EcoHealth 16, 682–693, 2019
https://doi.org/10.1007/s10393-019-01447-x

EcoHealth

Original Contribution

Laryngeal Demasculinization in Wild Cane Toads Varies with Land Use

Sara Zlotnik,[1,4] Marcos Gridi-Papp,[2] and Ximena E. Bernal[1,3]

[1]*Department of Biological Sciences, Purdue University, 915 West State Street, West Lafayette, IN 47907*
[2]*Department of Biological Sciences, University of the Pacific, 3601 Pacific Avenue, Stockton, CA 95211*
[3]*Smithsonian Tropical Research Institute, Apartado 0843-03092 Balboa, Republic of Panama*
[4]*School of Natural Resources and Environment, University of Florida, 103 Black Hall, Gainesville, FL 32611*

Abstract: Anthropogenic factors, including the spread of endocrine-disrupting chemicals, have been linked to alterations in the reproductive physiology, morphology, and behavior of wildlife. Few studies of endocrine disruption, however, focus on secondary sexual traits that affect mating signals, despite their importance for reproductive success. The larynx of many anurans (frogs and toads), for example, is larger in males than in females and is crucial for producing mating calls. We aim to determine if wild populations of cane toads (*Rhinella marina*) near sugarcane fields in Florida have demasculinized larynges when compared to populations near urban areas. We find evidence of demasculinization in both primary and secondary sexual traits in male toads living near sugarcane. Relative to body size, the laryngeal mass, vocal cord length, and dilator muscle width are all reduced in males from sugarcane regions compared to their urban counterparts. Strong correlations between primary and secondary male sexual traits indicate that demasculinization occurs in concert both within and across diverse organs, including the testes, larynx, and skin. Our results show that anurans near sugarcane fields have demasculinized reproductive systems, that this disruption extends to secondary sexual traits like the larynx, and that it is likely due to anthropogenic causes.

Keywords: Anuran amphibian, Mating signal, Endocrine disruptor, Ecophysiology, Vocal cords, *Rhinella marina* (*Bufo marinus*)

Introduction

Behavioral, morphological, and physiological differences between the sexes are common among sexual organisms, and these traits are often critical for reproductive success (Andersson 1994). Recent decades, however, have seen repeated deviations from typical patterns of sexual trait expression in many vertebrate animals (Harrison et al. 1997; Edwards et al. 2006). Such changes are frequently observed in altered landscapes that are contaminated with endocrine-disrupting chemicals, which mimic or block the actions of endogenous hormones (Hamlin and Guillete 2010), and are found in many human-made products (Caliman and Gavrilescu 2009; Schug et al. 2016). Labo-

Electronic supplementary material: The online version of this article (https://doi.org/10.1007/s10393-019-01447-x) contains supplementary material, which is available to authorized users.

Published online: October 18, 2019

Correspondence to: Sara Zlotnik, e-mail: zlotniks@ufl.edu

ratory experiments have provided a foundation for understanding the mechanisms of endocrine disruption, but often lack environmental relevance and rely on model organisms that are not representative of most wildlife (e.g., Carr et al. 2003; Hayes et al. 2010; Hoffmann and Kloas 2012). Field studies provide a more accurate picture of how wild animals in disturbed areas are affected by the cocktail of pollutants to which they are exposed (Orton and Tyler 2015).

Research on reproductive abnormalities, especially in field studies, is frequently focused on primary sexual traits: gametes, gonads, and genitalia (e.g., Mackenzie et al. 2003; Edwards et al. 2006; Orton and Tyler 2015). Secondary sexual traits—sexually dimorphic traits that develop later in life and are not directly involved in gamete production—can also be critical for reproductive success. In anuran amphibians (frogs and toads), for example, males typically use mating calls to attract females (Ryan 2001; Gerhardt and Huber 2002). The development of the larynx, the organ that produces these calls, is vital to male reproduction, and males generally grow much larger larynges than females as a result (Sassoon and Kelley 1986; McClelland and Wilczynski 1989; Guerra et al. 2014). Although both feminization (growth of female tissues in males), as well as demasculinization (reductions in male sexual traits), have been documented in anurans (Kloas et al. 2009; Mann et al. 2009; Hoskins and Boone 2018), few researchers have examined how laryngeal morphology is impacted by these phenomena.

To our knowledge, only one study has investigated the effects of pollutants on anuran larynges in the wild, and it did not find evidence of demasculinization (Smith et al. 2005). There have been several laboratory experiments testing the impacts of specific chemicals on laryngeal development, but they have yielded conflicting results (e.g., Hayes et al. 2002; Carr et al. 2003; Coady et al. 2005; Hayes et al. 2010). Furthermore, these studies focused on a single species: the African clawed frog, *Xenopus laevis* (e.g., Hayes et al. 2002; Smith et al. 2005; Qin et al. 2007). Although *Xenopus laevis* is a model organism in development and genetics, its laryngeal morphology is atypical for anurans (Sassoon and Kelley 1986; Colafrancesco and Gridi-Papp 2016) as this species is fully aquatic and calls underwater (Yager 1992). It is also unclear how similar the physiological responses to endocrine disruptors are among *Xenopus* and other anuran taxa (Pattersson and Berg 2007; Tamschick et al. 2016). Therefore, examination of the effects of pollutants on laryngeal development in a broad taxonomic range of anurans is clearly needed.

Our study investigates the laryngeal morphology, along with a suite of other sexual traits, of male cane toads (*Rhinella marina*) living near sugarcane fields in South Florida, USA. Male toads from sugarcane growing areas have more frequent gonadal abnormalities (McCoy et al. 2008a) and lower spermatogenesis (McCoy et al. 2017) than male toads from nearby urban areas. These differences in sexual traits between land-use regions are likely due to agricultural pollutants as sugarcane producers in Florida rely on a range of pesticides, including known endocrine disruptors (Mossler 2008). The herbicide atrazine, in particular, is applied to sugarcane fields in this area (McCoy et al. 2008b) and is known to affect sexual trait development in multiple anuran species (Hayes et al. 2010; Gunderson et al. 2011; Hoskins and Boone 2018). Other factors may also be involved in these gonadal abnormalities, however, as anuran reproductive physiology can be affected by other environmental conditions such as parasite presence (Sitja-Bobadilla 2009) and food availability (Girish and Saidapur 2000). Independent of the origin of these human-driven reproductive alterations, cane toad populations in Florida provide an ideal opportunity to investigate demasculinization and feminization across different organs. In addition to gonadal abnormalities, for instance, the dorsal coloration and nuptial pads of male toads near sugarcane fields also show signs of demasculinization (McCoy et al. 2008a). It is unclear, however, if and how their laryngeal morphology is affected.

Our first objective was to determine if male cane toad larynges display the same pattern of demasculinization in sugarcane growing areas as that documented in other sexual traits. As anuran laryngeal growth follows similar developmental timing as other secondary sexual traits (Sassoon and Kelley 1986; Guerra et al. 2014), we expected the larynx to become demasculinized under the same environmental conditions. We predicted, therefore, that male toads near sugarcane fields would have smaller larynges than those from urban areas. Urban areas were used as our baseline for comparison as cane toads in Florida are not frequently found in natural wetlands (Wilson 2016). We also measured the larynges of female toads to verify that differences between land-use regions were restricted to males and thus indeed evidence of demasculinization.

