A,B,C) indicate no significant differences among sites (p>0.05).

*ANOVA, F = 18.82, p = 0.0001.
**ANOVA, F = 88.86, p<0.0001.
***ANOVA, F = 57.58, p<0.0001.
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PPRs than females at both sites. A significantly larger proportion of male snapping turtles from Cootes Paradise had PPRs that overlapped female PPRs than did males from Lake Sasajewun (χ² = 12.50; p = 0.0004; df = 1) (Fig. 3). The proportion of female PPRs that overlapped male PPRs was not significantly different between Lake Sasajewun and Cootes Paradise (χ² = 3.20; p = 0.0737; df = 1) (Fig. 3). When we compared observer measurements within the same site (Cootes Paradise), the observers’ bias in measurement error was of a smaller magnitude than the differences in morphology among sites.

Testosterone. Table 3 summarizes the mean testosterone levels at different sites, although two females from Cootes Paradise were excluded as outliers because the estimated testosterone levels were two orders of magnitude larger than the rest. Only at Cootes Paradise were there sufficient observations to examine the relationship between testosterone levels and morphology. Not all observations were used in the following analyses because of occasional missing values from the morphological observations: testosterone levels of males increased linearly with Julian date [adjusted (adj) r² = 0.2038; F = 7.401; p = 0.0119; n = 26]; carapace length (adj r² = 0.3034; F = 11.888; p = 0.0021; n = 26); precloacal length (adj r² = 0.2730; F = 10.390; p = 0.0036; n = 26); and the PPR (adj r² = 0.1997; F = 7.478; p = 0.0113; n = 26). However, carapace length, precloacal length, and PPR all had low tolerances (0.04, 0.02, and 0.1, respectively), and thus were intercorrelated. Precloacal length was eliminated to reduce multicollinearity, and the relationship between the remaining factors and testosterone was assessed by a backward stepwise multiple regression. Testosterone had a significant relationship with both carapace length and Julian date (adj r² = 0.4957; F = 12.797; p = 0.0002; n = 25).

Julian date (adj r² = 0.0518; F = 2.913; p = 0.0970; n = 36), carapace length (adj r² = -0.0302; F = 0.0034; p = 0.9503; n = 35), precloacal length (adj r² = -0.0228; F = 0.3760; p = 0.5449; n = 29), and PPR (adj r² = -0.0361; F = 0.0240; p = 0.8781; n = 29) did not regress significantly with testosterone in females. Again, precloacal length had a low tolerance (0.08) and was eliminated, and a backward stepwise multiple regression showed no relationship between testosterone and carapace length and Julian date (adj r² = -0.0355; F = 0.4166; p = 0.6628; n = 35) in females.

Carapace length differed among sites in male snapping turtles (Table 1). Because carapace length and Julian date varied linearly with testosterone levels in males, differences in testosterone due to body size and time could be removed using carapace length and Julian date as covariates. There was a significant difference in mean testosterone levels among sites after adjustment for carapace length and Julian date for males (ANCOVA, F = 5.374; p = 0.0043; n = 32). Testosterone levels of males from Lake Sasajewun were significantly lower than levels of males from Jack Lake, but there were no other differences. Although body size varied among sites for female snapping turtles (Table 2), no covariates were used to compare mean testosterone levels among sites for females because there was no relationship between body size or Julian date with testosterone. There were no significant differences in mean testosterone levels of females (Table 3) among sites (ANOVA, F = 0.3347; p = 0.8003; n = 50).

Estrogen. Table 3 summarizes the mean estrogen levels at different sites. Only at Cootes Paradise were there sufficient observations to compare between estrogen levels and morphology. One male was excluded as an outlier, as his estimated estrogen level was an order of magnitude larger than in any other male. Not all observations were used in the following analyses because of occasional missing values from the morphological observations. In our multiple regression analyses, estrogen levels and all independent variables were transformed, using the reciprocal transformation, to normalize the residuals. There was no relationship between estrogen levels of males and Julian date (adj r² = 0.0005; F = 1.0118; p = 0.3245; n = 26), but there was a positive linear relationship between estrogen and carapace length (adj r² = 0.1411; F = 5.1076; p = 0.0332; n = 26), precloacal length (adj r² = 0.1627; F = 5.8566; p = 0.0235; n = 26), and PPR (adj r² = 0.2372; F = 8.7740; p = 0.0068; n = 26). A backward stepwise multiple regression of estrogen with Julian date, carapace length, and PPR eliminated all variables as insignificant except PPR. Precloacal length was removed due to multicollinearity.

