Suburbanization, estrogen contamination, and sex ratio in wild amphibian populations

Max R. Lambert[a,1], **Geoffrey S. J. Giller**[a], **Larry B. Barber**[b], **Kevin C. Fitzgerald**[b,2], **and David K. Skelly**[a]

[a]School of Forestry and Environmental Studies, Yale University, New Haven, CT 06511; and [b]U.S. Geological Survey, Boulder, CO 80303

Edited by James H. Brown, University of New Mexico, Albuquerque, NM, and approved July 28, 2015 (received for review January 22, 2015)

Research on endocrine disruption in frog populations, such as shifts in sex ratios and feminization of males, has predominantly focused on agricultural pesticides. Recent evidence suggests that suburban landscapes harbor amphibian populations exhibiting similar levels of endocrine disruption; however the endocrine disrupting chemical (EDC) sources are unknown. Here, we show that sex ratios of metamorphosing frogs become increasingly female-dominated along a suburbanization gradient. We further show that suburban ponds are frequently contaminated by the classical estrogen estrone and a variety of EDCs produced by plants (phytoestrogens), and that the diversity of organic EDCs is correlated with the extent of developed land use and cultivated lawn and gardens around a pond. Our work also raises the possibility that trace-element contamination associated with human land use around suburban ponds may be contributing to the estrogenic load within suburban freshwaters and constitutes another source of estrogenic exposure for wildlife. These data suggest novel, unexplored pathways of EDC contamination in human-altered environments. In particular, we propose that vegetation changes associated with suburban neighborhoods (e.g., from forests to lawns and ornamental plants) increase the distribution of phytoestrogens in surface waters. The result of frog sex ratios varying as a function of human land use implicates a role for environmental modulation of sexual differentiation in amphibians, which are assumed to only have genetic sex determination. Overall, we show that endocrine disruption is widespread in suburban frog populations and that the causes are likely diverse.

environmental sex determination | estrone | landscaping | lawn | phytoestrogen

Amphibians are a model animal system for studying endocrine disruption in nature (1). Wild amphibians show evidence (such as testicular oocytes) of feminizing endocrine disruption in human-modified environments (2–7). With few exceptions (6, 7), research on amphibians has focused on agricultural landscapes (2, 4, 5) and the effects of pesticides such as atrazine (2, 8). In parallel, endocrine disruption in fish has been observed in the context of chemical gradients associated with sources of endocrine disrupting chemicals (EDCs) such as municipal wastewater treatment facility discharges (9–11). These studies imply that endocrine disruption in aquatic systems is caused predominantly by industrial- or municipal-scale activities. However, recent work has revealed that suburban neighborhoods also may be hotspots for endocrine disruption, and green frogs [*Rana* (*Lithobates*) *clamitans*] from "backyard ponds" have been reported (6, 7) to exhibit higher frequencies of feminizing endocrine disruption (testicular oocytes in males) than frogs from other land uses (forest, agricultural, and urban). Endocrine disruption appears to be a ubiquitous phenomenon in suburban ponds, with testicular oocytes observed in male green frogs at all 34 suburban ponds studied to date (6, 7). The causes of endocrine disruption in suburban landscapes have not yet been studied. Although some sources of EDCs are obvious, as with agricultural landscapes and wastewater treatment facilities, EDC sources in suburban neighborhoods are less evident. Suburban ponds may be influenced by a variety of potential sources relative to undeveloped forested ponds (Fig. 1).

We compare ponds in landscapes ranging from highly developed suburban backyards to undeveloped forests (Fig. S1), and focused on sex ratios of green frogs (*R. clamitans*), which inhabit both environments and were previously evaluated for evidence of endocrine disruption (6, 7). Offspring sex ratio is an established metric of endocrine disruption (8, 12) and is ecologically relevant (13). Here, we address whether sex ratio in cohorts of metamorphosing green frogs varies with landscape structure surrounding ponds.

Human modification of the environment alters chemical sources and exposure pathways, providing a link between landscape structure and frog developmental biology. In parallel with frog sampling, we tested water from ponds to develop a profile of EDCs (both organic and inorganic) across suburban and forested landscapes. We hypothesized that the landscape structure surrounding study ponds would be related to patterns of detected EDCs, and explored the extent to which proportions of different land cover categories (Fig. 2) were associated with metamorph sex ratios and pond EDCs. Forested ponds are surrounded entirely by undeveloped forest vegetation. The landscape surrounding suburban ponds is composed of a heterogeneous mix of human residences, roads, sidewalks, and other structures (hereafter denoted "Developed Land Use") as well as lawns and other plantings (hereafter "Suburban Vegetation") in addition to small amounts of natural vegetation.

Here we show that sex ratios of frog metamorphs are sensitive to suburbanization intensity surrounding ponds, and also that increasing suburbanization is correlated with higher diversity of detected EDCs including biogenic steroidal hormones, phytoestrogens, and metalloestrogens. Without discounting potential contributions of pesticides or pharmaceuticals, which have been the focus of past studies on frog endocrine disruption and environmental

Significance

We focus on a critical issue, the influence of human-derived contaminants on wildlife populations. Endocrine disrupting chemicals that act through hormonal pathways are capable of having large influences even when concentrations are relatively low. While there is evidence that such endocrine disruption can result from the application of agricultural pesticides and through exposure to wastewater effluent, we have identified a diversity of endocrine disrupting chemicals within suburban neighborhoods. Sampling populations of a local frog species, we found a strong association between the degree of landscape development and frog offspring sex ratio. Our study points to rarely studied contamination sources, like vegetation landscaping and impervious surface runoff, that may be associated with endocrine disruption environments around suburban homes.

Author contributions: M.R.L., G.S.G., and D.K.S. designed research; M.R.L., G.S.G., L.B.B., and K.C.F. performed research; M.R.L., G.S.G., and K.C.F. analyzed data; M.R.L., G.S.G., L.B.B., and D.K.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1] To whom correspondence should be addressed. Email: max.lambert@yale.edu.

[2] Present address: AECOM, Louisville, KY, 40202.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1501065112/-/DCSupplemental.