Our second objective was to investigate the associations between laryngeal morphology and other sexual traits. We predicted that larynx size would be correlated with the expression of other sexual traits in males due to shared developmental pathways. We expected these associations to weaken near sugarcane fields, however, because different tissue types may vary in their sensitivity to pollutants or develop at life stages with different exposure rates. Due to the importance of the larynx for anuran reproduction, and in light of current rates of amphibian declines (Wake and Vredenburg 2008), it would be valuable to determine whether externally visible traits can be used to assess laryngeal demasculinization in the field.

METHODS

Toad Collection

Cane toads were introduced to Florida in the 1930s to control sugarcane pests and have been recorded near each of our collection sites dating back at least 50 years (Meshaka et al. 2004). The sugarcane fields of South Florida encompass a single region that is separated from urban areas by wetlands where cane toads are not frequently found. Our four sugarcane sites were, therefore, more clustered than our five urban sites, although the borders of all sites were at least ten kilometers apart (Fig. 1). Sugarcane sites (and number of toads per site) included Clewiston ($N = 31$), Lake Harbor ($N = 13$), Belle Glade ($N = 55$), and Canal Point ($N = 28$).

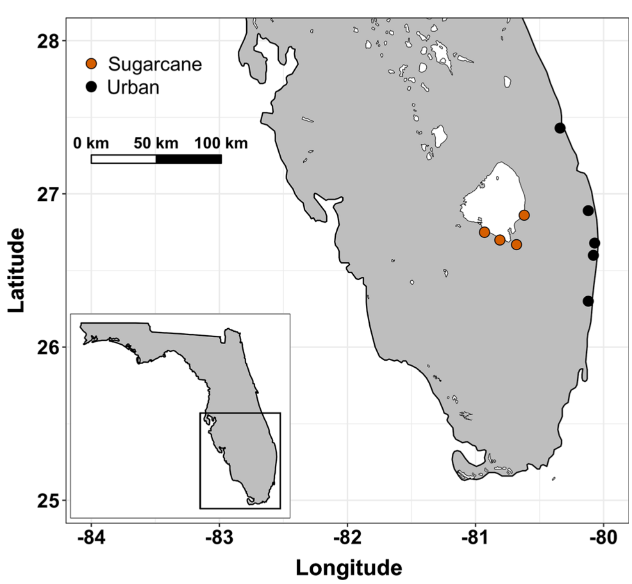

Figure 1. Collection sites of cane toads (*Rhinella marina*) in sugarcane (red) and urban (black) areas in South Florida. Sugarcane sites from west to east: Clewiston, Lake Harbor, Belle Glade, and Canal Point. Urban sites from north to south: Fort Pierce, Jupiter, West Palm Beach, Lake Worth, and Deerfield Beach (Color figure online).

Urban sites included Fort Pierce ($N = 19$), Jupiter ($N = 20$), West Palm Beach ($N = 28$), Lake Worth ($N = 11$), and Deerfield Beach ($N = 10$).

Although the dominant land-use region differed between sugarcane and urban sites, all toads were collected at night (between 21:00 and 24:00 h) in parks, sports fields, parking lots, and other residential areas. All collecting sites were close to streets, lawns, artificial lights, and either artificial or natural bodies of water. Therefore, although the urban sites had many freeways and shopping centers nearby whereas the sugarcane sites had extensive sugarcane fields nearby, the local habitats where the toads were collected were comparable across sites.

We collected and measured 215 toads: 96 sugarcane males, 61 urban males, 31 sugarcane females, and 27 urban females. Our collection dates ran from September 19, 2016, to October 24, 2016 (35 toads collected) and from June 20, 2017, to July 24, 2017 (180 toads collected). In 2017, we regularly alternated our collecting efforts among the nine sites so that both sugarcane and urban toads were consistently collected throughout this 35-day period. At all sites, only adults were collected, defined by a snout-vent length (SVL) of at least 90 mm (Zug and Zug 1979), and none were seen or collected while calling or in amplexus.

External Measurements

After collection, toads were euthanized with an overdose of 20% benzocaine gel followed by 30 min in a -20 °C freezer. We photographed the dorsum and right forearm of each toad using a digital camera (Canon PowerShot SX530 HS), measured SVL using digital calipers (Roktools Model DC-122A), and determined body mass with a portable scale (88 Adam Core Portable Compact Balance CQT 251). The toads were then preserved in 10% neutral buffered formalin and transported to Purdue University for dissection and additional measurements.

Female cane toads have mottled dorsal patterns characterized by small white spots whereas males are typically solid colored (Fig. 2a). To quantify the dorsal mottling, three independent observers used ImageJ (Schneider et al. 2012) to select the white spots in each dorsum photograph and measure their area. We then calculated the proportion of the dorsum made up of white spots, hereafter called the mottling score. The intraclass correlation coefficient (two-way mixed effects, consistency) was 0.84 ($F(214,428) = 6.20$, $P < 0.001$), indicating good reliability among the three observers (Koo and Li 2016).

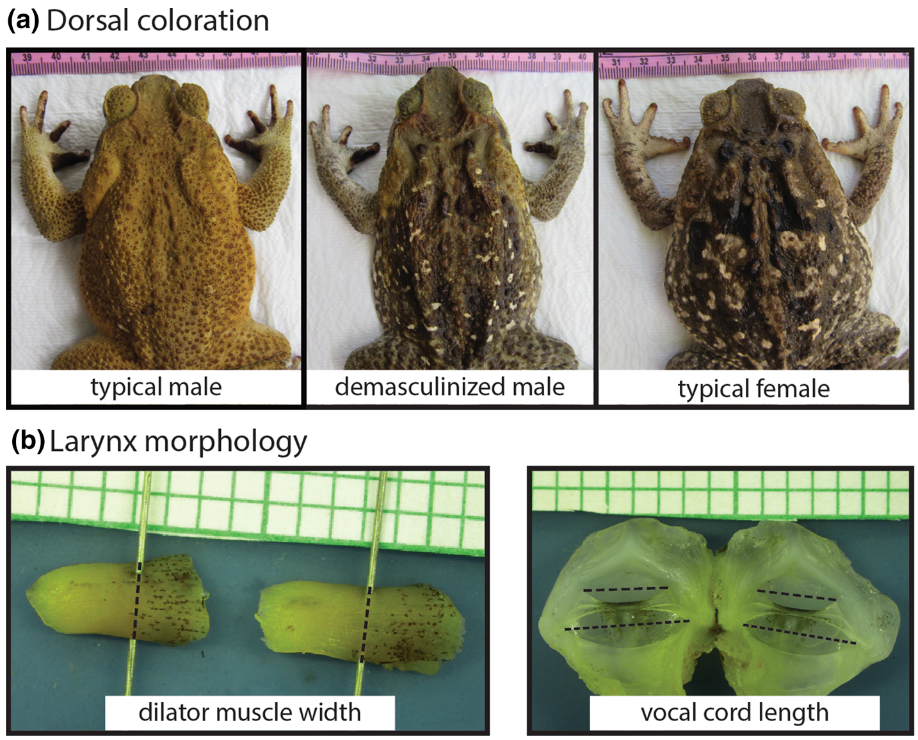

Figure 2. Photographs of cane toads collected in South Florida. Dorsal view photographs (**a**) illustrate the variation in coloration and nuptial pads between typical males, demasculinized males, and typical females. Photographs of the larynges (**b**) show the laryngeal dilator muscles, pinned at their midpoint, and arytenoid cartilages with vocal cords exposed. Grid paper shows millimeter ruler and dashed lines represent measurements taken from photographs.