There was no relationship between estrogen levels of females and Julian date.

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**Figure 2.** Frequency histogram of precloacal:posterior lobe ratios (PPRs) of snapping turtles at Cootes Paradise in 1994–1995 (males, n = 111; females, n = 125) and at Lake Sasajewun in 1987–1991 (males, n = 39; females, n = 111).

**Figure 3.** Proportion of male and female precloacal:posterior lobe ratios (PPRs) that overlap with the PPRs of the other sex at Cootes Paradise and Lake Sasajewun.
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differences between either Cootes Paradise and Lynde Creek or between Jack Lake and Lake Sasajewun except for \( p, p' \)-DDE and total PCB concentrations. However, we had low power to detect any differences due to our small sample sizes. Because there were significant relationships between testosterone concentrations and both carapace length and Julian date, a partial correlation was performed to estimate the effect that PCB and pesticide concentrations have upon testosterone levels at each site. After partia

Although 70.6% of the PCDDs/PCDFs and non-ortho PCBs detected in the pooled samples were higher in blood plasma collected at Jack Lake than at Cootes Paradise and 57.9% were higher at Jack Lake than at Lynde Creek Marsh, the concentrations were of the same magnitude (Table 6). If the concentrations were higher at either Cootes Paradise or Lynde Creek than at Jack Lake, then the concentrations differed by at least an order of magnitude. Generally, the contaminant levels were highest and similar in Cootes Paradise and Lynde Creek Marsh, lower in Jack Lake, and lowest in Lake Sasajewun. Similarly, there were no significant relationships between the pooled PCDDs/PCDFs and non-ortho PCBs from each site with the mean testosterone levels of either males or females.

### Discussion

Our hypothesis that the sexually dimorphic morphology of adult snapping turtles would be more feminized at contaminated sites was not supported. However, we did find a relationship between testosterone levels and latitude, which was consistent with previous studies. This suggests that exposure to contaminants may affect testosterone levels in snapping turtles, potentially influencing their sex expression.

#### Table 3: Mean concentrations ± SD of testosterone and estrogen in blood plasma (ng/ml) of snapping turtles from Cootes Paradise, Jack Lake, Lake Sasajewun, and Lynde Creek Marsh in 1995

| Hormone/site | Male | | | Female | | |
|--------------|-----|-----|-----|--------|-----|-----|
| Sex          | Mean ± SD | No. | Mean ± SD | No. |
| Testosterone | | | | | |
| Jack Lake (R) | 55.40 ± 16.40 | 7 | 0.69 ± 0.01 | 3 |
| Lake Sasajewun (R) | 11.45 ± 6.49 | 3 | 0.46 ± 0.12 | 7 |
| Cootes Paradise (Con) | 43.88 ± 21.50 | 27 | 0.59 ± 0.32 | 35 |
| Lynde Creek (Con) | 39.38 ± 6.47 | 2 | 0.47 ± 0.27 | 3 |
| Estrogen | | | | | |
| Jack Lake (R) | 0.62 ± 0.07 | 8 | 0.39 ± 0.11 | 3 |
| Lake Sasajewun (R) | 1.16 ± 0.98 | 2 | 0.62 ± 0.38 | 8 |
| Cootes Paradise (Con) | 1.95 ± 2.77 | 27 | 2.40 ± 6.25 | 33 |
| Lynde Creek (Con) | NA | 0 | 0.40 ± 0.11 | 3 |

Abbreviations: SD, standard deviation; R, reference site; Con, contaminated site; NA, not analyzed.