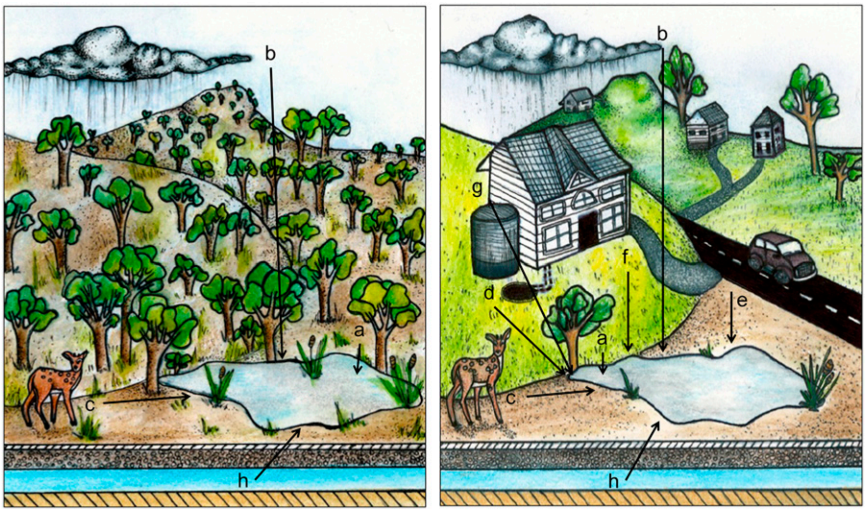

Fig. 1. Conceptual framework of potential endocrine-disrupting chemical sources in a forested pond (*Left*) and a developed suburban pond (*Right*). Sources include (a) lawn plants or forest vegetation (e.g., phytoestrogens), (b) atmospheric transport of pollutants (e.g., alkylphenol in precipitation), (c) biogenic hormone inputs from animals and plants (e.g., estrone excretion), (d) wastewater from domestic sewer lines or septic tanks (e.g., pharmaceuticals), (e) runoff from roads (e.g., antimony), (f) runoff from lawns (e.g., pesticides and fertilizers), (g) runoff from buildings (e.g., copper), and (h) altered surface water/groundwater redox states resulting in mobilization of trace elements (e.g., manganese). Figure courtesy of Corrine Edwards (Illustrator).

EDCs (2, 3), results presented here expand our understanding of endocrine disruption and suggest that human land uses expose wildlife to EDCs via previously unexplored pathways (e.g., vegetation biosynthesis and trace element contamination). Our results connect landscape structure with amphibian sex ratios, implying environmental modulation of sex differentiation in a taxon typically presumed to have only genetic sex determination (14).

Results

Sex Ratios. Offspring sex ratio varied as a function of land use. Overall, the percentage of females was greater (t test: $n = 13$, $t = -2.8125$, $P < 0.05$) in suburban ponds ($n = 9$, mean $= 50.7\%$ female, SE $= 2.98\%$) than in forested ponds ($n = 4$, mean $=$ 36.5% female, SE $= 4.0\%$). The average sex ratios for forested ponds and for suburban ponds adjacent to either septic systems or sanitary sewers are summarized in Fig. 5*A*. After model selection with a binomial GLM, only the percent of Developed Land Use was a significant predictor of frog sex ratios (Fig. 3); sex ratios became increasingly dominated by females as the landscape surrounding ponds became more developed ($D^2 = 0.37$, $P = 0.007$). Wastewater removal mode, water temperature, Suburban Vegetation, and water chemistry parameters all were nonsignificant ($P > 0.05$) and iteratively removed from the final model. This result suggests frog sex ratios may be influenced by landscape structure surrounding the ponds.

Organic EDC Detection. Of the six forested ponds, only one had any organic EDC detected. However, organic EDCs were detected in eight of nine septic suburban ponds and three of four sewered suburban ponds. Across six forested ponds, the only organic EDC detected was estrone. Among suburban ponds, we detected a variety of organic EDCs including classical estrogens (estrone, $n = 11$ ponds; 17β-estradiol, $n = 2$; equilenin, $n = 3$; equilin, $n = 1$), phytoestrogens (daidzein, $n = 6$; formononetin, $n = 2$; coumestrol, $n = 3$; prunetin, $n = 1$), and the androgen *trans*-androsterone ($n = 1$). The poisson GLM indicated that the number of EDCs detected exhibited positive relationships with suburbanization. Specifically, Developed Land Use (Fig. 4*A*) explained a significant portion of the variation in total number of classical estrogens detected at a pond ($D^2 = 0.23$, $P = 0.02$). However, the final model for the total number of classical estrogens detected did not include wastewater removal mode or Suburban Vegetation (both $P > 0.05$).

The final model for total phytoestrogens detected did not include wastewater removal mode or Developed Land Use (both $P > 0.05$). However, Suburban Vegetation (Fig. 4*B*) explained a significant amount of variation in the number of phytoestrogens detected ($D^2 = 0.30$, $P = 0.02$). This result indicates that residential landscaping may be linked to increased phytoestrogen diversity in suburban ponds.