Male toads have additional secondary sexual traits, including nuptial pads and enlarged forearms, that enable them to hold onto a female during amplexus (Wells 2007). We used the right-forearm photographs to measure these traits. We recorded the number of digits with visible nuptial pads (patches of dark, thick skin) and used ImageJ to measure the surface area covered by the nuptial pad on the first digit. We also estimated the average diameter of the right forearm by dividing the forearm area by its length.

Larynx Measurements

We removed the larynx from each preserved toad and transferred it to 70% ethanol. For each larynx, we marked the midpoints of the right and left dilator muscles and photographed them under a Nikon SMZ1000 microscope. Size of laryngeal dilator muscles has been previously used to assess laryngeal demasculinization in *Xenopus laevis* (Carr et al. 2003; Smith et al. 2005; Hayes et al. 2010). Using ImageJ, we measured the width of each muscle at its midpoint and averaged the right and left value for each toad (Fig. 2b, left). We then removed all muscle tissue, separated the arytenoid cartilages, and photographed the exposed vocal cords (Fig. 2b, right). We measured both edges of the right and left vocal cords and averaged all four measurements. Finally, we measured the total laryngeal mass using a Mettler Toledo AB104-S Analytical Balance.

Gonad and Fat Measurements

We removed and examined the gonads and attached fat bodies of all cane toads with testes, which we categorized as male regardless of other traits. The gonads of male cane toads include testes and Bidder's organs. Bidder's organs are found in most bufonids ("true toads") and are known to produce eggs in castrated males, although their typical functions are not well understood (Piprek et al. 2014). After preservation, the testes, Bidder's organs, and fat bodies were weighed using a Mettler Toledo AB104-S Analytical Balance. Fat body masses were recorded for 48 urban and 73 sugarcane males. For each Bidder's organ, we also recorded whether it grew as a single lobe, which is typical, or multiple lobes, which we interpreted as a sign of feminization, following McCoy et al. (2008a). Finally, we recorded the presence of either ovaries or oviducts in male toads.

Statistical Analyses

We ran generalized linear mixed models (GLMMs) using the package *lme4* (Bates et al. 2015) in R 3.4.3 (R Core Team 2017) to determine how sex and region affect each sexual trait. All models included region (sugarcane vs urban) or region and sex (sugarcane male, sugarcane female, urban male, urban female) as an independent fixed factor. We also included the year (2016 or 2017) as a fixed factor and collection site as a random factor to control for temporal and spatial differences in breeding condition. In models predicting length, area, or mass measurements, we controlled for body size by including either body mass or snout-vent length as a scaled covariate.

For models with a binomial dependent variable (single- or multi-lobed Bidder's organs; nuptial pads on one or multiple digits), we ran GLMMs with a binomial distribution. For models with continuous dependent variables, we ran GLMMs with a gamma distribution and a log link function as these variables had distributions that were positive and right skewed. For all models, we used log-likelihood ratio tests to determine the significance of sex and/or region on the dependent variable. We then applied Tukey's post hoc comparisons with the R package *emmeans* (Length 2018) to evaluate the differences between pairs of levels for models that included both male and female toads. For all models, we also re-ran the analyses with only the 180 toads from June to July 2017 (excluding the 35 toads from 2016), which represents a short collecting period in which breeding condition is unlikely to vary substantially. With one exception (explained in the "Results" section), we did not see qualitative changes in the results (i.e., P values did not change from below to above 0.05 or vice versa).

To investigate the degree of variation within each male sexual trait as well as the relationships between tissue types, we performed additional calculations on eight variables: laryngeal mass, vocal cord length, dilator width, testes mass, Bidder's organ mass, mottling score, nuptial pad size, and forearm diameter. For all variables except mottling score, we divided values by either body mass or SVL to get an index of relative trait expression. To determine whether sexual trait expression varies more in demasculinized populations, we calculated the coefficients of variation in each trait for (1) males from sugarcane sites and (2) males from urban sites. We then used nonparametric bootstrapping with 1000 replicates to determine the 95% confidence interval around each coefficient of variation using the package *boot* (Canty and Ripley 2017). Lastly, we calculated the Spearman's rank correlation coefficient for all pairs of the eight scaled variables using the package *Hmisc* (Harrell and Dupont 2018). We calculated separate correlations for sugarcane and urban males as we predicted that sexual traits would be less strongly correlated in sugarcane areas due to inconsistent impacts of endocrine disruption.

Results

External Measurements

The body sizes of toads did not differ between urban and sugarcane areas for males (mass: $z = 0.98$, $P = 0.763$; SVL: $z = 0.83$, $P = 0.841$) or females (mass: $z = 0.23$, $P = 0.996$; SVL: $z = 0.66$, $P = 0.912$). Females were larger than males in urban areas (mass: $z = 4.02$, $P < 0.001$; SVL: $z = 5.25$, $P < 0.001$), but not in sugarcane areas (mass: $z = 1.64$, $P = 0.356$; SVL: $z = 1.89$, $P = 0.231$).

We found evidence of demasculinization of both the dorsal skin and the nuptial pads. Sugarcane males had more dorsal mottling than urban males ($z = 4.40$, $P < 0.001$), but less than either urban females ($z = 5.55$, $P < 0.001$) or sugarcane females ($z = 7.75$, $P < 0.001$). Mottling scores did not differ between regions for females ($z = 0.54$, $P = 0.950$). Compared to urban males, male toads from sugarcane areas had smaller nuptial pads ($\chi^2(1) = 8.93$, $P = 0.003$; Fig. 4c) and were less likely to have nuptial pads on more than one digit per limb ($\chi^2(1) = 15.93$, $P < 0.001$). In contrast, the average forearm diameter did not differ between sugarcane and urban males ($z = 1.94$, $P = 0.213$) or between sugarcane and urban females ($z = 0.36$, $P = 0.984$). The forearm diameter was sexually dimorphic, however, as it was larger in males than in females in both sugarcane ($z = 11.22$, $P < 0.001$) and urban areas ($z = 12.34$, $P < 0.001$).

