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Amphibian intersex in suburban landscapes

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Abstract. Within the last decade, reproductive abnormalities have been discovered in wild amphibian populations of multiple species and in a variety of regions in North America. Predominantly, these field studies have focused on agricultural landscapes. In this study, we worked in suburban environments based on preliminary evidence showing that amphibian populations can display surprisingly high frequencies of reproductive deformities, including intersex traits. Here, we report results from 28 suburban ponds located near onsite septic systems as well as those located in sewered neighborhoods. Caffeine, an indicator of domestic wastewater contamination, was detected in more than 70% of all ponds; prevalence of contamination was indistinguishable for ponds in sewered neighborhoods and those served by onsite septic systems. Among green frogs (*Rana* (=*Lithobates*) *clamitans*) collected from the same ponds, intersex was detected in each population; on average, one male in five showed evidence of intersex. This frequency was insensitive to wastewater treatment mode. Given prior findings that intersex is absent or rare in less developed landscapes, our results suggest that domestic wastewater contamination in suburban contexts may be more widespread than is generally appreciated and should be investigated as a contributor to intersex in wild amphibians. This hypothesis is consistent with abundant prior research on wild riverine and estuarine fish populations associating reproductive deformities with wastewater exposure.

Key words: caffeine; chemical pollution; frog; oocyte; septic systems; sewer; wastewater; wetland.

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Introduction

Chemical pollution is an important conservation concern for freshwater species (e.g., Stuart et al. 2004, Dudgeon et al. 2006). Agricultural pesticides and fertilizers, industrial effluent and runoff from roads are known to contribute to pollution of freshwaters (Kolpin et al. 2002, Schwarzenbach et al. 2006). There is corresponding evidence that aquatic species can be negatively influenced by exposure to contaminants (Purdom et al. 1994, Jobling et al. 1998, Boone and James 2003, Relyea 2005, Rohr and Crumrine 2005, Davidson and Knapp 2007, Kloas et al. 2009). In this study we focus on amphibians. Intersex in amphibians has received widespread notice since laboratory studies beginning a decade ago linked abnormal sexual development with exposure to the pesticide atrazine (Hayes et al. 2002*a*). Subsequent field studies have revealed that intersex can be common in wild populations (Hayes et al. 2002*b*, Smith et al. 2005, Murphy et al. 2006, McCoy et al. 2008, McDaniel et al. 2008). This research has supported a link between elevated incidence of sexual abnormalities and proximity to agricultural practices. However, amphibian field studies were inspired by the results of pesticide exposure experiments in the laboratory. As a consequence nearly all of the amphibian field studies of sexual abnormalities have used agricultural versus reference sites or gradients of agricultural intensity as the foundation for sampling strategies. This approach has generally meant that the non-agricultural portions of the landscape have not been closely characterized and that common landscape types have remained largely unstudied.

A first effort to estimate responses across a wider diversity of land cover types suggests a more complex picture for the natural history of amphibian sexual abnormalities (Skelly et al. 2010). In that study, sexual abnormalities in green frogs (*Rana* (=*Lithobates*) *clamitans*) were discovered in all developed landscape types (agricultural, suburban, urban) and were entirely absent within undeveloped (forested) landscapes. Surprisingly, frequencies of abnormalities were up to three times higher in densely settled landscapes (suburban, urban) compared with agricultural contexts. These findings imply that landscapes like suburban neighborhoods can be hotspots for sexual abnormalities in wildlife, but there is currently no evidence regarding causative agents.

One potential explanation for this landscape pattern is that contamination of amphibian habitat by endocrine disruptors derived from domestic sources such as wastewater contributes to elevated frequencies of intersex. The possibility that wastewater contaminates suburban ponds and that it may cause abnormal sexual development in amphibians has not been explored previously. However, treated wastewater is widely implicated in the observation of elevated frequencies of sexual abnormalities in fish living in rivers and estuaries around the world (Kloas et al. 2009). The well developed understanding of wastewater mediated abnormalities within fish coupled with the recent discovery of amphibian abnormalities in developed landscapes prompted the study described here. We focus specifically on an examination of suburban ponds and resident amphibian populations. Our goals were (1) to sample extensively across ponds located in suburban neighborhoods to gain a better understanding of the prevalence and intensity of sexual abnormalities in this context, (2) to evaluate whether these responses were differentially associated with different modes of treating wastewater, and (3) to directly evaluate whether suburban surface waters are being contaminated by domestic wastewater. To achieve this last goal, we sampled ponds for the presence of caffeine, an indicator of wastewater contamination (Buerge et al. 2003, Hillebrand et al. 2012). To maintain comparability with our earlier study, and because they are widely distributed in suburban ponds, we used green frogs as our focal species.