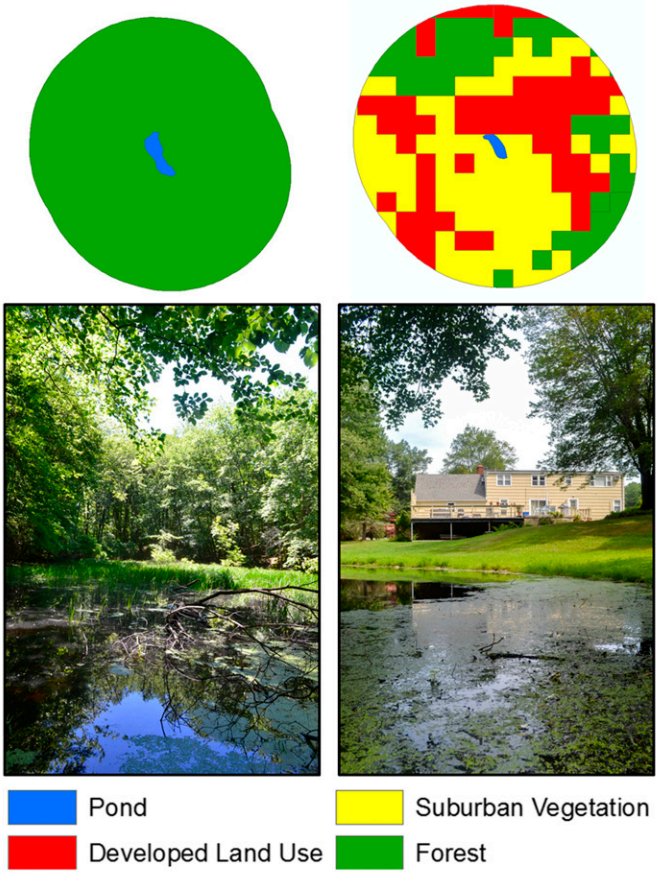

Fig. 2. Examples of an undeveloped forested pond (*Left*) and a developed suburban pond (*Right*). Images at top illustrate differences in land cover between the two categories. Forested ponds were composed of 100% forest (coniferous, deciduous, mixed forest, scrub shrub, and forested wetland land-cover types). Suburban ponds were a composite of at least 70% Suburban Vegetation ("Open Spaces Developed;" lawns and landscape plantings) and Developed Land Use (composed of "High-Intensity Developed," "Medium-Intensity Developed," and "Low-Intensity Developed").

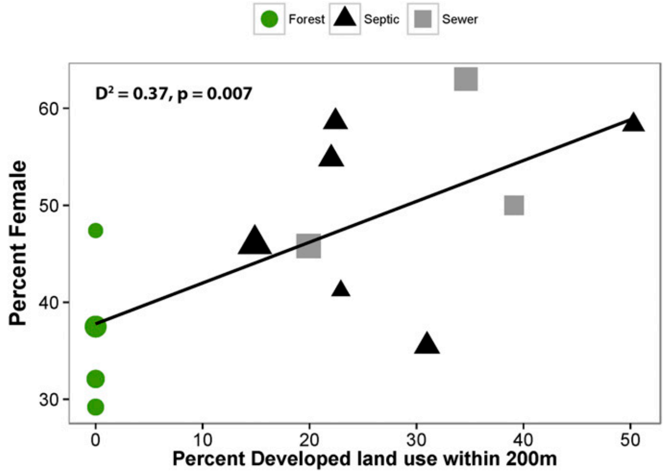

Fig. 3. Relationship between the percent of Developed Land Use at each of 13 ponds and green frog metamorph sex ratios (percent female). A binomial generalized linear model indicates that sex ratios contain a higher proportion of females as the landscape surrounding ponds becomes increasingly developed. Point size indicates the samples size (*n* = 17–55) of metamorphs collected from each pond.

The final model for total number of organic EDCs (classical estrogens + phytoestrogens) detected only included wastewater removal mode ($D^2 = 0.32$, $P = 0.009$) and not Suburban Vegetation or Developed Land Use (both $P > 0.05$). Tukey's HSD indicates that ponds with septic systems have more total EDCs than forested ponds ($P = 0.02$), that ponds with sewer systems are trending toward having more EDCs than forested ponds ($P = 0.09$), and that ponds with sewer and septic systems do not differ in their total EDC occurrence ($P = 0.59$). This result indicates that we detected more organic EDCs in suburban ponds but that wastewater removal mode did not influence the number of total number of organic EDCs detected (Fig. 4*C*).

Estrogenic Equivalencies. Organic EDCs detected here exhibit a range of relative estrogen potencies (REPs, standardized to 17β-estradiol, Table S1). Relative estradiol equivalency quotients (EEQs, see *Materials and Methods*) are summarized in Fig. 5 *B* and *C* for estrogens and phytoestrogens, respectively, and compare contamination intensities among ponds. Inorganic metalloestrogenicity relative EEQs were calculated in a similar manner using REPs for select trace elements (Table S1) and are summarized in Fig. 5*D*.

Organic EEQs did not exhibit any correlations with land use characteristics (all $P > 0.05$). Metalloestrogenicity model selection indicated that Suburban Vegetation and Developed Land Use were not important (both $P > 0.05$). However, wastewater removal mode (Fig. 4*D*) was significant ($P < 0.001$) and Tukey's HSD indicated that both ponds with septic systems ($P < 0.001$) and ponds with sewer systems ($P = 0.002$) had higher metalloestrogenicity than forested ponds, but that the ponds with septic and sewer systems had statistically indistinguishable metalloestrogenicities ($P = 0.12$).

The EDCs detected are only a subset of all putative EDCs in the study ponds, so we are cautious in interpreting the magnitude of differences among EEQs across different types of EDCs as well as among ponds (*SI Materials and Methods*).

Discussion

Frog sex ratios varied with human land use characteristics, and specifically a suburbanization gradient. This result, coupled with studies illustrating that amphibian sex differentiation is sensitive to estrogen exposure in the laboratory (8, 12, 15, 16), challenges the assumption that amphibian sex determination is strictly genetic (14) and implies a potential role for environmental sex modification. Offspring sex ratio is generally poorly studied in metamorphosing amphibians from wild populations, though it is commonly understood that amphibians have genetic sex determination (14). Although laboratory studies indicate that amphibian sex ratios can be environmentally manipulated (8, 12), these prior results provide no indication of normal sex ratio variation in wild populations from different environments. Limited evidence from a single natural frog population indicates that normal metamorph sex ratios can sometimes be skewed toward one sex (17). Based on results from the present study, green frog sex ratios appear to be naturally male-biased and become increasingly feminized with suburbanization. This pattern is consistent with prior work on fish that shows feminization of natural populations exposed to treated wastewater (10). Further examination of wild frog sex ratio patterns along environmental gradients will be important for improving our understanding of potential environmental influences on amphibian sex differentiation.