Larynx Measurements

For all the three larynx measurements, urban males had larger larynges than sugarcane males (mass: $z = 7.02$, $P < 0.001$; vocal cord: $z = 5.17$, $P < 0.001$; dilator: $z = 3.08$, $P = 0.011$; Fig. 3). Sugarcane males, however, had larger larynges than both urban females (mass: $z = 10.30$, $P < 0.001$; vocal cord: $z = 15.18$, $P < 0.001$; dilator: $z = 5.99$, $P < 0.001$) and sugarcane females (mass: $z = 13.76$, $P < 0.001$; vocal cord: $z = 15.92$, $P < 0.001$; dilator: $z = 6.78$, $P < 0.001$). Females from urban and sugarcane areas did not differ in larynx size (mass: $z = 2.29$,

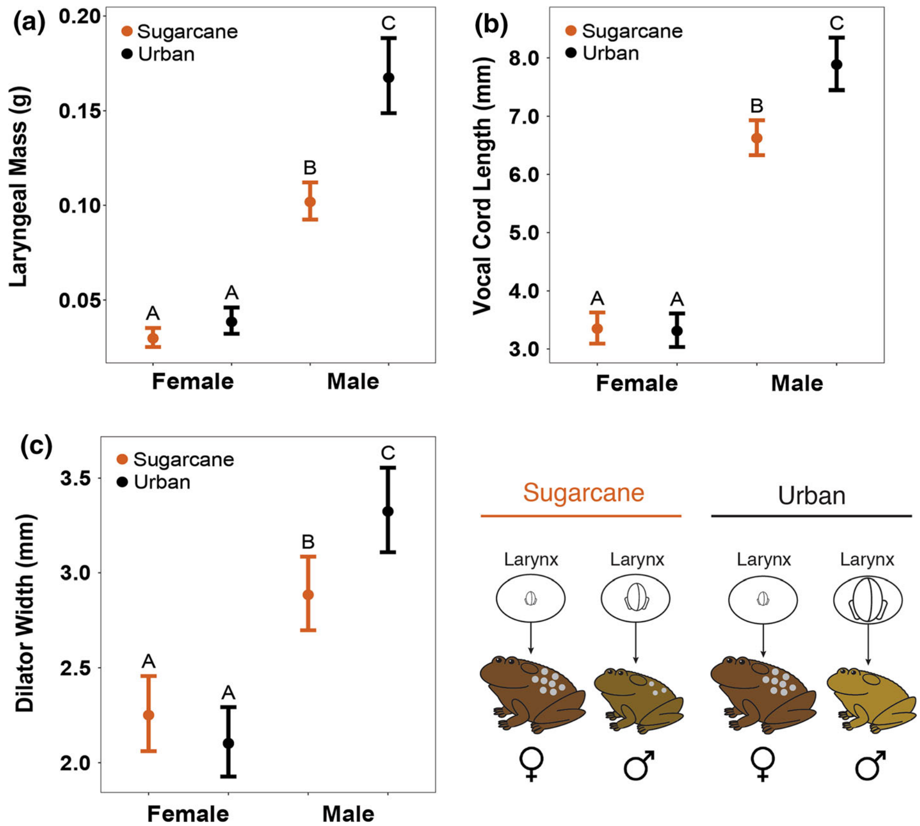

Figure 3. Comparisons of **a** laryngeal mass, **b** vocal cord length, and **c** dilator muscle width across sex and land-use region in cane toads. Plots show estimated marginal means with 95% confidence intervals derived from generalized linear mixed models that include body size as a covariate; letters denote significant differences (Tukey's HSD; $P < 0.05$). Panel **d** illustrates the differences in laryngeal and external morphology between toads from sugarcane and urban areas.

$P = 0.101$; vocal cord: $z = 0.22$, $P = 0.996$; dilator: $z = 1.18$, $P = 0.641$).

Gonad and Fat Measurements

Male cane toads from sugarcane growing areas had smaller testes ($\chi^2(1) = 5.72$, $P = 0.017$) and larger Bidder's organs ($\chi^2(1) = 4.28$, $P = 0.039$), relative to body mass, than male toads from urban areas (Fig. 4a, b). In the model including only the toads from June to July 2017, however, the difference in Bidder's organ mass between sugarcane and urban toads was not significant ($\chi^2(1) = 3.02$, $P = 0.082$). Sugarcane males were also more likely to have multi-lobed Bidder's organs than urban males ($\chi^2(1) = 6.34$, $P = 0.012$). Of the 157 adult males, however, only 17 (10.8%) had ovaries or oviducts, five from urban sites (8.2% of urban males), and 12 from sugarcane sites (12.5% of sugarcane males). Finally, male toads near sugarcane fields had larger fat bodies than male toads from urban areas ($\chi^2(1) = 8.30$, $P = 0.004$; Fig. 4d).

Variation and Correlations Between Traits

As predicted, the coefficients of variation in sexual trait expression were all either higher or equivalent in male toads from sugarcane growing areas compared to those from urban areas. The bootstrapped 95% confidence intervals showed greater variation in sugarcane areas for laryngeal mass (sugarcane: 42.7–54.6%, urban: 23.5–38.5%), testes mass (sugarcane: 38.2–48.6%, urban: 24.3–34.7%), mottling score (sugarcane: 55.5–88.8%, urban: 41.8–59.6%), and nuptial pad size (sugarcane: 44.2–60.0%, urban: 30.2–46.9%). Other traits had greatly overlapping confidence intervals, indicating little difference in variation between regions: vocal cord length (sugarcane: 21.0–26.6%, urban: 11.8–25.3%), dilator width (sugarcane: 16.3–20.9%, urban: 13.0–22.1%), Bidder's organ mass (sugarcane: 40.9–54.2%, urban: 45.4–67.3%), and forearm diameter (sugarcane: 7.8–10.4%, urban: 7.4–10.3%).

In contrast to our prediction that associations between traits would weaken near sugarcane fields, we found significant correlations ($P < 0.05$) for nearly all pairs of

