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Original Contribution

Intersex Frogs Concentrated in Suburban and Urban Landscapes

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Abstract: The occurrence of intersex characteristics in amphibians has been linked to pesticide exposure in the laboratory and proximity to agricultural activity within natural populations. But, overall, the ecology of amphibian intersex is poorly known and, specifically, its occurrence in many landscape types and regions is unstudied. We offer the first analysis of the frequency of amphibian intersex across a range of land covers representing the major landscape types within a region. We used remotely sensed information to characterize land cover surrounding 4774 potential sampling locations within the Connecticut River Valley. From among these, we selected 24 ponds to collect postmetamorphic green frogs (*Rana clamitans*) from four land cover types: undeveloped, agricultural, suburban, and urban. Collected males were preserved and, then, prepared gonadal tissue samples were screened for the presence of testicular oocytes. A total of 233 animals was examined. Thirteen percent of all male green frogs had gonads containing testicular oocytes. Sexual abnormalities were not randomly distributed among sites or landscape types. Suburban landscapes had the highest frequency of abnormalities (21%), and both suburban and urban land covers were positively associated with the presence of abnormalities within a population. There was no evidence of a positive association with agricultural land cover. Examination of amphibian intersex across a range of contexts reveals that developed landscapes may be hotspots for abnormal sexual development. This new finding suggests that other mechanisms, not previously considered, could contribute to intersex in natural amphibian populations.

Keywords: amphibian, hermaphroditism, free living, frog, intersex, landscape

Introduction

Abnormalities in vertebrate sexual development can involve the presence of both male and female traits within a single individual. While intersex conditions are known in a range of vertebrate groups, their development has been particularly well studied within amphibians (Witschi, 1921a, 1921b; Cheng, 1929; Storrs-Mendez and Semlitsch, 2010). Interest in intersex frogs was raised considerably after publications linking abnormal sexual development with exposure to an agricultural herbicide, atrazine (Hayes et al., 2002, 2003). Subsequently, researchers conducting field studies have detected sexual abnormalities in free-living amphibian populations of several species (Hayes et al., 2002; Smith et al., 2005; Murphy et al., 2006; McCoy et al., 2008; McDaniel et al., 2008). Most studies have been conducted with a focus on agricultural land uses. Agricul-

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tural sites have been compared with nonagricultural reference sites (e.g., McDaniel et al., 2008), or a gradient of agricultural intensity, defined by land use or pesticide application rate, has been evaluated (e.g., McCoy et al., 2008). Collectively, these studies have yielded two primary results. First, amphibian intersex in wild populations is more common than previously recognized, and perhaps more common than it has been historically (Reeder et al., 2005). Second, most studies are consistent with enhanced risk to amphibians when living in proximity to agricultural activities. These results have been used to conclude that exposure to atrazine, or other chemical agents used in agricultural landscapes, may be responsible for elevated frequencies of abnormal development in amphibians (e.g., Hayes et al., 2002; Murphy et al., 2006; McCoy et al., 2008; Mann et al., 2009).

In this study, we expand the study of intersex in wild amphibians to encompass the range of land covers encountered within a region. Because prior field studies were motivated by particular results from laboratory exposure experiments, or for other unspecified reasons, previous researchers have not addressed broader questions regarding the geography of amphibian intersex. It is possible that by focusing on a portion of the range of landscape types in which amphibians live, these previous studies do not offer a full picture of the distribution of abnormal sexual development in amphibians. A broader perspective could either reinforce the need to evaluate agricultural exposures, or conversely, highlight previously undocumented geographic patterns of sexual deformities. In the latter case, there may be reasons to expand the range of hypotheses being evaluated.

Methods

We collected green frogs (*Rana clamitans*) at 24 Connecticut wetlands within the watershed of the Connecticut River. All sampled wetlands appeared on National Wetland Inventory (NWI) digital maps and were categorized as palustrine, permanent/semipermanent, unconsolidated bottom (NWI category: PUB). Wetlands were selected for sampling from a pool of 4774 PUB wetlands initially screened by first assigning all wetlands to one of four landscape types based on the predominant land covers: undeveloped, agricultural, suburban, urban. Land cover classes were estimated within a buffer of 200 m surrounding the pond shoreline using the Connecticut Department of Environmental Protection 1995 Land Use Land Cover Database. For each of the four landscape categories, several land covers were combined to estimate total percentage.