Data presented here show significant associations between Suburban Vegetation (i.e., lawn and ornamental plants) and

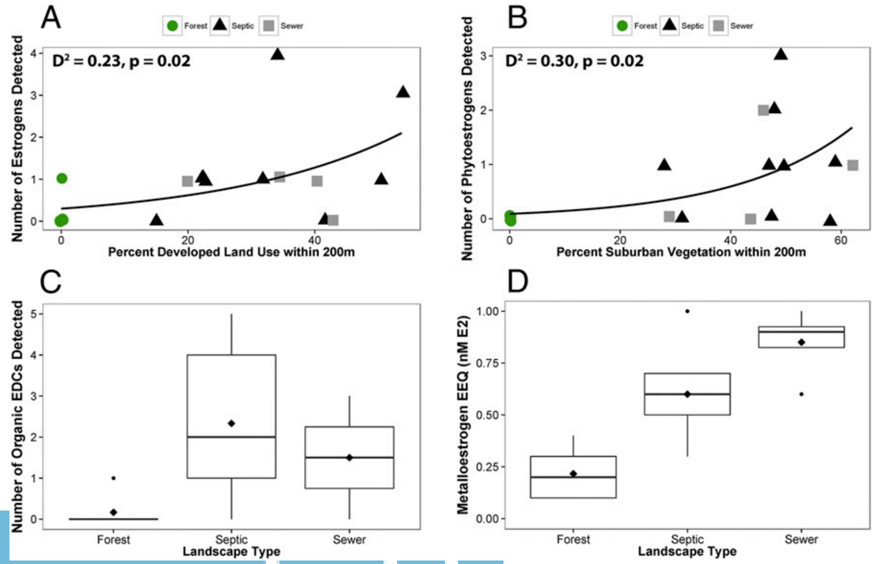

Fig. 4. Relationships between the number of classical estrogens, the number of phytoestrogens, the total number of organic endocrine disrupting chemicals (EDCs), and the metalloestrogen estradiol equivalency quotient (EEQ; Metalloestrogenicity) with land use attributes. Poisson generalized linear models indicate that the total number of classical estrogens (*A*) detected is correlated with Developed Land Use, whereas the number of phytoestrogens (*B*) detected is correlated with Suburban Vegetation. The total number of organic EDCs (*C*) detected is higher in suburban ponds than forested ponds but does not differ between ponds adjacent to septic systems and those adjacent to sanitary sewers. The relative metalloestrogen EEQ (*D*) is higher in suburban ponds than forested ponds but does not differ between ponds adjacent to septic systems and those adjacent to sanitary sewers. Metalloestrogen EEQ values were calculated with relative estrogen potencies (Table S1) and the concentrations measured in the ponds (Dataset S1) of 16 trace elements.

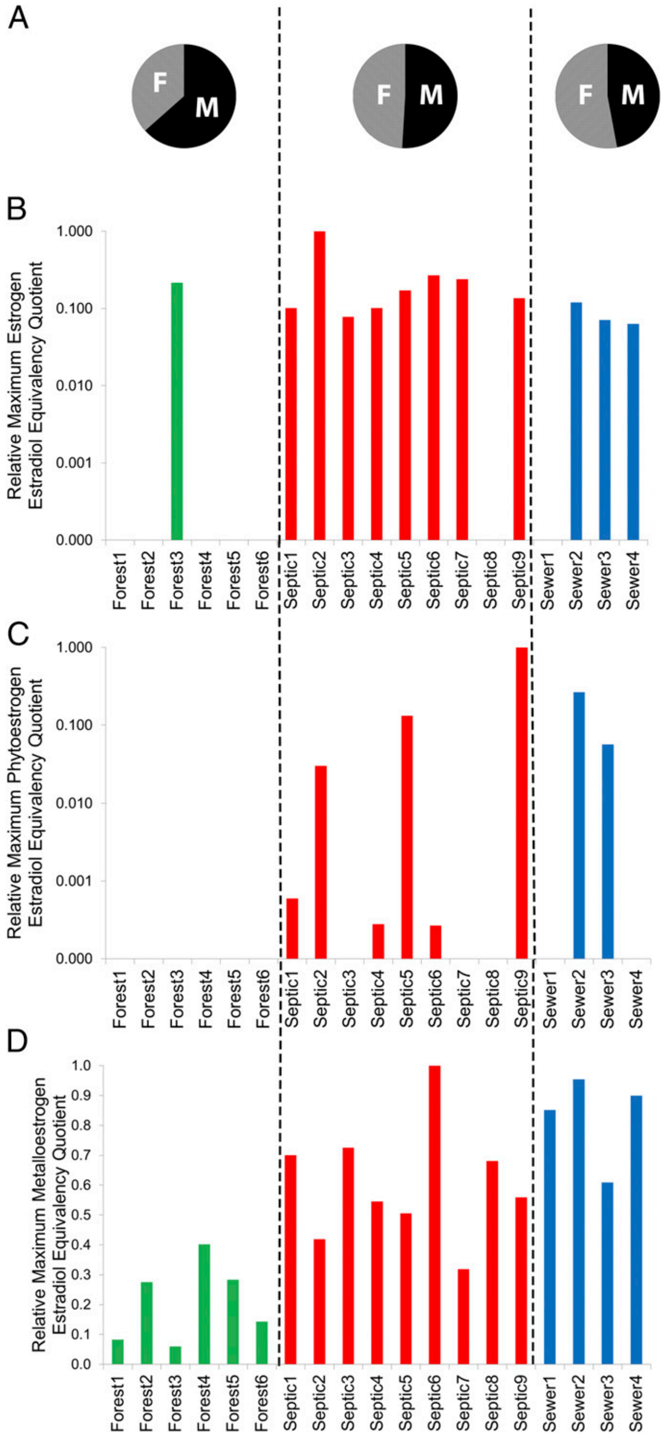

Fig. 5. Comparison of average green frog metamorph sex ratio (*A*) and relative water exposure to three classes of endocrine disrupting chemicals (EDCs) [classical estrogens (*B*), phytoestrogens (*C*), and metalloestrogens (*D*)]. Data are arrayed by land use: forested ponds are shown in green; suburban ponds adjacent to septic systems are shown in blue; suburban ponds adjacent to sewer lines are shown in red. Sex ratios are displayed as the average proportion of males (M, black) and females (F, gray). Relative classical estrogen and phytoestrogen estradiol equivalency quotients (EEQs; see *SI Materials and Methods*) are normalized to the highest EEQ for each class of EDC and are on a log scale. Each pond can only be compared with other ponds within a given panel. Vertical dashed lines separate forested, suburban septic, and suburban sewer ponds. Frogs were not collected from Forest 1, Forest 4, Septic 2, Septic 5, Septic 6, Septic 8, Sewer, 1, and Sewer 2.

organic EDC contamination. This finding suggests vegetation landscaping may be important modifiers of the local hydrogeochemical environment, potentially affecting surface waters through multiple processes (e.g., plant biosynthesis or chemical cycling). The relationship between Suburban Vegetation and the diversity of phytoestrogens suggests that changes in vegetation species in suburban areas may initiate a cascade of biological and chemical processes that increases EDC diversity and loading.