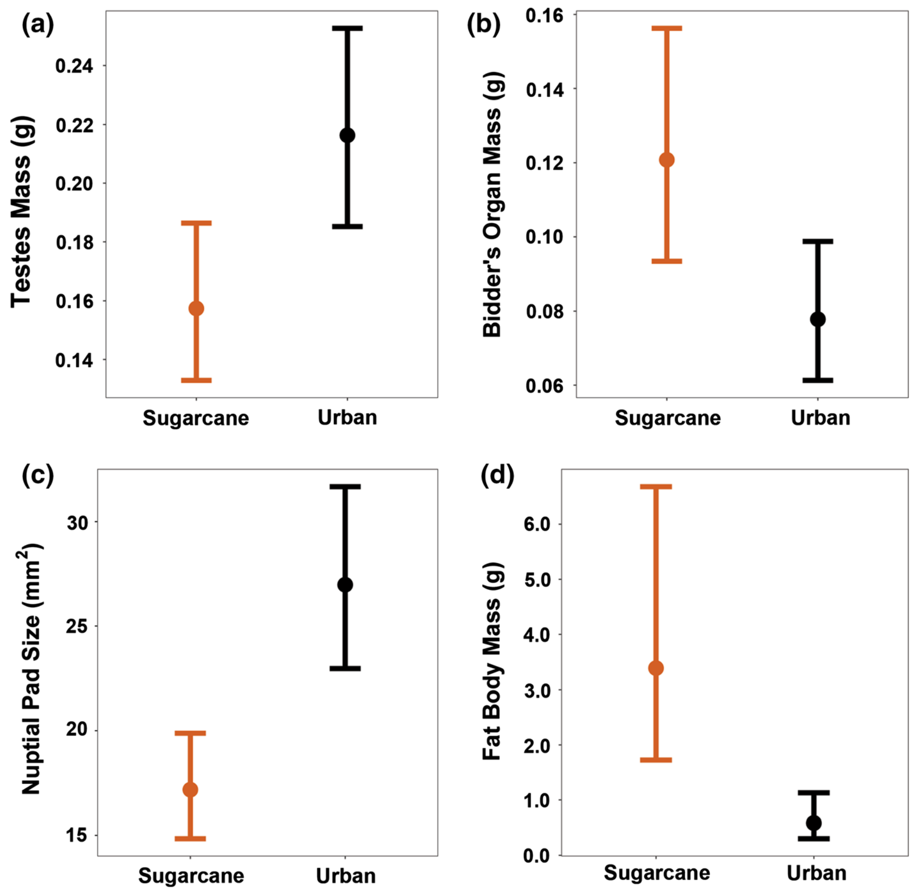

Figure 4. Comparisons between male cane toads from sugarcane (red) and urban (black) areas for four sexual traits: **a** testes mass, **b** Bidder's organ mass, **c** nuptial pad size, and **d** fat body mass. Plots show estimated marginal means with 95% confidence intervals derived from generalized linear mixed models that include body size as a covariate. Male toads showed significant differences between land-use regions for each of the four traits (LRT; $P < 0.05$) (Color figure online).

sexual traits in male toads from sugarcane areas. Only three of 28 pairs of traits were not significantly correlated: testes mass and dilator width; testes mass and mottling score; and Bidder's organ mass and mottling score. In contrast, male toads from urban areas only had nine significant correlations between the 28 pairs of traits, likely due to lower variation in trait expression. The three measures of larynx size were positively correlated with each other and negatively correlated with Bidder's organ mass; testes mass was positively correlated with nuptial pad size and negatively correlated with Bidder's organ mass; and nuptial pad size was positively correlated with vocal cord length.

Discussion

We confirmed that male cane toads living near sugarcane fields in South Florida show demasculinization of both primary and secondary sexual traits, including the size of the larynx, an organ that is critical to male fitness. Male toads from sugarcane areas have smaller laryngeal masses, shorter vocal cords, and thinner dilator muscles than urban males, despite no differences in overall body size. All three measures are strongly correlated in both regions, representing a concerted shift in larynx size, but not shape, in sugarcane populations. Female larynges, in contrast, do not differ between urban and sugarcane areas. The observed reductions in male larynx size, therefore, provide evidence for a demasculinizing process associated with sugarcane fields. To our knowledge, this study is the first to document laryngeal demasculinization in wild anuran amphibians, a process that could adversely affect the attractiveness of male mating calls and thus overall reproductive success.

The reductions in male larynx size that we observed stand in contrast with the lack of differences in laryngeal morphology across maize and non-maize growing regions seen in wild-caught *Xenopus laevis* from South Africa (Smith et al. 2005). Other evidence of demasculinization of the anuran larynx comes from laboratory studies, which showed demasculinizing effects of specific chemicals, including atrazine (Hayes et al. 2002, 2010; but see Carr et al. 2003; Coady et al. 2005) and polychlorinated biphenyls (Qin et al. 2007). To our knowledge, however, we are the first to investigate this question in a species other than *Xenopus laevis* and the first to find evidence of laryngeal demasculinization in the field. Our results, therefore, highlight the importance of considering a diverse range of species when predicting how anthropogenic factors impact an entire taxonomic group. Amphibians are a particularly important group in which to examine these effects as they are declining worldwide (Wake and Vredenburg 2008) and are particularly vulnerable to aquatic pollutants (Kloas et al. 2009; Egea-Serrano et al. 2012).

It is not clear how the observed changes in larynx size affect call characteristics and attractiveness in male toads near sugarcane fields. Anuran females, including cane toads, generally prefer lower frequency calls (Ryan 2001; Yasumiba et al. 2015; Muller and Schwarzkopf 2017). Given that the dominant frequency of anuran calls is negatively associated with larynx size (Gingras et al. 2013) and vocal cord mass (Martin 1971), demasculinized males may produce less attractive, higher frequency calls. Laboratory studies with *Xenopus laevis* found that exposure to estrogenic and antiandrogenic chemicals caused males to produce fewer and less complex advertisement calls, which were less attractive to females (Hoffmann and Kloas 2010, 2012). It is unclear, however, whether these effects were purely behavioral, due to morphological changes in the larynx, or both. Additional research is needed to determine how anuran call properties change with laryngeal morphology to ultimately understand the fitness consequences of demasculinization. Furthermore, demographic field studies are necessary for determining whether endocrine disruption of mating calls could result in population declines rather than solely affecting individual-level mate attraction. These data are crucial for understanding the implications of these morphological alterations for amphibian conservation at a regional and global scale.

Male cane toads near sugarcane fields also differed in gonad size compared to those from nearby urban areas. Relative to body size, male toads near sugarcane fields had smaller testes and larger, often multi-lobed Bidder's organs, compared to male toads from urban areas. The Bidder's organs are sometimes considered rudimentary ovaries as they develop eggs if the testes are removed (Piprek et al. 2014). An increase in Bidder's organ size, therefore, suggests feminization, although the consequences for male reproduction are unknown. Similar to McCoy et al. (2008a), we also found that male toads near sugarcane fields had greater dorsal mottling, smaller nuptial pads, and fewer digits with visible nuptial pads, compared to urban males. Nuptial pads are important for male anurans as they must hold onto a female for hours during amplexus prior to fertilizing her eggs (Wells 2007). Therefore, reductions in nuptial pad size may decrease reproductive success. Our results add to the previous studies that show negative impacts of endocrine-disrupting pollutants on reproductive output (McCoy et al. 2017), offspring fitness (Bókony et al. 2018), and thus overall population viability of wild toads.

Our study is one of the first to examine anuran fat bodies in the context of endocrine disruption, and the first to find evidence of increased fat content relative to body size (Langerveld et al. 2009; Orton et al. 2014). In cane toads, fat body growth reflects seasonal changes in breeding condition, with trade-offs observed between gonad size and fat body size in both sexes (Yasumiba et al. 2016). Given that the toads we sampled were in similar reproductive states, however, our results suggest that the smaller testes and larger fat bodies seen in sugarcane sites indicate a more permanent resource allocation strategy away from reproduction. Such a strategy could have implications for toad survival during severe disturbances as anuran fat bodies can be depleted during periods of dormancy (Fitzpatrick 1976). While the role of hormones on lipid deposition and storage in overwintering anurans has received considerable attention (Boutilier et al. 1997, Tattersall and Ultsch 2008), less is known about how endocrine disruptors affect those pathways. Overall, our findings suggest that understanding how habitat alteration affects wildlife populations requires consideration of a range of traits.