Methods

Study design

We focused on wetlands within the town of Avon, Connecticut, USA, for which we had access to information on the locations of sanitary sewer lines and onsite septic treatment systems. Avon covers an area of 58.5 km^2 and has a population of 18,098 people (U.S. Census Bureau 2010). The town is suburban in character: 71% of the households live in detached single-family homes (U.S. Census Bureau 2010). Correspondingly, there is very little agricultural activity within the town of Avon and none near any of our sampling locations. Within Avon, we identified potential sampling locations using digital National Wetland Inventory (NWI) maps. To maximize the chance of finding our study species, the green frog, we focused on palustrine, permanent/semi-permanent, unconsolidated bottom ponds (NWI code: PUBH/F). This screen yielded a total of 96 ponds. We eliminated very large wetlands from consideration (>25,000 m^2), netting 89 ponds. These 89 locations were then listed in random order. We visited ponds in listed order and included them in our sample if the wetland existed as categorized on the NWI map, and if we could obtain landowner permission. Our efforts yielded 28 sampled ponds.

Sampling

We collected one-liter water samples from each pond on two dates between 6/16/2009 and 10/29/2009. Samples were collected from just below the water surface in clean Nalgene bottles. Following collection, samples were placed on ice and returned the same day to the laboratory, where they were stored at 4°C until analysis for caffeine. Each pond was photographed, and we recorded pond size, pH, conductivity, dissolved oxygen concentration, and water temperature.

We searched each pond for green frogs and successfully collected animals (using dip nets and hand capture) from 19 of the 28 sampling locations. All frogs were collected between 7/22/09 and 9/28/09 and all ponds were visited between at least 4 occasions. Each amphibian sampling visit lasted 30 to 120 minutes. Within each pond we attempted to collect 20 adult or juveniles showing outward morphological structures indicative of males (tympanum size, thumb width, throat pigmentation). We averaged 17 individuals per pond. At 3 ponds we collected fewer than 3 individuals. While establishing that a species is entirely absent is challenging (Werner et al. 2007), most of the study ponds are relatively small and green frog larvae are unusual in having larvae present year round. In each of the ponds where we failed to find metamorphosed green frogs, neither was there any evidence of larval stage green frogs. Following collection, individuals were placed in coolers with ice packs and returned the same day to the laboratory.

Caffeine analysis

We tested for the presence of caffeine within each pond to provide an indicator of contamination by domestic wastewater (Hillebrand et al. 2012). Note that we do not hypothesize that caffeine plays a role in abnormal sexual development of amphibians. Analytical methods were modified from those described in Buerge et al. (2003). All pond water samples and field blanks were filtered prior to extraction using GF/C filter membranes. Solid phase extraction with Oasis HLB cartridges was used to extract caffeine from the water samples. Procedural blanks (1 L DI water) were run periodically to check for caffeine contamination during extraction. Caffeine was recovered from the cartridges with 5 ml of methanol. The extracts were then concentrated down to 1 mL under a gentle flow of N^2 gas. Extracts were transferred by pipette to autosampler vials, and 10 μL of 100 ppm 13C-caffeine internal standard were added to the extracts (for a concentration of 1 ppb 13C-caffeine in the autosampler vial).

Analysis was conducted on a Hewlett Packard G1800C GC/MS, which was run in selected ion monitoring mode. The peak produced by the 197 ion was used to estimate concentration of the 13C-caffeine internal standard (qualifier ion = 111); the peak produced by the 194 ion was used to estimate concentration of the natural 12C-caffeine (qualifier ion = 109). The instrument detection limit for prepared caffeine standards was 0.3 ppb per injection, however, due to noise in the baseline of chromatograms from some of the pond samples, the detection limit was closer to 1 ppb/injection in some cases. To assess the extraction efficiency of caffeine, 1-L samples of DI water with caffeine concentrations of 20 ppt, 50 ppt, 100 ppt, and 1 ppb were extracted, concentrated, and analyzed, yielding percent recoveries between 64% and 130%. On two separate occasions, pond water samples were spiked with caffeine and analyzed in order to assess the extraction efficiency of caffeine from natural water samples (approx. 65% recovery). Field and procedural blanks showed caffeine concentrations between 0.3 and 30.0 ppt. For the purposes of our analyses we declared detection of caffeine as an estimate of >30.0 ppt within at least one of the samples from a given pond.

