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Giant toads (*Rhinella marina*) living in agricultural areas have altered spermatogenesis

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Abstract

Across diverse taxa, germ cell development is controlled by an intricate cascade of processes that are tightly controlled by the hypothalamic-pituitary-gonadal axis. Endocrine disturbances, such as those induced by endocrine disrupting chemicals (EDCs) can negatively affect spermatogenesis. Here, we investigate whether spermatogenesis is altered in the giant toad, *Rhinella marina*, living in agricultural areas where EDCs are used relative to suburban areas. We also ask if reductions in spermatogenesis were associated with developmental gonadal abnormalities (intersex) found in the same frogs. We found that toads in agricultural areas exhibited reduced spermatogenesis relative to non-agricultural animals, and that those reductions were not associated with gross gonadal abnormalities. All toads living in agricultural areas had reduced spermatogenesis relative to those living in non-agricultural areas regardless of whether they had gonadal abnormalities originating during development. Similarities in reproductive dysfunction among diverse taxa living in agricultural areas, including humans, suggest that many vertebrate taxa living in agricultural areas around the globe are likely experiencing some level of reproductive dysfunction.

Graphical abstract

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Keywords

agriculture; giant toad; pesticides; reproductive abnormalities; spermatogenesis

Introduction

Reproductive function in both humans and wildlife has declined over the past 50-60 years as a result of elevated environmental contaminant exposure [reviewed in (Bergman et al., 2013)]. For example, testicular dysgenesis syndrome in humans is associated with four conditions: cryptorchidism, hypospadias, impaired spermatogenesis and testis cancer, which are all manifestations of disturbed prenatal testicular development. Each of these manifestations of testicular dysgenesis syndrome have been linked to exposure to endocrine disrupting chemicals (EDCs) (Bay et al., 2006). Copious studies across taxa have found a diversity of reproductive effects induced by EDC exposure (Edwards et al., 2006; Lambert et al., 2015; McCoy et al., 2016; Skelly et al., 2010; Yeung et al., 2011).

Although the exact dysfunction can differ among diverse taxa due to exposure, or species differences in reproductive system development, we expect analogous effects of exposure to EDCs across taxa because many aspects of the endocrine system are highly conserved (Diamanti-Kandarakis et al., 2009; Edwards et al., 2006). For example, the effects of atrazine, a commonly used pesticide, on male reproductive development are consistent across all vertebrate classes examined (Hayes et al., 2011). In addition, sperm concentration and motility were reduced in men living in semirural and agricultural areas relative to more urban and less agriculturally exposed areas (Le Moal et al., 2014; Swan et al., 2003a). Later work showed that pesticides, including atrazine, contributed to the poor semen quality seen in men from more agricultural areas (Mehrpour et al., 2014; Swan et al., 2003b), and in men from sub-fertile couples seeking infertility diagnosis (Meeker et al., 2004). This relationship clearly shows the link between pesticide exposure and reduced fertility. It is now known, that alterations of hypothalamic-pituitary circuitry, hormone mediated endocrine regulation, or

the microenvironments within seminiferous tubules can result in transient or long-term disturbance of spermatogenesis (Yeung et al., 2011).

The route of exposure between humans and free-living wildlife are similar. In humans, exposure occurs through drinking water, air, food, or through contacting contaminated soil (Swan et al., 2003a). All of these routes are also important for wildlife living in contaminated environments, but exposure in wildlife is likely more strongly linked to their habitat relative to humans because they live in the environment exclusively. That is, all of their food and water comes from their area of habitation; whereas humans can obtain food and water from diverse sources while living in the same area.

The EDC exposure profiles of wildlife vary with habitat, and effects should, thus, scale with land use patterns. For example, endocrine disruption in suburban frog populations was associated with a mixture of different EDCs including classical estrogens, phytoestrogens, and metalloestrogens (Lambert et al., 2015). Effects found downstream of wastewater treatment plants are typically associated with exposure to estrogenic wastewater effluent. Effects documented in agricultural areas are most often related to pesticides and potentially phytoestrogens (Lambert et al., 2015; Lambert and Skelly, 2016).

Amphibians are known to be sensitive to pesticide exposure and do bio-accumulate pesticides through dermal exposure from contaminated soil or overspray of pesticide (Van Meter et al., 2015; Van Meter et al., 2016; Van Meter et al., 2014). For example, chorus frogs downwind of intense pesticide spraying conducted in Central Valley California concentrate current use pesticides (Smalling et al., 2013).