#### Table 4: Mean concentrations ± standard deviation of PCB and organochlorine pesticide (ng/g wet weight) in blood plasma of male snapping turtles from Cootes Paradise, Lynde Creek, Jack Lake, and Lake Sasajewun, Ontario, in 1995

| Compound | Cootes Paradise (Con) | Lynde Creek (Con) | Jack Lake (R) | Lake Sasajewun (R) |
|----------|-----------------------|-------------------|--------------|-------------------|
| \( n = 9 \) | | | | |
| \( \text{trans-Nonachlor} \) | \( 10.8 \pm 11.3 \) | \( 6.2 \pm 1.6 \) | \( 0.3 \pm 0.4 \) | \( 0.1 \pm 0.1 \) |
| \( \text{p,p'-DDE} \) | \( 10.1 \pm 4.3 \) | \( 21.7 \pm 3.0 \) | \( 0.7 \pm 0.3 \) | \( 0.2 \pm 0.4 \) |
| \( \text{p'-Mirex} \) | \( 3.8 \pm 3.4 \) | \( 5.2 \pm 0.9 \) | \( 0.4 \pm 0.1 \) | \( 0.2 \pm 0.3 \) |
| \( \text{Mirex} \) | \( 8.3 \pm 7.9 \) | \( 10.0 \pm 2.1 \) | \( 0.4 \pm 0.2 \) | \( 0.4 \pm 0.1 \) |
| \( \alpha\text{-HCH} \) | \( 0.5 \pm 0.7 \) | \( 0.7 \pm 1.0 \) | \( 1.4 \pm 0.7 \) | \( 0.5 \pm 1.0 \) |
| \( \gamma\text{-HCH} \) | ND | ND | 0.3 ± 0.8 | ND |
| \( \text{Oxychlordane} \) | \( 7.0 \pm 8.8 \) | \( 2.4 \pm 0.7 \) | 0.2 ± 0.3 | 0.2 ± 0.3 |
| \( \text{cis-Chlordane} \) | \( 0.8 \pm 0.5 \) | \( 0.7 \pm 0.4 \) | ND | ND |
| \( \text{cis-Nonachlor} \) | \( 5.3 \pm 8.0 \) | \( 2.6 \pm 0.7 \) | ND | ND |
| \( \text{Heptachlor epoxide} \) | \( 0.6 \pm 1.2 \) | \( 0.9 \pm 0.2 \) | ND | 1.5 ± 2.0 |
| \( \text{Dieldrin} \) | \( 0.9 \pm 1.2 \) | \( 3.8 \pm 1.9 \) | \( 0.3 \pm 0.8 \) | ND |
| \( \text{Total PCB} \) | \( 414.8 \pm 351.7 \) | \( 263.3 \pm 116.2 \) | 17.8 ± 7.8 | 18.2 ± 12.5 |

Abbreviations: PCB, polychlorinated biphenyl; Con, contaminated site; R, reference site; CB, chlorinated biphenyl; HCB, hexachlorobenzene; OCS, octachlorostyrene; HCH, hexachlorocyclohexane; ND, not detected.

Similar letters (A,B,C) indicate no significant differences in concentrations among sites (p<0.05).

*Number of plasma samples used in analysis.
Table 5. Partial correlations of organochlorine concentrations with testosterone levels in blood plasma of male snapping turtles from Cootes Paradise and Jack Lake after removing the effects of body size and Julian date

| Compound          | Cootes Paradise (Con) | Jack Lake (R) | Cootes Paradise (Con) | Jack Lake (R) |
|-------------------|-----------------------|---------------|-----------------------|---------------|
|                   | Partial $r^2$         | $p$-Value     | Partial $r^2$         | $p$-Value     |
| 1,2,4,5-CB        | -0.5224               | 0.1941        | -0.1376               | 0.8253        |
| 1,2,3,4-CB        | -0.0671               | 0.8747        | 0.3804                | 0.512         |
| HCB               | -0.6995               | 0.0535        | NA                    | NA            |
| OCS               | -0.3254               | 0.4316        | NA                    | NA            |
| trans-Nonachlor   | 0.1159                | 0.7845        | 0.2862                | 0.6406        |
| $p,p'$-DDE        | 0.401                 | 0.3248        | 0.112                 | 0.8577        |
| $p'$-Mirex        | 0.0759                | 0.8582        | 0.113                 | 0.7109        |
| Mirex             | 0.0763                | 0.8575        | -0.2238               | 0.5026        |
| $\alpha$-HCH     | -0.5967               | 0.1194        | 0.6244                | 0.2962        |
| $\gamma$-HCH     | NA                    | NA            | 0.816                 | 0.2985        |
| Oxychlordane      | 0.427                 | 0.2913        | -0.0674               | 0.9142        |
| cis-Chlordane     | 0.0213                | 0.96          | NA                    | NA            |
| cis-Nonachlor     | 0.3436                | 0.4043        | NA                    | NA            |
| Heptachlor epoxide| 0.0754                | 0.6952        | NA                    | NA            |
| Dieldrin          | -0.3868               | 0.3439        | -0.0974               | 0.9142        |
| Total PCB congeners| 0.2434               | 0.5614        | -0.0657               | 0.2257        |