Investigating multiple sexual traits also yielded insights into how individuals vary in their degree of demasculinization. As predicted, several traits (laryngeal mass, testes mass, dorsal mottling, and nuptial pad size) showed greater variation in males from sugarcane areas than in those from urban areas, indicating that demasculinization does not act uniformly across individuals. The morphological variation seen in males from sugarcane areas may be due to their variability in microhabitat use, and thus chemical exposure, or in physiological susceptibility to demasculinization. Other anurans have shown variation in the effects of chemicals within and across populations (Bridges and

Semlitsch 2000). The differences in sexual trait variation between land-use types, however, may be explained by potential differences in the proportions of males along various points in their nonbreeding condition. More long-term field studies are needed to better understand the variance in sexual trait expression and ultimately disentangle the effects of endocrine-disrupting pollutants from typical seasonal changes in anuran reproductive physiology.

We also found significant correlations between most pairs of sexual traits in male toads from sugarcane areas, indicating that demasculinization affects diverse tissues to similar degrees within an individual. These findings contrast with our prediction that male toads in sugarcane areas would show weaker associations between sexual traits due to inconsistent effects of demasculinization among organs. If this process instead acts in concert across sexual traits, we could predict impacts on the larynx and testes based on externally visible morphological characteristics such as dorsal mottling and nuptial pad size (Orton et al. 2014). Such noninvasive methods of detecting demasculinization could be used for widespread field surveys of wildlife reproductive health.

The demasculinizing effects on the larynx and other sexual traits seem most likely due to chemical contamination of water bodies near sugarcane fields, although further experimental studies are required to test this hypothesis. Pesticides and herbicides are frequently applied to sugarcane in Florida (Mossler 2008), and many are known or suspected endocrine disruptors (see McCoy et al. 2008b for a list of chemicals). While urban water bodies also contain contaminants, the concentrations of many agricultural pollutants, such as the herbicide atrazine, are much lower in cities than near sugarcane or other crop fields (Miles and Pfeuffer 1997; Hoffman et al. 2000). Atrazine is an estrogenic endocrine-disrupting chemical that has been shown to have feminizing and demasculinizing effects in male anurans of two species: *Xenopus laevis* (Hayes et al. 2002, 2010; but see Solomon et al. 2008) and *Acris blanchardi* (Hoskins and Boone 2018). Although other differences between sites could contribute to the observed morphological changes, the correlated impacts on diverse primary and secondary male sexual traits point to endocrine disruption as the underlying mechanism. Studies that directly examine the exposure of toads to endocrine-disrupting chemicals in these populations and investigate the effects of such chemicals under laboratory conditions are necessary to test for causation.

Overall, our study indicates that the reproductive morphology of amphibian species may be more strongly affected by human land use than would be expected based solely on the studies of gonadal abnormalities. These findings are particularly relevant in anurans as aquatic pollutants, including endocrine-disrupting chemicals, are thought to contribute to global amphibian declines (Egea-Serrano et al. 2012; Regnault et al. 2018). Wildlife monitoring would benefit from a more complete understanding of how a range of sexual traits are altered across species and habitats. Anthropogenic habitat degradation can have extensive effects on vertebrate reproduction, ultimately impacting many or all aspects of reproductive physiology and morphology.

Acknowledgements

We are grateful to N. Anderson, J. Manzano Alvarez, J. Peniston, B. Leavell, and H. Legett for collecting toads; G. Darnell, J. Lam, and E. Terry for assisting with dissections and measurements; and R. Knepp, J. Rusk, and C. Garcia Botero for analyzing photographic data. We are also thankful to K. McCoy, J. Lucas, and S. Johnson for providing valuable advice throughout the project as well as D. Carrillo and G. Nuessly for providing logistical support in Florida. Finally, we thank B. Muller, E. Bledsoe, and E. Khazan for providing comments that improved this manuscript, as well as J. Peniston, for designing figures.

Funding

This work was supported by funds from the Department of Biological Sciences at Purdue University to XEB and to SZ (Lindsey Graduate Fellowship) and by a student research grant from the Animal Behavior Society to SZ. SZ was supported by the Purdue Doctoral Fellowship from the Purdue University Graduate School. XEB was supported by the National Science Foundation (IOS #1433990).

Data Availability

All data generated or analyzed during this study are included in the electronic supplementary materials.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval Experiments were approved by the Purdue Animal Care and Use Committee (Protocol #1405001073). All applicable institutional and/or national guidelines for the care and use of animals were followed.

References

Andersson M (1994) *Sexual Selection*, Princeton, NJ: Princeton University Press

Bates D, Maechler M, Bolker B, Walker S (2015) Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* 67(1):1–48. https://doi.org/10.18637/jss.v067.i01

Bókony V, Üveges B, Ujhegyi N, Verebélyi V, Nemesházi E, Csíkvári O, Hettyey A (2018) Endocrine disruptors in breeding ponds and reproductive health of toads in agricultural, urban, and natural landscapes. *Science of the Total Environment* 634:1335–1345. https://doi.org/10.1016/j.scitotenv.2018.03.363

Boutilier RG, Donohoe PH, Tattersall GJ, West TG (1997) Hypometabolic homeostasis in overwintering aquatic amphibians. *The Journal of Experimental Biology* 200:387–400

Bridges CM, Semlitsch RD (2000) Variation in pesticide tolerance of tadpoles among and within species of Ranidae and patterns of amphibian decline. *Conservation Biology* 14(5):1490–1499. https://doi.org/10.1046/j.1523-1739.2000.99343.x

Caliman FA, Gavrilescu M (2009) Pharmaceuticals, personal care products and endocrine disrupting agents in the environment—a review. *Clean* 37:277–303. https://doi.org/10.1002/clen.200900038

Canty A, Ripley B (2017) boot: Bootstrap R (S-Plus) Functions. R package version 1.3-20.