Beyond associations between land use and contamination by EDCs, we investigated whether mode of wastewater removal (sewer or septic) was associated with EDC detections. Other studies have shown leaching of EDCs from septic tanks (18, 19), and "exfiltration" from sanitary sewer systems into groundwater (20, 21). The fact that EDC profiles between ponds located in sewered neighborhoods and those served by septic systems are essentially indistinguishable is a striking finding. Sanitary sewer systems are widely held to be a superior means of protecting the environment from pollution associated with high-density septic-tank waste. Within our study ponds, no such advantage was detected; ponds adjacent to either sewers or septic systems had equivalent numbers of detected EDCs. Although both septic tanks and sewer lines are known to exude contaminants in groundwater, we found no unambiguous evidence of wastewater chemicals, such as the synthetic pharmaceuticals 17α-ethynylestradiol, in the suburban pond waters. We did detect equilin and equilenin. Although both compounds are used as pharmaceuticals, they also are produced by vertebrates (22) and so their natural in situ production in ponds cannot be discounted. These results, with the outcome that ponds with septic systems and ponds with sewer systems do not differ in the diversity of organic EDCs detected, suggest that wastewater discharge may not be the dominant source of endocrine disruption in suburban pond water. However, the dominant organic EDCs detected were estrone and phytoestrogens, chemicals that are likely derived from animal and plant sources within the ponds rather than septic tanks or sewer systems.

Estrone was found in almost every suburban pond, conceivably due to local vertebrate biosynthesis. Although 17β-estradiol occurs less frequently, it can be oxidized to estrone (22), potentially contributing to the ubiquity of estrone. Estrone is typically associated with vertebrates (23), but also may be considered a phytoestrogen, as it has been isolated from plant species (24–26). In addition to natural environmental sources, estrone can be associated with human residential wastewater effluent (18, 19). Although prevalent and yielding a high REP (Table S1), the sources of estrone are difficult to untangle.

Plant-derived phytoestrogens can be potent EDCs (27, 28) and their diversity as well as frequency of detection in suburban ponds indicates that phytoestrogens may be ecologically relevant. Recent evidence (29) indicates that roots of certain plants produce highly estrogenic chemicals. It may be that plant-root-derived phytoestrogens, from lawn plants like clover, may reach local aquatic systems. Further exploration of phytoestrogen sources and impacts is greatly needed.

Trace element metalloestrogenicity also may contribute to the endocrine disrupting activity of suburban water. Trace elements come from a multitude of natural and anthropogenic sources including altered redox states of surface water/groundwater systems in developed areas (30) and runoff inputs from impervious surfaces and lawns. Potential endocrine disrupting effects from exposure to complex trace element mixtures in aquatic environments are poorly understood and highlight another opportunity for research.

This interdisciplinary field study provides an integrated framework based on landscape ecology design, GIS land use analysis, frog biology, and organic and inorganic water chemical characterization (Dataset S1). Nevertheless, it is important to acknowledge that our study is observational in its approach and our analyses are based on exploring associations. We have uncovered strong correlations between land use variables and both the distribution of potential EDCs as well as a key biological end point. Observational studies such as this one provide critical context for targeted experimental exploration of the influences of individual EDCs as well as mixtures that may be prevalent in natural environments. Whatever approaches are adopted, there is a strong incentive to follow up on the initial findings described here. Landscape conversion to suburban residential use is outpacing population growth in the United States (31), making developed neighborhoods an important research venue. The surprising pattern of endocrine disruption uncovered in this study may arise directly from the heterogeneity inherent in suburban landscapes. Suburban ecosystem modifications likely produce complex interactions and feedbacks among the built, hydrological, geochemical, and biological components. Unraveling these relationships is a critical task in discovering the causes of endocrine disruption in human-modified environments.

Materials and Methods

Site Selection. We compared small, freshwater ponds proximate to human residences to those in forests. When in suburban neighborhoods we targeted ponds directly down-gradient and less than 20 m from a septic-tank leaching field or a sanitary sewer line. Note, however, that our hypothesis regarding contamination is not restricted to wastewater sources. We ensured that all forested ponds were located in mature forests and at least 200 m from the nearest road or household.

We searched for ponds (within a 35-km radius) in the greater New Haven, Connecticut area (Fig. S1). To determine suburban (treatment) and forested (control) ponds, we calculated land cover metrics around the freshwater ponds using a geographic information system (GIS – ESRI ArcMap 9.6). Specifically, we used 2010 US Fish and Wildlife Service National Wetland Inventory data (www.fws.gov/wetlands/) to identify all ponds within our study area as well as National Oceanic and Atmospheric Administration (NOAA) 2006 land cover data (32) to calculate the relative proportion of each land cover type (defined in *SI Materials and Methods*) within a 200-m radius of each pond. To minimize anthropogenically derived contaminants, control ponds were identified that had 200-m buffers containing 100% forested land cover (coniferous, deciduous, mixed forest, scrub shrub, and forested wetland). If a pond buffer contained a combined land cover that was 70% or greater of Developed Land Use (High-intensity developed, Medium-intensity developed, and Low-intensity developed) and Suburban Vegetation (Open Spaces developed) land cover types it was considered a suburban pond. We further stratified the suburban ponds using University of Connecticut 1998 Sewer Service Areas GIS data (magic.lib.uconn.edu/). A pond was assumed to be located near a sewer system if it was within 1 km of a sewer line and near a septic system if further than 1 km from a sewer line. Septic and sewer classifications were verified with landowners. We randomized the lists of forested and suburban ponds and visited them in the indicated order. We were able to collect both frog and water samples from most ponds. However, we were only able to collect either just frogs or just water from a subset of ponds.