Abbreviations: R, reference site; Con, contaminated site; CB, chlorinated biphenyl; HCB, hexachlorobenzene; OCS, octachlorostyrene; HCH, hexachlorocyclohexane; PCB, polychlorinated biphenyl; NA, not applicable.

Table 6. Concentrations (ng/kg wet weight) of non-ortho PCB, PCDD, and PCDF congeners in blood plasma of female snapping turtles from Cootes Paradise, Lynde Creek, Jack Lake, and Lake Sasajewun, Ontario, in 1995.

| Compound          | Cootes Paradise (Con) | Lynde Creek (Con) | Jack Lake (R) | Lake Sasajewun (R) |
|-------------------|-----------------------|-------------------|---------------|---------------------|
|                   |                     |                   |               |                     |
| Non-ortho PCB congener |                     |                   |               |                     |
| PCB-37            | 2.88                 | 3.87              | 4.02          | 2.13                |
| PCB-81            | 3.61                 | 2.47              | 0.37          | ND                  |
| PCB-77            | 3.16                 | 3.36              | 4.77          | 1.78                |
| PCB-126           | 54.24                | 57.68             | 3.27          | 2.05                |
| PCB-169           | 2.46                 | 4.57              | 0.39          | ND                  |
| PCB-189           | ND                   | 0.13              | ND            | ND                  |
| PCDD congener     |                      |                   |               |                     |
| 2,3,7,8-TCDD      | 0.73                 | 1.73              | 0.11          | ND                  |
| 1,2,3,7,8-PentaCDD| 0.33                 | 2.64              | 0.33          | 0.19                |
| 1,2,3,4,7,8-HexaCDD| 0.12                | 0.26              | 0.42          | ND                  |
| 1,2,3,6,7,8-HexaCDD| 0.33                | 2.38              | 0.32          | ND                  |
| 1,2,3,7,8,9-HexaCDD| 0.1                 | 0.26              | 0.39          | ND                  |
| 1,2,3,4,6,7,8-HeptaCDD| 0.41              | 0.17              | 0.67          | ND                  |
| OctaCDD           | 0.91                 | 0.86              | 4.59          | 0.22                |
| PCDF congener     |                      |                   |               |                     |
| 1,2,4,7,8-PentaCDF| ND                   | 0.21              | ND            | ND                  |
| 1,3,3,7,8-PentaCDF| 0.07                 | ND                | 0.18          | ND                  |
| 2,3,4,7,8-PentaCDF| 0.33                 | 0.88              | 0.29          | ND                  |
| 1,2,4,6,7,8-HexaCDF| ND                   | ND                | 0.07          | ND                  |
| 1,2,4,6,8,9-HexaCDF| ND                   | ND                | 0.11          | ND                  |
| 1,2,3,4,7,8-HexaCDF| 0.09                | 0.08              | 0.23          | ND                  |
| 1,2,3,6,7,8-HexaCDF| 0.12                | 0.34              | 0.25          | ND                  |
| 1,2,3,7,8,9-HexaCDF| 0.14                | 0.28              | 0.01          | ND                  |
| 2,3,4,6,7,8-HexaCDF| 0.31                | 0.37              | 0.56          | ND                  |
| 1,2,3,4,6,7,8-HeptaCDF| 0.13              | 0.15              | 0.42          | ND                  |
| 1,2,3,4,7,8,9-HeptaCDF| 0.09                | 0.04              | 0.35          | ND                  |
| OctaCDF           | 0.47                 | 0.36              | 1.31          | 0.34                |

Abbreviations: R, reference site; Con, contaminated site; ND, not detected; PCB, polychlorinated biphenyl; PCDD, polychlorinated dibenzoepin; PCDF, polychlorinated dibenzofuran.