Clearly pesticides and other EDCs are used in urban and suburban areas, but there are substantial differences in the milieus of pesticides across land use types. Urban and suburban areas are typically polluted with a variety of chemicals including polyaromatic hydrocarbons (PAHs) associated with road runoff (Crosbie and Chow-Fraser, 1998; Dorchin and Shanas, 2010; Maltby et al., 1995; Mitsova et al., 2011); whereas agricultural areas are more often polluted with mixtures of pesticides (Crosbie and Chow-Fraser, 1999; Kreuger, 1998; Rasmussen et al., 2011). This association is so strong that PAH and pesticide (i.e. metolachlor) concentrations have been used to estimate the proportion of urbanized and agricultural land, respectively, among different watersheds (Crosbie and Chow-Fraser, 1998).

In the current study, we test the hypothesis that spermatogenesis is altered in wild giant toads (*Rhinella marina*) living in agricultural areas where endocrine disrupting pesticides are known to be used (see appendix in McCoy et al., 2008b) relative to suburban areas where other pollutants occur (Crosbie and Chow-Fraser, 1998; Crosbie and Chow-Fraser, 1999; Dorchin and Shanas, 2010; Kreuger, 1998; Maltby et al., 1995; McCoy et al., 2008b; Mitsova et al., 2011; Rasmussen et al., 2011). We previously demonstrated that endocrine disruption (e.g., altered steroidogenesis), and a variety of reproductive abnormalities in *Rhinella marina* (*R. marina*) in South Florida increase with percentage of agricultural land use (McCoy et al., 2008a), but that nephrotoxicity did not (McCoy et al., 2008b). These incongruous results suggest that although animals in both urban and agricultural areas were

exposed to toxicants (exhibited nephrotoxicity), only the agricultural toads experienced endocrine disruption that affected their reproductive system. We exploit the same differences in human activity patterns in this study to investigate how spermatogenesis is altered across land use patterns. We also address whether toads living in agricultural areas with gonadal abnormalities, many of which are developmental in origin (i.e. intersex gonads), have reductions in spermatogenesis relative to morphologically “normal” agricultural individuals, or if reductions in spermatogenic indices are independent of gonadal morphology (developmental abnormality). For instance, we ask whether site differences in spermatogenesis are driven by agricultural individuals with altered gonadal morphology (e.g., intersexes) or if agricultural toads generally have altered spermatogenesis regardless of gross gonadal morphology. Similarities in reproductive dysfunction among diverse taxa, such as humans and amphibians, living in agricultural areas would suggest that most, if not all, vertebrate taxa, living in similar landscapes could be affected.

Methods

Land use characterization

Five sites in south Florida were surveyed that varied in land-use in order to investigate the association between intensity of agriculture and reproductive abnormalities in *R. marina* (Table 1, Figure 1). Methods used to determine land use are detailed in (McCoy et al., 2008a). Briefly, Google Earth digital satellite images and a distance key of each site were downloaded into Image Pro-Plus (Media Cybernetics Inc) image analysis program. We then centered a 5.6 km² grid (with 9~622 m² cells) over each collection site. The home range of *R. marina* is ~2 km² (Zug et al., 1975), so this grid includes all of the area experienced by a toad from each local site. Then percentage of agricultural land within each cell of the grid was estimated and the mean percentage of agriculture for each site was calculated. Agricultural intensity of each site was ranked from 1 to 5 based on the total % of agricultural land. Lake Worth (LW-0%) and Wellington (WT-0%), had zero agriculture and were the most suburban sites and included residential, commercial, transportation, urban recreational areas, or industrial land. Lake Worth, however, was 37 km away from intensive agricultural areas and was ranked 1, whereas Wellington was only 14 km away from intensive agriculture and was ranked 2. Homestead (HS-34% rank of 3) had mixed land use and included agricultural fields (34% agriculture), buildings, and roadways, and thus, consisted of both suburban and agricultural land. Agricultural areas included Canal Point (CP-51% rank of 4), which was largely agricultural but was bordered on one side by Lake Okeechobee, whereas Belle Glade (BG-93% rank of 5) was almost entirely agricultural land.

At least 20 sexually mature adult individuals (> 91mm) (Cohen and Alford, 1993; Meshaka et al., 2006) were collected from each site during the summers of 2005 and 2006. Each individual was euthanized with an overdose of the anesthetic MS222 (0.3% Tricaine Methanesulfonate, pH 7). Gonads were removed and fixed in 10% neutral buffered formalin for gross morphological (McCoy et al., 2008a) and histological analyses (reported here). All procedures herein were approved by University of Florida's institutional animal care and use committee (permit #D699).