Frog Sampling. We focused on *R. clamitans* because they exist in suburban and forested landscapes and exhibit long larval periods (∼9 mo). Prolonged larval development allows more than sufficient time for complete gonadal differentiation by metamorphosis (33). Where possible, we collected prometamorphic frogs from ponds by targeting individuals that were completing metamorphosis with the development of all four limbs and the presence of a reduced tail (Gosner stages 43–46; ref. 34). Across ponds, we sampled 17–55 individuals (n = 424, average = 33, Dataset1). We euthanized frogs within 24 h with tricaine methanesulfonate (MS-222) and immediately dissected the frogs. We recorded the sex upon the inspection of the gonad and the macrostructure of a testis or ovary. Our research was conducted under approval of Yale Institutional Animal Care and Use Committee (IACUC) protocol 2012–10361 and Connecticut Department of Energy and Environmental Protection permit 0112019d.

Water Collection. All ponds used in this study are permanent water bodies, although the Forest 3 site experienced significant drying. Before collecting surface water samples, we rinsed all waders and supplies thoroughly in nanopure deionized water. We wore new nitrile gloves whenever collecting water samples. At each site, we filled a 1-L HDPE plastic bottle with surface water for analysis of steroidal hormones, phytoestrogens, and trace elements. For consistent sampling, we waded or canoed to the deepest possible portion of each pond, triple-rinsed each bottle, held each container ∼15 cm beneath the surface until full, and capped the bottle underwater leaving no head space. After collection, we cooled the samples on ice and shipped them overnight to the USGS laboratory in Boulder, Colorado. Subsamples for trace element analysis were filtered through 0.45-μm membranes and preserved with high purity nitric acid. Samples were stored at 4 °C (trace elements) or frozen (steroidal hormones) until analysis.

Organic Chemical Analysis. We assayed a diversity of organic chemicals using ELISA and gas chromatography tandem mass spectrophotometry (GC-MS/MS). The herbicide atrazine, and the pharmaceuticals compounds carbamazepine and sulfamethoxazole were analyzed via ELISA according to the manufacturer's directions (Abraxis). Because of the biological potency of sex hormones such as the classical estrogens 17β-estradiol and estrone, and the lack of information on their occurrence and impact in suburban ponds, a suite of steroidal hormones and phytoestrogens (details below) were analyzed by GC-MS/MS as described (35). Other potential anthropogenic EDCs, including alkylphenols and bisphenol A, also were measured but not included in this paper.

All samples were spiked with 25 ng/L of d$_5$ *cis*-androsterone, d$_4$ estrone, d$_4$ 17β-estradiol, d$_4$ 17α-ethynylestradiol, and d$_7$ cholesterol as surrogate standards immediately before extraction. The unfiltered, spiked samples were passed through an octadecylsilica solid-phase-extraction disk, eluted with methanol, derivatized with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), containing d$_{12}$ chrysene and d$_{12}$ perylene as internal standards, to form trimethylsilyl ethers, and analyzed by GC-MS/MS in multiple reaction monitoring (MRM) mode.

The following compounds were measured: estrogens (17β-estradiol, 17α-estradiol, estrone, estriol, equilin, equilenin, 17α-ethynylestradiol, 19-norethistrone, and mestranol), androgens (*cis*-androsterone, *trans*-androsterone, 5α-androstan-17β-ol-3-one, 4-androstene-3,17-dione, *epi*-testosterone, and testosterone), progestogens (progesterone and medroxyprogesterone), phytoestrogens (equol, biochanin A, coumestrol, daidzein, formononetin, prunetin, and glycitein), and sterols (cholesterol, dihydrocholesterol, and 3β-coprostanol). We calculated concentrations of compounds using internal standard techniques if the following criteria were met: The retention time of the compound did not deviate more than 0.05 min from the expected retention time, the signal/noise ratio was greater than 3, the two qualifying ions were detected and had response ratios ± 20% of the expected values, and the calculated concentration was within the range of the calibration curve.

Major and Trace Element Chemistry. To understand the potential inorganic chemical sources across the land-use gradient, we measured the concentrations of 58 major and trace elements using inductively coupled-plasma/atomic emission spectrometry (36) and inductively coupled-plasma/mass spectrometry (37).

Organic Estradiol Equivalency Quotient (EEQ) Calculations. To compare the relative estrogenicity for our study ponds, we derived EEQs (see *SI Materials and Methods* for detail) based on published relative estrogen potencies (REPs, Table S1). EEQ is defined as the hypothetical molar concentration of 17β-estradiol that is equivalent to the detected molar concentration of a compound of interest (38). The REP value of the focal compound is used to calculate an estrogen equivalency (EE) in terms of equivalent molar concentrations of 17β-estradiol, and is estimated as the ratio of the 50% effect concentrations (38–40) values (see *SI Materials and Methods* for detail). We used published REP values (38–43) and the molar concentrations of detected EDCs to estimate individual compound EE values, which are summed to give the EEQ.

Inorganic EEQ Calculations (Metalloestrogenicity). There is a limited literature on the estrogenic effects of inorganic chemicals reporting 17β-estradiol REPs determined from in vitro tests (44). Although the mechanisms of action are not well characterized, trace elements could conceivably contribute to endocrine disruption effects in suburban waters. We used the measured concentrations of 16 trace elements (Dataset S1) from our ponds and their published REPs (Table S1) to determine inorganic metalloestrogen EEQs (metalloestrogenicity, see *SI Materials and Methods* for details).