Samples were pooled within each site.

Supported. Not only were the precausal length and PPR smaller at contaminated sites than at reference sites but also the degree of sexual dimorphism was less pronounced at one of the contaminated sites to such an extent that errors in sexing adult turtles using only sexually dimorphic morphology were possible. We have no evidence that any of these turtles whose PPRs overlapped were intersexes, which occasionally occurs after embryonic turtles are exposed to organochlorines (10) or hormonal treatment (7,8). Many of the males whose PPRs overlapped with females had what appeared to be normal penises. We sexed the turtles with intermediate PPRs by the presence of the penis. The differences in morphology appeared to be better correlated with local levels of contamination than with latitude. If latitude has an effect on morphology, the relationships between PPR or precausal length would be positive. However, when the regressions between PPR and precausal length were performed separately within contaminated sites and then within control sites only two of the relationships were positive and six were negative or nonsignificant, suggesting that latitude does not predict morphology.

Our hypothesis that snapping turtles from contaminated sites would have lower levels of testosterone in blood plasma compared to those from control sites was not supported. In absolute levels, the testosterone concentrations were approximately equivalent at Cootes Paradise and Jack Lake, and the lowest values were at the second reference site (Lake Sasajewun). We were surprised that males from Lake Sasajewun had lower testosterone levels than those from Jack Lake, especially because both are reference sites and Lake Jack was the closest study site to Lake Sasajewun. However, the sample size of testosterone measurements of males from Lake Sasajewun was small, so the mean estimate may not have been reliable. Although it is rare that relationships between hormone levels and contaminant concentrations are found within sites, differential sex steroid levels have been found among sites with different contaminant levels in fish and reptiles (12,13,28). However, differences in activity patterns could confound comparisons between northern less-contaminated sites, and southern more heavily contaminated sites. Snapping turtles emerge from hibernation and become active sooner in southern populations (29). Testosterone levels have been correlated with mating activity in snapping turtles (30) and estrogen levels have been correlated with the ovarian cycle (31-33); thus, activity levels, and hormone levels, may be asynchronous between the populations of different latitudes. An implicit assumption of the analysis of covariance is that the regression coefficients (i.e., the relationship of time with hormone levels) are the same for each treatment. Latitudinal differences in behavior and physiology make such comparisons difficult.

Our results are at odds with previous studies examining testosterone profiles of male snapping turtles. Testosterone levels of male snapping turtles from Wisconsin peaked in May and then declined until there was a second, smaller peak in October.
(30,33). We did not find any apparent peak in any of the hormone profiles; instead, the testosterone levels kept increasing until August, when sampling stopped. Other studies have found that estrogen levels in snapping turtles are lowest in June during ovulation and the postovulatory phase, and increase again in the fall during vitellogenesis (31,32). However, we found that mean estrogen levels were constant over time, probably because we caught female snapping turtles mostly during the ovulatory and postovulatory period.

There was no evidence that contaminants had any effect upon testosterone or estrogen levels. Although many partial correlations were negative, there were no significant relationships between testosterone levels and any of the PCBs and pesticides within either Cootes Paradise or Jack Lake. Similarly, there was no relationship between testosterone or estrogen and either PCBs or pesticides among sites. Surprisingly, although the relationships were all nonsignificant, most were positive, which is the opposite of what was predicted. As expected, the concentration of most of the PCBs and pesticides measured were lower in turtles from Jack Lake or Lake Sasajewun than in those from Cootes Paradise or Lyndee Creek. Nevertheless, 12 of the 16 PCBs and pesticides tested for were found, albeit at low concentrations, in the samples from the control sites. We did not find the same trend with PCDDs/PCDFs and non-ortho PCBs. Although the pooled levels of PCDDs/PCDFs and non-ortho PCBs were lowest at Lake Sasajewun, there were no differences among Jack Lake, Cootes Paradise, or Lyndee Creek, and for many of these contaminants, the levels were highest at Jack Lake. These results are likely confounded by pooling samples; thus, we could not adjust our analyses with covariation with body size.