We defined agricultural land cover as the total area covered by Exposed Soil, Exposed Soil/Cropland, Pasture & Hay/Cropland, Pasture & Hay/Exposed Soil, and Shade-Grown Tobacco. We defined urban land cover as the total area covered by Commercial & Industrial & Pavement, and Residential & Commercial categories. We defined suburban land cover as the total area covered by Rural Residential, and Turf & Tree Complex categories. Finally, we defined undeveloped land cover as the total area within a buffer covered by Coniferous Forest, Deciduous Forest, and Mixed Forest categories.

After characterizing land covers in buffers, all ponds were ranked based on their cumulative covers in each of the types. The top 50 ranked wetlands in each landscape type (and the top 100 for agricultural landscapes) were placed in a list. The list was then randomized and ponds on each list were visited in that order. We visited a total of 136 ponds including at least 12 ponds on each of the four lists.

Within each landscape type, wetlands were sampled if the wetland existed, if permission for sampling was granted by the landowner, and if green frogs were present within the wetland. Uneven numbers of sampled wetlands among landscape types reflected sharp differences in the prevalence of green frogs. Most notably, green frogs were particularly uncommon within undeveloped (forested) landscapes, a pattern also noted in a survey of Connecticut wetlands (Skelly et al., 2006).

All sampling visits took place between July 9 and September 30, 2005. At each wetland, we verified in person that the major land cover classes surrounding a wetland were accurately reflected by our prior GIS. We targeted a collection total of 20 postmetamorphic (juvenile or adult) green frogs. Following collection, animals were placed on ice, and returned to the lab where they were euthanized using MS-222. For each individual, a small incision through the skin was made just caudal of the forearm. This incision allowed the fixative (Bouin's solution) to penetrate the body cavity. After 48 h in fixative, specimens were transferred to 70% ethanol saturated with 2–3% saturated lithium carbonate. After 24 h, they were placed in fresh ethanol/lithium carbonate.

In this study, we focus on individuals identified as males from their gross external morphology. Because males were absent from specimens collected at one pond, this study is based on a total of 23 sampled ponds (233 individuals total). Each individual was dissected by opening the body cavity to expose the gonads. The left gonad was removed and placed in a tissue cassette and stored in ethanol/lithium carbonate solution. Each entire gonad was embedded in paraffin and sagitally sectioned every 5 μm. Every 20th section was retained, stained with hematoxylin and eosin, and then examined for the presence of testicular oocytes.

We conducted statistical analyses relating landscape-based predictors to pond-wide responses reflecting the presence and frequency of testicular oocytes. In all cases, the frequency of testicular oocytes was angular transformed prior to analyses. We used a one-way Analysis of Variance (ANOVA) and pairwise comparisons (Fisher's Least Significant Difference) to evaluate whether testicular oocyte frequency varied among landscape types. We used logistic regression to estimate the relationship between the proportion of the 200-m landscape buffer within different land covers and the frequency of testicular oocytes. Specifically, we compared all possible combinations of agricultural, suburban, and urban land covers as predictors. Undeveloped land cover was excluded from the models to prevent problems of collinearity. We evaluated models based on Akaike's Information Criterion (AIC). Finally, we evaluated spatial autocorrelation in the frequency of testicular oocytes using a Mantel test. All analyses were run in XLStat (version 2010.2.03).

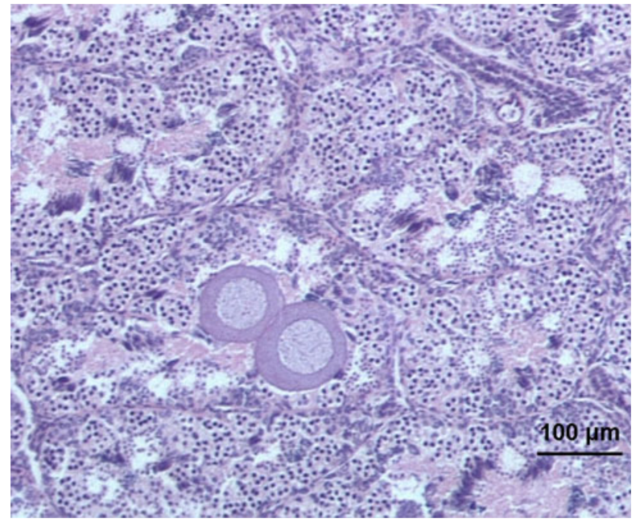

Figure 1. Photomicrograph of two oocytes within a stained section of testicular tissue from an adult green frog.