Carr JA, Gentles A, Smith EE, Goleman WL, Urquidi LJ, Thuett K, Kendall RJ, Giesy JP, Gross TS, Solomon KR, Van Der Kraak G (2003) Response of larval *Xenopus laevis* to atrazine: Assessment of growth, metamorphosis, and gonadal and laryngeal morphology. *Environmental Toxicology and Chemistry* 22(2):396–405. https://doi.org/10.1002/etc.5620220222

Coady KK, Murphy MB, Villeneuve DL, Hecker M, Jones PD, Carr JA, Solomon KR, Smith EE, Van Der Kraak G, Kendall RJ, Giesy JP (2005) Effects of atrazine on metamorphosis, growth, laryngeal and gonadal development, aromatase activity, and sex steroid concentrations in *Xenopus laevis*. *Ecotoxicology and Environmental Safety* 62:160–173. https://doi.org/10.1016/j.ecoenv.2004.10.010

Colafrancesco KC, Gridi-Papp M (2016) Vocal sound production and acoustic communication in amphibians and reptiles. In: *Vertebrate Sound Production and Acoustic Communication*, Suthers RA, Fitch WT, Fay RR, Popper AN (editors), New York, NY: Springer, pp 83–117

Edwards TM, Moore BC, Guillette LJ (2006) Reproductive dysgenesis in wildlife: A comparative view. *International Journal of Andrology* 29:109–121. https://doi.org/10.1111/j.1365-2605.2005.00631.x

Egea-Serrano A, Relyea RA, Tejedo M, Torralva M (2012) Understanding of the impact of chemicals on amphibians: A meta-analytic review. *Ecology and Evolution* 2(7):1382–1397. https://doi.org/10.1002/ece3.249

Fitzpatrick LC (1976) Life history patterns of storage and utilization of lipids for energy in amphibians. *Integrative and Comparative Biology* 16:725–732. https://doi.org/10.1093/icb/16.4.725

Gerhardt HC, Huber F (2002) *Acoustic Communication in Insects and Anurans: Common Problems and Diverse Solutions*, Chicago, IL: University of Chicago Press

Gingras B, Boeckle M, Herbst CT, Fitch WT (2013) Call acoustics reflect body size across four clades of anurans. *Journal of Zoology* 289:143–150. https://doi.org/10.1111/j.1469-7998.2012.00973.x

Girish S, Saidapur SK (2000) Interrelationship between food availability, fat body, and ovarian cycles in the frog, *Rana tigrina*, with a discussion of the role of fat body in anuran reproduction. *Journal of Experimental Zoology* 286(5):487–493. https://doi.org/10.1002/(sici)1097-010x(20000401)286:5<487::aid-jez6>3.0.co;2-z

Gunderson MP, Veldhoen N, Skirrow RC, Macnab MK, Ding W, van Aggelen G, Helbing CC (2011) Effect of low dose exposure to the herbicide atrazine and its metabolite on cytochrome P450 aromatase and steroidogenic factor-1 mRNA levels in the brain of premetamorphic bullfrog tadpoles (*Rana catesbeiana*). *Aquatic Toxicology* 102:31–38. https://doi.org/10.1016/j.aquatox.2010.12.019

Guerra MA, Ryan MJ (2014) Cannatella DC (2014) Ontogeny of sexual dimorphism in the larynx of the túngara frog. *Physalaemus pustulosus. Copeia* 1:123–129. https://doi.org/10.1643/cg-13-051

Hamlin HJ, Guillette LJ (2010) Birth defects in wildlife: the role of environmental contaminants as inducers of reproductive and developmental dysfunction. *Systems Biology in Reproductive Medicine* 56:113–121. https://doi.org/10.3109/19396360903244598

Harrell FE, Dupont C et al. (2018) Hmisc: Harrell Miscellaneous. R package version 4.1-1

Harrison PTC, Holmes P, Humfrey CDN (1997) Reproductive health in humans and wildlife: Are adverse trends associated with environmental chemical exposure? *Science of the Total Environment* 205:97–106. https://doi.org/10.1016/S0048-9697(97)00212-X

Hayes TB, Collins A, Lee M, Mendoza M, Noriega N, Stuart AA, Vonk A (2002) Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. *Proceedings of the National Academy of Sciences of the USA* 99(8):5476–5480. https://doi.org/10.1073/pnas.082121499

Hayes TB, Khoury V, Narayan A, Nazir M, Park A, Brown T, Adame L, Chan E, Buchholz D, Stueve T, Gallipeau S (2010) Atrazine induces complete feminization and chemical castration in male African clawed frogs (*Xenopus laevis*). *Proceedings of the National Academy of Sciences of the USA* 107(10):4612–4617. https://doi.org/10.1073/pnas.0909519107

Hoffman RS, Capel PD, Larson SJ (2000) Comparison of pesticides in Eight U.S. Urban Streams. *Environmental Toxicology and Chemistry* 19(9):2249–2258. https://doi.org/10.1897/1551-5028(2000)019<2249:copieu>2.3.co;2

Hoffmann F, Kloas W (2010) An environmentally relevant endocrine-disrupting antiandrogen, vinclozolin, affects calling behavior of male *Xenopus laevis*. *Hormones and Behavior* 58(4):653–659. https://doi.org/10.1016/j.yhbeh.2010.06.008

Hoffmann F, Kloas W (2012) Estrogens can disrupt amphibian mating behavior. *PLoS ONE* 7(2):1–8. https://doi.org/10.1371/journal.pone.0032097

Hoskins TD, Boone MD (2018) Atrazine feminizes sex ratio in Blanchard's cricket frogs (*Acris blanchardi*) at concentrations as low as 0.1 μg/L. *Environmental Toxicology and Chemistry* 37(2):427–435. https://doi.org/10.1002/etc.3962

Kloas W, Urbatzka R, Opitz R, Würtz S, Behrends T, Hermelink B, Hofmann F, Jagnytsch O, Kroupova H, Lorenz C, Neumann N, Pietsch C, Trubiroha A, Van Ballegooy C, Wiedemann C, Lutz I (2009) Endocrine disruption in aquatic vertebrates. *Annals of the New York Academy of Sciences* 1163:187–200. https://doi.org/10.1111/j.1749-6632.2009.04453.x

Koo TK, Li MY (2016) A guideline of selecting and reporting intraclass correlation coefficients for reliability research. *Journal of Chiropractic Medicine* 15:155–163. https://doi.org/10.1016/j.jcm.2016.02.012

Langerveld AJ, Celestine R, Zaya R, Mihalko D, Ide CF (2009) Chronic exposure to high levels of atrazine alters expression of genes that regulate immune and growth-related functions in developing *Xenopus laevis* tadpoles. *Environmental Research* 109:379–389. https://doi.org/10.1016/j.envres.2009.01.006

Length R (2018) Emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.1

Mackenzie CA, Berrill M, Metcalfe C, Pauli BD (2003) Gonadal differentiation in frogs exposed to estrogenic and antiestrogenic compounds. *Environmental Toxicology and Chemistry* 22(10):2466–2475. https://doi.org/10.1897/02-173

Mann RM, Hyne RV, Choung CB, Wilson SP (2009) Amphibians and agricultural chemicals: Review of the risks in a complex environment. *Environmental Pollution* 157:2903–2927. https://doi.org/10.1016/j.envpol.2009.05.015

Martin WF (1971) Mechanics of sound production in toads of the genus *Bufo*: Passive elements. *Journal of Experimental Zoology* 176(3):273–293. https://doi.org/10.1002/jez.1401760304

McClelland BE, Wilczynski W (1989) Sexually dimorphic laryngeal morphology in *Rana pipiens*. *Journal of Morphology* 201:293–299. https://doi.org/10.1002/jmor.1052010308

McCoy KA, Bortnick LJ, Campbell CM, Hamlin HJ, Guillette LJ, St. Mary CM (2008) Agriculture alters gonadal form and function in the toad *Bufo marinus*. *Environmental Health Perspectives* 116(11):1526–1532. https://doi.org/10.1289/ehp.11536