There were significant regressions between carapace length and PCB or pesticide concentrations in 8 of the 17 compounds examined from the Cootes Paradise turtles, and every regression but one (non-significant) was positive. Conversely, none of the 13 regressions between carapace length and PCB or pesticide concentrations from Jack Lake was significant. Bishop et al. (34) found no relationship between female body size and organochlorine contaminant concentrations in snapping turtle eggs. However, there was a positive relationship between body size and organochlorine concentration in body tissues (23,33). We found that larger turtles had higher concentrations of PCBs and pesticides in blood plasma, at least when the level of contamination was already high. This suggests that the trends in organochlorine contamination in blood plasma follow the same trends as do other tissues. Because turtles from Jack Lake did not show a relationship between body size and contaminant concentration, either we did not have sufficient power to detect the relationship or the contaminants were being metabolized or removed at a rate equal to the rate of uptake. A power analysis (36) with a medium effect size of $F^2$ estimated 1 - $\beta = 0.1911$ ($R[1,8]$ = 5.3177; $\lambda = 1.5$) for the regressions between carapace length and PCB and pesticide concentrations. Therefore the nonnegative regressions were inconclusive (37), as we did not have enough power to detect a difference unless the effect size was very large.

Our two main results seem contradictory. There is no evidence that testosterone production has been impaired or estrogen production enhanced in snapping turtles with high levels of organochlorine contaminants. However, there is evidence that there is some feminization occurring at the contaminated sites. $p,p\text{-DDE}$ inhibits sexual development in rats without affecting plasma testosterone levels (38). The concentration of $p,p\text{-DDE}$ that could inhibit androgen receptor activity in the rat was 63.6 ng/g ww (38), which is of the same magnitude as the mean concentration of $p,p\text{-DDE}$ in the plasma of adult Cootes Paradise turtles (10.1 ng/g ww), total DDT in snapping turtle muscle tissue from the Niagara peninsula in 1989 (164.60 ng/g ww) (23), and $p,p\text{-DDE}$ in snapping turtle eggs from Cootes Paradise in 1991 (5,910 ng/g ww) (4). Previous studies have shown that $p,p\text{-DDE}$ is an androgen receptor antagonist and raises the possibility that the changes in sexual characteristics could relate to antiandrogenic activity. These concentrations of $p,p\text{-DDE}$ are sufficiently high to inhibit androgen receptor transcription ability in vitro in rats (38). Similarly, PCBs have been shown to induce changes in sexual development without alterations to testosterone levels (39). Our study suggests that external sexual development may be more sensitive to exposure to environmental contaminants than testosterone production or metabolism of adults. Continued exposure during sexual development of juveniles may also contribute to alterations in sexually dimorphic morphology, particularly since the dose of the exposure increases as the turtle gets larger.

Biomonitoring often involves either sampling body tissue (2,5,23) or eggs (4,5,21,22). Unfortunately, although snapping turtles make good environmental indicators, snapping turtle populations are highly susceptible to increased adult mortality (40,41). Repeated or large-scale lethal tissue sampling of snapping turtle adults is undesirable, particularly if the population is already in decline. Although measuring contaminant levels in eggs would have a much smaller effect upon the adult population, contaminant levels in eggs may better represent recent dietary intake just prior to egg production (35) rather than long-term accumulation in body tissue. Using blood plasma samples as a measure of bioaccumulation may be a preferable method for biomonitoring, as it is nonlethal and probably causes little harm to adults. Although lipid levels were generally undetectable in the plasma samples and contaminant levels are proportional to lipid levels, random error may be introduced, but the estimate would be unbiased using this method. Because contaminant concentrations present in blood plasma are proportional to body size, blood sampling could be used to measure long-term bioaccumulation of contaminants.

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