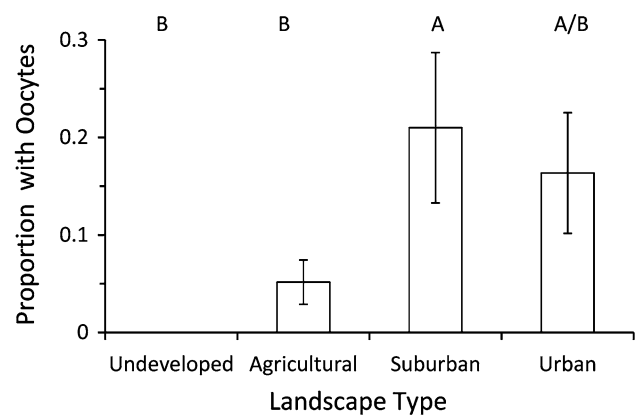

Figure 2. Proportion of individual green frogs with testicular oocytes across 23 Connecticut ponds located in four wetland types. Averages are calculated across 2 undeveloped ponds, 11 agricultural ponds, 6 suburban ponds, and 4 urban ponds. *Letters* represent groups with indistinguishable frequencies according to a Fisher's Least Significant Difference test. *Error bars* denote 1 SE.

Results

Each of the 24 sampled ponds was categorized and selected for sampling based on the predominating land cover type. In all, we sampled 12 agricultural ponds, 6 suburban ponds, 4 urban ponds, and 2 undeveloped ponds. No male frogs were recovered from one of the agricultural ponds; our analyses focused on the remaining 23 ponds. Of 233 male green frogs evaluated, examination of sectioned and stained gonad tissue revealed testicular oocytes (Fig. 1) in 31 cases (13%). The frequency of testicular oocytes varied among landscape categories (one-way ANOVA: $F_{3,19} = 3.34$; $P = 0.041$), ranging from 0% in undeveloped landscapes to an average of 21% in suburban contexts (Fig. 2). Urban landscapes were associated with an abnormality frequency of 16%, and the corresponding frequency in agricultural landscapes was 6%. The frequency of abnormalities in suburban landscapes differed from those in undeveloped ($P = 0.03$) and agricultural ($P = 0.02$) landscapes (Fisher's Least Significant Difference test). Abnormal gonads were observed in 5 of 6 suburban ponds, 3 of 4 urban ponds, 4 of 11 agricultural ponds, and 0 of 2 undeveloped ponds.

Across three of the four landscape types, nearly all of our sampled ponds were surrounded by buffers comprised of multiple land cover types (undeveloped ponds were surrounded by completely forested buffers). We further examined the potential contributions of land covers to amphibian reproductive development using logistic regressions of the presence of testicular oocytes against land

Table 1. Logistic regressions of the presence of testicular oocytes within a green frog population against land cover proportion within 200-m buffers surrounding 23 Connecticut ponds[a]

Variables	Likelihood		
	Ratio	Probability	AIC
Suburban	25.80	0.014	31.80
Urban/suburban	23.20	0.013	31.20
Urban/suburban/agricultural	21.11	0.013	31.11

AIC Akaike's Information Criterion.

[a]Included land covers were categorized as suburban, urban, and agricultural. Undeveloped land cover was not included to avoid collinearity within models. For one, two, and three factors, the best-fit models are displayed along with their AIC values.

cover predictors (fraction of the buffer in suburban, urban, and agricultural covers). Among all combinations of three or fewer predictors, three models of testicular oocyte presence based on three predictors (suburban, urban, and agriculture), two predictors (suburban, urban), and one predictor (suburban) were roughly equivalent based on AIC scores (Table 1). Overall, the presence of testicular oocytes within a green frog population tended to be positively related to the fraction of the buffer in suburban and urban land covers, and negatively related to the fraction in agricultural land cover. Finally, a Mantel test revealed no significant association between the frequency of testicular oocytes and distances among ponds ($P = 0.85$).

Discussion

Previous studies of sexual development in free-living amphibians have concluded that agricultural landscapes foster increased risk of abnormalities (e.g., Hayes et al., 2002; Murphy et al., 2006; McCoy et al., 2008; McDaniel et al., 2008). In this study, we found that, while abnormalities were present in agricultural landscapes, abnormal sexual development tended to be concentrated in landscapes with higher densities of humans. Suburban landscapes yielded frequencies of testicular oocytes in male green frogs three times higher (21% vs. 6%) than those found in agricultural landscapes. Because 200-m landscape buffers around most ponds were comprised of more than one land cover type, we estimated the associations of different land covers with the presence of testicular oocytes in male green frogs. These analyses showed that increased suburban and urban covers were associated with increased likelihood of abnormalities; there was no evidence of a positive association for agricultural land covers.