McCoy KA, Hoang LK, Guillette LJ, St. Mary CM (2008) Renal pathologies in giant toads (*Bufo marinus*) vary with land use. *Science of the Total Environment* 407:348–357. https://doi.org/10.1016/j.scitotenv.2008.09.008

McCoy KA, Amato CM, Guillette LJ, Mary CMS (2017) Giant toads (*Rhinella marina*) living in agricultural areas have altered spermatogenesis. *Science of the Total Environment* 609:1230–1237. https://doi.org/10.1016/j.scitotenv.2017.07.185

Meshaka WE, Butterfield BP, Hauge JB (2004) *The Exotic Amphibians and Reptiles of Florida*, Malabar, FL: Krieger Publishing Company

Miles CJ, Pfeuffer RJ (1997) Pesticides in canals of South Florida. *Archives of Environmental Contamination and Toxicology* 32:337–345. https://doi.org/10.1007/s00244900194

Mossler M (2008) Florida Crop/Pest Profile: Sugarcane. UF/IFAS Extension PI-171:1-14

Muller BJ, Schwarzkopf L (2017) Success of capture of toads improved by manipulating acoustic characteristics of lures. *Pest Management Science* 73:2372–2378. https://doi.org/10.1002/ps.4629

Orton F, Baynes A, Clare F, Duffus ALJ, Larroze S, Scholze M, Garner TWJ (2014) Body size, nuptial pad size and hormone levels: Potential non-destructive biomarkers of reproductive health in wild toads (*Bufo bufo*). *Ecotoxicology* 23:1359–1365. https://doi.org/10.1007/s10646-014-1261-3

Orton F, Tyler CR (2015) Do hormone-modulating chemicals impact on reproduction and development of wild amphibians? *Biological Reviews* 90(4):1100–1117. https://doi.org/10.1111/brv.12147

Pattersson I, Berg C (2007) Environmentally relevant concentrations of ethynylestradiol cause female-biased sex ratios in *Xenopus tropicalis* and *Rana temporaria*. *Environmental Toxicology and Chemistry* 26(5):1005–1009. https://doi.org/10.1897/06-464r.1

Piprek RP, Kloc M, Kubiak JZ (2014) Bidder's organ – Structure, development and function. *International Journal of Developmental Biology* 58:819–827. https://doi.org/10.1387/ijdb.140147rp

Qin ZF, Qin XF, Yan L, Han-Ting L, Zhao XR, Xu XB (2007) Feminizing/demasculinizing effects of polychlorinated biphenyls on the secondary sexual development of *Xenopus laevis*. *Aquatic Toxicology* 84:321–327. https://doi.org/10.1016/j.aquatox.2007.06.011

R Core Team (2017) *R: A language and environment for statistical computing*, Vienna, Austria: R Foundation for Statistical Computing

Regnault C, Usal M, Veyrenc S, Couturier K, Batandier C, Bulteau AL, Lejon D, Sapin A, Combourieu B, Chetiveaux M, Le May C, Lafond T, Raveton M, Reynaud S (2018) Unexpected metabolic disorders induced by endocrine disruptors in *Xenopus tropicalis* provide new lead for understanding amphibian decline. *Proceedings of the National Academy of Sciences of the USA* 115(19):E4416–E4425. https://doi.org/10.1073/pnas.1721267115

Ryan MJ (2001) *Anuran Communication*, Washington, DC: Smithsonian Institution Press

Sassoon D, Kelley DB (1986) The sexually dimorphic larynx of *Xenopus laevis*: Development and androgen regulation. *American Journal of Anatomy* 177:457–472. https://doi.org/10.1002/aja.1001770404

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* 9(7):671–675. https://doi.org/10.1038/nmeth.2089

Schug TT, Johnson AF, Birnbaum LS, Colborn T, Guillette LJ, Crews DP, Collins T, Soto AM, vom Saal FS, McLachlan JA, Sonnenschein C, Heindel JJ (2016) Minireview: Endocrine disruptors: Past lessons and future directions. *Molecular Endocrinology* 30(8):833–847. https://doi.org/10.1210/me.2016-1096

Sitja-Bobadilla A (2009) Can myxosporean parasites compromise fish and amphibian reproduction? *Proceedings of the Royal Society B* 276:2861–2870. https://doi.org/10.1098/rspb.2009.0368

Solomon KR, Carr JA, Du Preez LH, Giesy JP, Kendall RJ, Smith EE, Van Der Kraak GJ (2008) Effects of atrazine on fish, amphibians, and aquatic reptiles: A critical review. *Critical Reviews in Toxicology* 38(9):721–772. https://doi.org/10.1080/10408440802116496

Smith EE, Du Preez LH, Gentles A, Solomon KR, Tandler B, Carr JA, Van Der Kraak GL, Kendall RJ, Giesy JP, Gross TS (2005) Assessment of laryngeal muscle and testicular cell types in *Xenopus laevis* (Anura: Pipidae) inhabiting maize and non-maize growing areas of South Africa. *African Journal of Herpetology* 54(1):69–76. https://doi.org/10.1080/21564574.2005.9635519

Tamschick S, Rozenblut-Koscisty B, Ogielska M, Lehmann A, Lymberakis P, Hoffmann F, Lutz I, Kloas W, Stock M (2016) Sex reversal assessments reveal different vulnerability to endocrine disruption between deeply diverged anuran lineages. *Scientific Reports* 6:23825. https://doi.org/10.1038/srep23825

Tattersall GJ, Ultsch GR (2008) Physiological ecology of aquatic overwintering in ranid frogs. *Biological Reviews* 83:119–140. https://doi.org/10.1111/j.1469-185X.2008.00035.x

Wake DB, Vredenburg VT (2008) Are we in the midst of the sixth mass extinction? A view from the world of amphibians *Proceedings of the National Academy of Sciences of the USA* 105:11466–11473. https://doi.org/10.1073/pnas.0801921105

Wells KD (2007) *The Ecology and Behavior of Amphibians*, Chicago, IL: The University of Chicago Press

Wilson AC (2016) *Distribution of cane toads* (Rhinella marina) *in Florida and their status in natural areas* (Master's Thesis). Retrieved from https://ufdc.ufl.edu/ufetd

Yager DD (1992) Underwater acoustic communication in the African pipid frog *Xenopus borealis*. *Bioacoustics* 4:1–24. https://doi.org/10.1080/09524622.1992.9753201

Yasumiba K, Alford RA, Schwarzkopf L (2015) Why do male and female cane toads, *Rhinella marina*, respond differently to advertisement calls? *Animal Behaviour* 109:141–147. https://doi.org/10.1016/j.anbehav.2015.08.015

Yasumiba K, Alford RA, Schwarzkopf L (2016) Seasonal reproductive cycles of cane toads and their implications for control. *Herpetologica* 72(4):288–292. https://doi.org/10.1655/herpetologica-d-15-00048.1

Zug GR (1979) Zug PB (1979) The marine toad, *Bufo marinus*: a natural history resumé of native populations. *Smithsonian Contributions to Zoology* 284:1–58. https://doi.org/10.5479/si.00810282.284