Histological preparation

Green frogs were euthanized in an overdose of buffered tricaine solution (MS-222). A small incision was made under the arm, to allow fluids to penetrate the body cavity. Animals were fixed in Bouin Fixation Fluid for 48 hrs, then transferred to a preservative made from 70% ethanol with saturated lithium carbonate (20–30 mL LiCO3 per 1 L 70% EtOH). Preservative was refreshed after 24 hrs.

Animals were examined under a dissecting microscope (Leica MZ12.5) to measure snout-vent length (SVL), to confirm gender identification, and to record any gross morphological abnormalities. We then surgically opened the ventral surface of the body exposing the internal organs. After photographing the intact organs and examining gonads for evidence of abnormal morphology, we dissected out the left gonad (in a small number of cases the left gonad was damaged or rendered unusable through a mistake in histological preparation; in those cases we used the right gonad). Dissected gonads were placed in tissue cassettes and immersed in preservative. Excised gonads were embedded in paraffin. For large individuals (>40 mm SVL),

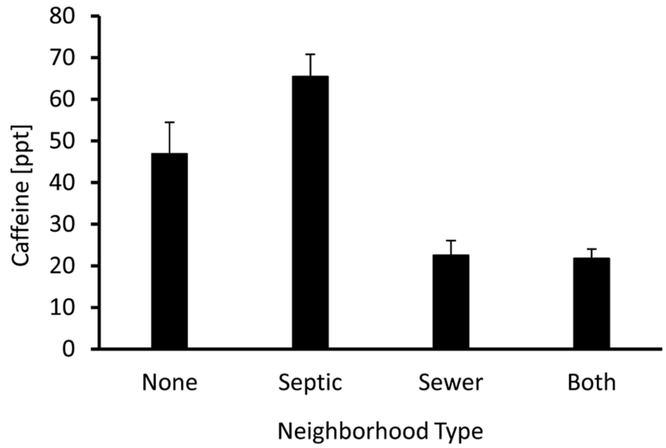

Fig. 1. Fraction of ponds in which caffeine was detected from at least one of two water samples. The 28 ponds are categorized as being within 100 m of the nearest sewer line ($n = 4$), septic system ($n = 14$), neither ($n = 5$), or both ($n = 5$).

gonads were sagitally sectioned every 5 μm. Every 20th section was mounted and stained with H & E yielding a representative section for every 100 μm of tissue and a total of 8 sections. For smaller individuals (SVL = 20–40 mm), gonads were sectioned every 3 μm and every 20th section was mounted and a total of 4 sections were examined for each individual.

Tissue sections on each slide were first screened on a Leica MZ12.5 microscope at 100× magnification for identification of testicular oocytes. The number of oocytes per section was recorded, and all oocytes were photographed using a Leica DC 200 digital camera. Each slide was then screened a second time on a Leitz compound scope at 500× magnification to ensure detection of small or partial testicular oocytes. We estimated the frequency of abnormalities as the proportion of animals collected from a given pond which had testicular oocytes within their gonads.

Neighborhood characterization

Wastewater treatment in Avon is completed through both sanitary sewers and onsite septic treatment systems. We characterized neighborhoods surrounding ponds as sewer, septic, or both based on the presence of sewer lines and onsite septic treatment systems within 100 m of the shoreline of a focal pond. Across the 28 ponds from which we collected water samples, 14 were adjacent to at least one septic system, 4 were located adjacent to a sewer line, 5 ponds were adjacent to both sewer lines and septic systems, and 5 ponds for which septic systems and sewer lines located at least 100 m or more from the shoreline. For our analysis of testicular oocyte frequencies across the subset of 16 ponds from which sufficient green frogs were collected, we combined sewer ($n = 2$ ponds) and both ($n = 1$ pond) into a single category resulting in three categories: none, septic, sewer/both.

Results

Caffeine was detected in more than 70% (20 of 28) of ponds and averaged 48 ppt (range: 0–286 ppt; SE = 11). We categorized neighborhoods as being adjacent to septic systems ($n = 14$ ponds), sanitary sewers ($n = 4$), both septic and sewer ($n = 5$), or neither located within 100 m ($n = 5$). Caffeine was detected in ponds within each of the neighborhood types and there was no evidence that caffeine concentration varied among ponds in neighborhoods of different types (Fig. 1; one-way ANOVA: $F_{3,24} = 1.043$, $P = 0.39$).