Statistical Analysis. We conducted all statistical analyses with R (version 2.15.2, R Core Team) and considered a result significant at $\alpha = 0.05$. No data required transformations before analysis. We used a *t* test to assess differences in sex ratio [reported as "percent female" in a given pond; calculated as ((# Females in pond)/(# Females + # Males in pond) × 100)] in frog samples from suburban and forested sites. We were conservative in our analysis of sex ratios and only used ponds with at least 17 individuals sampled so as to maximize sample size and the statistical power of our analyses. Two ponds had only 10 and 12 metamorphs collected and were eliminated from the analyses because of reduced statistical power.

We then modeled whether sex ratios varied as a function of suburban land use characteristics using a generalized linear model (GLM) with a binomial error and logit link function. A binomial GLM is ideal for studying proportion data such as sex ratios. We included the wastewater removal mode (septic, sewer, or none), the percent of Developed Land Use within 200 m of the pond, percent of Suburban Vegetation within 200 m of the pond, water temperature, total classical estrogens (17β-estradiol, equilenin, equilin, and estrone) detected, total phytoestrogens (coumestrol, daidzein, formononetin, and prunetin) detected, total organic EDCs detected (classical estrogens + phytoestrogens), and metalloestrogens detected as variables in the model. We iteratively removed nonsignificant terms from the model, validating the reduced model with the larger, more saturated model using the "anova" function in R (45). We stopped when we had a model with all significant terms. For non-Gaussian GLMs, we calculated the amount of deviance explained (D^2), similar to the R^2 in Gaussian regression models (46).

For chemistry data, we used a similar modeling approach as described above for sex ratios except we used a Poisson model, which is ideal for count data, and did not include water temperature in the model. We separately modeled the total number of classical estrogens detected, the total number of phytoestrogens detected, and the total number of organic EDCs detected as a function of land use. If there were significant differences among wastewater removal modes, we used a Tukey's Honestly Significant Difference (HSD) test using the "glht" function in the package multcomp.

For metalloestrogenicity, we used similar modeling as described above except we used a Gaussian GLM for continuous data.

ACKNOWLEDGMENTS. We thank the homeowners who provided access to the "backyard" pond sites, the South Central Connecticut Regional Water Authority for access to forested sites, Y. Argueyla, B. Johnson, O. Malik, and G. Mount for field assistance, as well as S. Keefe for laboratory assistance. We also thank E. Giller, L. Jarett, M. Klein, and H. and V. Lambert. This project was conducted with support from the U.S. Geological Survey National Research and Toxics Substances Hydrology Programs, and funding provided by a crowdsource grant through PetriDish.org, Carpenter/Sperry Matching Fund, Schiff Fund, the Williams Internships Fund, the Hixon Center for Urban Ecology, the Yale Center for Reproductive Ecology, and the Yale Institute for Biospheric Studies.