The strong distinction in results between this and prior studies may stem from multiple causes. But one contributing factor is the range of environments examined. Prior studies were focused on the role of agriculture and associated pesticide exposures. Site selection strategies used in most studies have relied on comparisons between agricultural and reference sites or on gradients of agricultural intensity (e.g., Hayes et al., 2002; McCoy et al., 2008; McDaniel et al., 2008). In most cases, the land covers within reference sites were either not reported or were not closely characterized. In this study, we used GIS analysis to screen landscape buffers surrounding more than 4000 ponds. Our goal was to select, from that large pool, a set of wetlands that collectively represented the characteristic landscape types within a region. This approach allows us to conclude where, on the variety of landscapes, sexual abnormalities occur, and where they may be concentrated. Our study is the first, to our knowledge, to conclude that amphibian sexual abnormalities can be concentrated in developed landscapes, like suburban neighborhoods, in part because this is the first study to representatively sample across landscape types.

Abnormalities were recorded at modest frequencies in agricultural landscapes in this study (average = 6% of males with testicular oocytes). Abnormalities were encountered in just 4 of 11 agricultural ponds sampled, and the maximum frequency encountered in an agricultural pond was 20% (compared with 56% in a suburban pond). It is reasonable to ask whether these results stem from some difference between agriculture in Connecticut as opposed to agricultural environments sampled in prior studies (e.g., Florida, Iowa, Michigan, Nebraska, South Africa). While such an effect is certainly possible, of our 11 ponds, corn (*Zea mays*) was being grown within the buffer of 9 ponds. Shade tobacco (*Nicotiana tabacum*) was the next most common row crop (three ponds), followed by vegetable crops (two ponds). The predominance of corn cultivation within agricultural areas in this study matches that found in prior studies (e.g., Murphy et al., 2006; McCoy et al., 2008; McDaniel et al., 2008), suggesting that the difference does not stem from the cultivation of different crops with attendant differences in pesticide regimes.

While our study was not designed to discover causes of sexual abnormalities in amphibians, our findings do prompt questions about the factors that may elevate risk of sexual dysgenesis in settled landscapes. The range of substances capable of disrupting normal sexual development in vertebrates is broad (Sumpter and Johnson, 2005). Prior studies have focused on agricultural landscapes because laboratory results suggested that low concentrations of an agricultural pesticide, atrazine, may promote sexual abnormalities in amphibians, including testicular oocytes (Hayes et al., 2002; but see Kloas et al., 2009). While it is possible that atrazine exposure contributed to elevated frequencies of abnormalities in suburban and urban environments in this study (atrazine is sometimes used in residential settings for lawn-care applications), it is then difficult to explain much lower frequencies in a large set of agricultural ponds dominated by those in corn-growing areas where atrazine use is likely. If we expand the view to consider other potential disruptors of sexual development, there are many candidates, but one possibility stands out in particular as being worthy of further investigation. Synthetic estrogens are likely to be concentrated in areas of higher human density. These compounds (e.g., EE2, an active ingredient in some birth control and prostate medications) are known to promote sexual abnormalities in male vertebrates, including amphibians (e.g., Mackenzie et al., 2003; Pettersson and Berg, 2007), and are known to be much more potent than many xenoestrogens (e.g., bisphenol A, 4-octylphenol; Sumpter and Johnson, 2005). In fact, synthetic estrogens are often used as positive controls in exposure experiments, such as those focused on the potential effects of atrazine (e.g., Kloas et al., 2009). EE2 and other synthetic estrogens have been detected in surface and ground waters (e.g., Desbrow et al., 1998; Ying et al., 2002; Singh et al., 2010), but their presence within our study region and most other landscapes is unknown and in need of further study.

The primary goal of this study was to estimate associations between the presence and frequency of amphibian sexual abnormalities and variation across a set of representative landscape types. We were motivated by a concern that conclusions about contributing factors were being drawn without first broadly characterizing the phenomena of sexual abnormalities in wild populations. The geography of sexual dysgenesis revealed in our study ponds is difficult to reconcile with the prevailing hypothesis. This conclusion leads us to make two recommendations. First, additional studies should be carried out in which sexual development in amphibians is characterized across landscapes. It will be particularly informative if such studies are carried out in regions for which elevated frequencies of sexual abnormalities have already been discovered in agricultural contexts. And second, other hypotheses consistent with enhanced risk in areas of higher human density should be explored. While it is possible that atrazine or other pesticides could be at the base of the patterns we discovered, there are additional possibilities (e.g., exposure to estrogenic compounds leaching from septic and sewer systems) that also merit attention. At base, we can expect the most rapid progress toward an understanding of abnormal amphibian sexual development in nature, if multiple working hypotheses are entertained within research programs incorporating information from strong field-sampling efforts.

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