A total of 256 male green frogs were collected from 19 ponds but were rare (≤3 individuals recovered) in three of them. Within the remaining 16 ponds (≥9 individuals recovered), we discovered abnormal sexual development in the form of testicular oocytes within males in each of the green frog populations. All neighborhood types

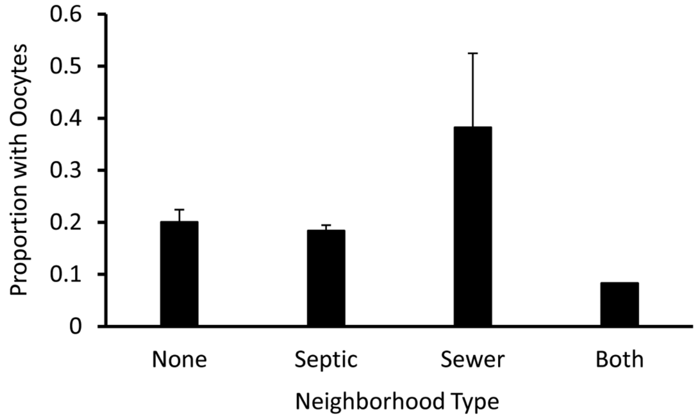

Fig. 2. Frequency of testicular oocytes within male Green frogs collected from ponds located within 100 m of septic systems ($n = 9$), sewer lines ($n = 2$), neither ($n = 4$), or both($n = 1$). Frogs were collected from a total of 16 ponds. Error bars represent 1 SE.

were represented in the subset of ponds from which we recovered adequate male green frog samples: septic ($n = 9$), sewer lines ($n = 2$), neither ($n = 4$), or both ($n = 1$). Estimated frequency of abnormalities across ponds ranged from 6 to 58% of animals examined (mean = 21%). We evaluated the association between abnormality frequency and neighborhood type. Because of the limited number of ponds in sewer and both categories, we combined them to create three neighborhood categories: septic ($n = 9$), neither ($n = 4$), and sewer/both ($n = 3$). Among these three categories, frequency did not vary by neighborhood type (Fig. 2; one-way ANOVA: $F_{2,13} = 0.60$, $P = 0.56$). Neither was there evidence that caffeine concentration differed between green frog present ($n = 16$) versus absent ($n = 9$) ponds (t-test: df = 23, $t = 0.96$, $P = 0.35$).

Discussion

Conservation biologists have published extensively on threats associated with suburbanization of landscapes (e.g., McDonnell and Pickett 1990, Gibbs et al. 2005, McDonald et al. 2008). Typically, conversion and fragmentation of habitat have been considered closely together with the challenges presented by roads and other features of converted landscapes (Trombulak and Frissell 2001, Miller and Hobbs 2002). In spite of widespread concern about pollution, studies of chemical contamination in suburban contexts have focused more often on the pattern of contamination as opposed to its potential influences (e.g., Eckhardt and Stackelberg 1995, Standley et al. 2008). In this study we follow up on an analysis of amphibian intersex across several towns and covering multiple landscape types (undeveloped, agricultural, suburban, urban), in which suburban landscapes yielded populations with the highest frequencies of males displaying intersex characteristics (Skelly et al. 2010). Among several questions raised by this pattern, one is whether contamination of freshwater ponds by domestic wastewater may contribute to intersex in amphibians. For our study we asked how frequently intersex was encountered in suburban ponds, and specifically whether intersex responses varied among amphibian populations living in neighborhoods that differed in the proximity to sanitary sewers versus residential onsite septic treatment systems. In addition, we sampled each pond for the presence of caffeine as an indicator of wastewater contamination. We found that intersex in suburban green frogs was both widespread among sampled ponds and frequent within populations. In fact, we detected intersex within each of the 16 ponds from which we collected sufficient samples of green frogs. Overall, one male in five was found to harbor testicular oocytes, a frequency comparable to that seen in a much smaller sample of suburban ponds in our previous study (Skelly et al. 2010). A review of prior studies shows that the relative ubiquity of intersex in suburban ponds stands in sharp contrast to the near total absence of intersex recorded in undeveloped or reference landscapes (Reeder et al. 2005, Skelly et al. 2010, Papoulias et al. 2012; D. K. Skelly, *unpublished data*). Across Avon's ponds, we found relatively modest variation in intersex frequencies among populations even though the ponds we sampled differed in the potential nearby sources of wastewater. This pattern was echoed in the results of our caffeine sampling which also revealed widespread evidence of wastewater contamination (caffeine was detected in >70% of all ponds sampled).