1. Kloas W (2002) Amphibians as a model for the study of endocrine disruptors. *Int Rev Cytol* 216:1–57.
2. Hayes T, et al. (2003) Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (Rana pipiens): Laboratory and field evidence. *Environ Health Perspect* 111(4):568–575.
3. Park BJ, Kidd K (2005) Effects of the synthetic estrogen ethinylestradiol on early life stages of mink frogs and green frogs in the wild and in situ. *Environ Toxicol Chem* 24(8):2027–2036.
4. Murphy MB, et al. (2006) Atrazine concentrations, gonadal gross morphology and histology in ranid frogs collected in Michigan agricultural areas. *Aquat Toxicol* 76(3-4): 230–245.
5. McDaniel TV, et al. (2008) Potential endocrine disruption of sexual development in free ranging male northern leopard frogs (Rana pipiens) and green frogs (Rana clamitans) from areas of intensive row crop agriculture. *Aquat Toxicol* 88(4):230–242.
6. Skelly DK, Bolden SR, Dion KB (2010) Intersex frogs concentrated in suburban and urban landscapes. *EcoHealth* 7(3):374–379.
7. Smits AP, Skelly DK, Bolden SR (2014) Amphibian intersex in suburban landscapes. *Ecosphere* 5(1):1–9, 10.1890/ES13-00353.1.
8. Langlois VS, et al. (2010) Low levels of the herbicide atrazine alter sex ratios and reduce metamorphic success in Rana pipiens tadpoles raised in outdoor mesocosms. *Environ Health Perspect* 118(4):552–557.
9. Jobling S, Nolan M, Tyler CR, Brighty G, Sumpter JP (1998) Widespread sexual disruption in wild fish. *Environ Sci Technol* 32(17):2498–2506.
10. Vajda AM, et al. (2008) Reproductive disruption in fish downstream from an estrogenic wastewater effluent. *Environ Sci Technol* 42(9):3407–3414.
11. Barber LB, Vajda AM, Douville C, Norris DO, Writer JH (2012) Fish endocrine disruption responses to a major wastewater treatment facility upgrade. *Environ Sci Technol* 46(4):2121–2131.
12. Pettersson I, Berg C (2007) Environmentally relevant concentrations of ethynylestradiol cause female-biased sex ratios in Xenopus tropicalis and Rana temporaria. *Environ Toxicol Chem* 26(5):1005–1009.
13. Sakisaka Y, Yahara T, Miura I, Kasuya E (2000) Maternal control of sex ratio in Rana rugosa: Evidence from DNA sexing. *Mol Ecol* 9(11):1711–1715.
14. Hayes TB (1998) Sex determination and primary sex differentiation in amphibians: Genetic and developmental mechanisms. *J Exp Zool* 281(5):373–399.
15. Hayes TB, Menendez KP (1999) The effect of sex steroids on primary and secondary sex differentiation in the sexually dichromatic reedfrog (Hyperolius argus: Hyperolidae) from the Arabuko Sokoke Forest of Kenya. *Gen Comp Endocrinol* 115(2): 188–199.
16. Bögi C, et al. (2003) Endocrine effects of environmental pollution on Xenopus laevis and Rana temporaria. *Environ Res* 93(2):195–201.
17. Papoulias DM, Schwarz MS, Mena L (2013) Gonadal abnormalities in frogs (Lithobates spp.) collected from managed wetlands in an agricultural region of Nebraska, USA. *Environ Pollut* 172:1–8.
18. Swartz CH, et al. (2006) Steroid estrogens, nonylphenol ethoxylate metabolites, and other wastewater contaminants in groundwater affected by a residential septic system on Cape Cod, MA. *Environ Sci Technol* 40(16):4894–4902.
19. Standley LJ, et al. (2008) Wastewater-contaminated groundwater as a source of endogenous hormones and pharmaceuticals to surface water ecosystems. *Environ Toxicol Chem* 27(12):2457–2468.
20. Reynolds JH, Barrett MH (2007) A review of the effects of sewer leakage on groundwater quality. *Water Environ J* 17(1):34–39.
21. Rutsch M, et al. (2008) Towards a better understanding of sewer exfiltration. *Water Res* 42(10-11):2385–2394.
22. Bhavnani BR, Short RV, Solomon S (1969) Formation of estrogens by the pregnant mare. I. Metabolism of 7-3H-dehydroisoandrosterone and 4-14C-androstenedione injected into the umbilical vein. *Endocrinology* 85(6):1172–1179.
23. Bentley PJ (1998) *Comparative Vertebrate Endocrinology* (Cambridge University Press, Cambridge, United Kingdom).
24. Gawienowski AM, Gibbs CC (1969) The isolation of estrone from apple seeds. *Phytochemistry* 8(3):685–686.
25. Hewitt S, Hillman JR, Knights BA (1980) Steroidal oestrogens and plant growth and development. *New Phytol* 85(3):329–350.
26. Janeczko A, Skoczowski A (2005) Mammalian sex hormones in plants. *Folia Histochem Cytobiol* 43(2):71–79.
27. Benlhabib E, Baker JI, Keyler DE, Singh AK (2002) Composition, red blood cell uptake, and serum protein binding of phytoestrogens extracted from commercial kudzu-root and soy preparations. *J Med Food* 5(3):109–123.
28. Skipor J, Misztal T, Piskula M, Wiczkowski W, Thiery JC (2012) Phytoestrogens and thyroid hormone levels in the cerebrospinal fluid of ewes fed red clover silage. *Small Rumin Res* 102(2-3):157–162.
29. Morgan HE, Dillaway D, Edwards TM (2014) Estrogenicity of soybeans (Glycine max) varies by plant organ and developmental stage. *Endocr Disruptors (Austin)* 2:e28490.
30. Shenker M, Seitelback S, Brand S, Haim A, Litaor MI (2005) Redox reactions and phosphorous release in re-flooded soils of an altered wetland. *Eur J Soil Sci* 56(4): 515–525.
31. Theobald DM (2005) Landscape patterns of exurban growth in the USA from 1980 to 2020. *Ecol Soc* 10:32–60.
32. Dobson JE, et al. (1995) *NOAA Coastal Change Analysis Program (C-CAP): Guidance for Regional Implementation. NOAA Technical Report NMFS 123* (U.S. Department of Commerce, Seattle, WA).
33. Ogielska M, Kotusz A (2004) Pattern and rate of ovary differentiation with reference to somatic development in anuran amphibians. *J Morphol* 259(1):41–54.
34. Gosner KL (1960) A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16(3):183–190.
35. Foreman WT, et al. (2012) Determination of steroid hormones and related compounds in filtered and unfiltered water by solid-phase-extraction, derivatization and gas chromatography with tandem mass spectrometry. *U.S. Geological Survey Techniques and Methods* (U.S. Geological Survey, Washington, DC). Book 5, chap. 9B.
36. Garbarino JR, Taylor HE (1979) An inductively-coupled plasma atomic-emission spectrometric method for routine water quality testing. *Appl Spectrosc* 33(3):220–226.
37. Garbarino JR, Taylor HE (1996) *Inductively coupled Plasma-Mass Spectrometric Method for the Determination of Dissolved Trace Elements in Natural Water*, U.S. Geological Survey Open-File Report (U.S. Geological Survey, Washington, DC), Vol 94-358.
38. Bovee TFH, Schoonen WGEJ, Hamers ARM, Bento MJ, Peijnenburg AA (2008) Screening of synthetic and plant-derived compounds for (anti)estrogenic and (anti) androgenic activities. *Anal Bioanal Chem* 390(4):1111–1119.
39. Cherdshewasart W, Mahapanichkul T, Boonchird C (2010) Estrogenic and anti-estrogenic activities of the Thai traditional herb, Butea superba Roxb. *Biosci Biotechnol Biochem* 74(11):2176–2182.
40. de Haan L, Hooijerink D, Bor G, Murk AJ, Brouwer A; Hoogenboom LAP (2001) Estrogenic activity of estradiol and its metabolites in the ER-CALUX assay with human T47D breast cells. *APMIS* 109(2):101–107.
41. Bhavnani BR, Tam SP, Lu X (2008) Structure activity relationships and differential interactions and functional activity of various equine estrogens mediated via estrogen receptors (ERs) ERalpha and ERbeta. *Endocrinology* 149(10):4857–4870.
42. Markiewicz L, Garey J, Adlercreutz H, Gurpide E (1993) In vitro bioassays of non-steroidal phytoestrogens. *J Steroid Biochem Mol Biol* 45(5):399–405.
43. Blair RM, et al. (2000) The estrogen receptor relative binding affinities of 188 natural and xenochemicals: Structural diversity of ligands. *Toxicol Sci* 54(1):138–153.
44. Choe SY, et al. (2003) Evaluation of estrogenicity of major heavy metals. *Sci Total Environ* 312(1-3):15–21.
45. Zuur PJ, Ieno EN, Walker NJ, Saveliev AA, Smith GM (2009) *Mixed Effects Models and Extensions in Ecology with R* (Springer, New York).
46. Guisan A, Zimmerman NE (2000) Predictive habitat distribution models in ecology. *Ecol Modell* 135(2-3):147–186.
47. Kuiper GGJM, et al. (1998) Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. *Endocrinology* 139(10):4252–4263.
48. Soto AM, et al. (1995) The E-SCREEN assay as a tool to identify estrogens: An update on estrogenic environmental pollutants. *Environ Health Perspect* 103(Suppl 7): 113–122.