Our study is one of the first to examine contamination and intersex in 'backyard' ponds. Nevertheless, the patterns we discovered are consistent with the few studies of lakes carried out in residential contexts. Standley et al. (2008) found contamination including steroid hormones (estrone, progesterone) within 3 kettlehole lakes on Cape Cod surrounded by high residential density; contamination was less prevalent in three kettlehole lakes surrounded by lower residential density. A study of Minnesota lakes similarly revealed widespread contamination (Writer et al. 2010); estrogenic contaminants (e.g., bisphenol A, 17β-estradiol, estrone, 4-nonylphenol) were detected in 90% of sampled lakes. In some lakes, endocrine disrupting compounds were detected at concentrations near those reported for wastewater treatment plant effluent. These examples are representative of an emerging sense that pharmaceuticals and other chemicals of domestic origin commonly reach surface waters (e.g., Heberer 2002, Ying et al. 2002, Spongberg and Witter 2008, Wu et al. 2008).

Future amphibian studies will benefit from consideration of the relevant research on fish in which a chain of causation has been established between estrogenic compounds within surface waters subject to inputs of treated wastewater and sexual abnormalities in wild populations (reviewed by Kloas et al. 2009, Metcalfe et al. 2010). This work has revealed that estrogenic compounds are present and sufficiently concentrated in surface waters subject to inflows of treated wastewater (Spengler et al. 2001, Drewes et al. 2005) to promote intersex (e.g., Blazer et al. 2007, Kidd et al. 2007, Watanabe et al. 2009). Correspondingly, numerous studies have shown that the frequency of abnormal sexual development, including intersex traits, is strongly elevated at riverine sites downstream of wastewater treatment outfalls (e.g., Vajda et al. 2008) to the degree that the abnormalities have been quantitatively associated with upstream human population density (DesForges et al. 2010). This body of research has demonstrated that even treated wastewater, in which the concentrations of estrogens have been substantially depleted, is capable of promoting abnormal development. The situation for suburban amphibians in small ponds is likely to differ somewhat since wastewater would either be untreated (e.g., leaking from sanitary sewer lines) or has been treated using domestic onsite systems that, in general, are likely to yield effluent that retains many organic contaminants (Kolpin et al. 2002, Verstraeten et al. 2005, Swartz et al. 2006, Standley et al. 2008). Leakage from sanitary sewers, known as exfiltration, is a documented phenomenon which can lead to significant contamination but the overall extent of the problem is poorly characterized (Rutsch et al. 2008).

While contamination of suburban surface waters may be more common than is generally appreciated, is there evidence that amphibians are affected by the compounds they are likely to encounter at the prevailing concentrations? This is a critical question because, even in the better studied fish systems, individual contaminants are frequently encountered at concentrations below no-effect thresholds, implying that animals may be responding to the influence of mixtures. This interpretation is supported by studies of cultured tissues and whole organisms showing that, when exposures involve combinations of compounds, the concentrations of individual compounds need not exceed the scale of ng/L (Silva et al. 2002). The Minnesota lakes study (Writer et al. 2010) yielded a remarkable result: plasma samples from male fathead minnows (*Pimephales promelas*) caged within each lake for 21 days yielded vitellogenin, a protein associated with egg production and normally absent in males. Amphibians also are known to be sensitive to estrogenic compounds found in wastewater. In one example, Northern leopard frogs (*Rana pipiens*) raised in a range of dilutions of municipal wastewater effluent (estrogenic activity equivalent up to 2 ng/L estradiol) showed delays in time to metamorphosis, as well as an increased incidence of testicular oocytes among males (Sowers et al. 2009). Further research will be needed to reveal whether contaminants found in suburban ponds are capable of producing sexual abnormalities in amphibians, and whether those abnormalities are correlated with functional consequences to breeding individuals. It is also important to note that the patterns we uncovered do not discount the potential role of other types of contaminants such as pesticides. While agricultural activity is largely absent within the study area, suburban landscapes are subject to a great variety of pesticide applications (Bormann et al. 2001).

Our findings suggest that future studies of amphibians and other wildlife in suburban environments are critically needed. There is a particular demand for field studies evaluating the identities and concentrations of relevant contaminants (including both wastewater contaminants as well as pesticides and other potential types of endocrine disruptors) alongside studies exploring the functional consequences to individuals displaying intersex characteristics. More broadly, our findings suggest that consideration of the conservation implications of chemical contamination, while highly relevant to agricultural landscapes, may extend beyond them. Suburban and urban landscapes are recipients of an enormous diversity of chemical contaminants for which the fate and influences on natural communities remain poorly understood.

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