Male Category Comparisons

To test the hypothesis that differences in spermatogenesis are associated with gonadal abnormalities toads were grouped into four categories based on gross gonadal morphology and their occurrence in non-agricultural (WT, and LW) or agricultural (BG and CP) areas (Table 2): 1) non-agricultural males (NAGM), 2) agricultural males (AgM), 3) agricultural males with Bidders organ abnormalities (Bidders Male), 4) agricultural intersex toads. Toads defined as “Males” had normal Bidder’s organs and lacked female reproductive tissue. “Bidder’s males” had normal testes based on gross morphology but abnormal Bidder’s organs and no ovaries or oviducts. Bidder’s organs were classified as abnormal if they showed accumulation of vitellogenin at a gross morphological scale or occurred multiply on at least one side of the body [see (McCoy et al., 2008b) Figure 1]. Vitellogenin accumulation in male Bidder’s organ is only known to occur after castration (Brown et al., 2002; Pancak-Roessler and Norris, 1991; Zaccanti et al., 1994). “Intersex toads” had testes along with ovaries or oviducts. The number of spermatogenic cells was compared among these four male categories. For this analysis we ask if spermatogenesis is associated with gonadal morphology (e.g., altered gonadal development) or agricultural land use (present environment). For male category comparisons, we did not include toads from Homestead, the intermediate site because, it could not be classified as exclusively non-agricultural or agricultural (see (McCoy et al., 2008a).

Histological preparations

Fixed tissues were cut in half across the transverse plane, dehydrated in alcohol, embedded in paraffin, and sectioned so that the middle portion of each individual’s testis was cross-sectioned and analyzed. Sections were 8 µm thick, and were stained using standard Hematoxylin and Eosin (Presnell and Schreibman, 1997). To reduce collection bias, slides were randomized and the observer was blind to site.

Quantification of Spermatogenesis

Five slides per animal were examined using an Olympus BX50 microscope, and one section was evaluated per slide. Sections examined were approximately 180µm apart so that the same cells were not evaluated in different sections. Spermatogenic cells in five lobules (Figure 2A) per section were quantified, yielding a total of 25 lobules evaluated for each individual. For each lobule the number of spermatocysts containing primary spermatocytes, secondary spermatocytes, or spermatids (both round and elongate) were quantified (Figure 2B). The quantity of spermatozoa (loose and organized) per lobule was categorized as 0 (approximately 0-25% of the total area of the lobule), 1 (25-50%), 2 (50-75%), or 3 (75-100%).

Statistical tests

Statistical analyses were carried out using SPSS version 15.0. The composition of spermatogenic cell types was evaluated by site and male category using separate multivariate analysis of variance (MANOVA) tests. Ranked sperm abundances were evaluated among sites and male category using Kruskal-Wallis one-way analysis of variance tests. When 3 or more groups are evaluated in a Kruskal-Wallis test and when the number of observations

within groups exceeds 5 (as in this study) the distribution of Kruskal-Wallis approximates the Chi square distribution, thus the chi square statistic is output by SPSS and reported here (Siegel and Castellan, 1988).

Results

Site Comparisons

Spermatogenesis was reduced in toads living in agricultural areas (Wilk's $\Lambda_{12, 235.7}=0.602$; $p<0.001$; Figure 3). The number of primary spermatocytes ($F_{4, 91}=8.59$, $p<0.001$; Figure 3) was significantly lower at Belle Glade and Canal Point (site with highest agricultural activity) relative to Lake Worth (most suburban). Secondary spermatocytes ($F_{4,91}=7.13$, $p<0.001$; Figure 3) and spermatids ($F_{4,91}=8.90$, $p<0.001$; Figure 3) were also significantly reduced in toads from Belle Glade and Canal Point (most agricultural sites) compared to the less agricultural and more suburban sites Homestead, Wellington, and Lake Worth. Spermatozoa quantity also varied across sites ($X^2_4=14.978$, $p=0.005$; Figure 4). Lake Worth and Wellington, both non-agricultural sites, had a higher percentage of individuals with median sperm ranks two and three, or 50-100% lobule coverage whereas agricultural sites had higher incidence of sperm ranks equal to one (25-50% coverage) (Figure 4).

Male Category Comparisons

Spermatogenesis differed significantly among types of males ($F_{9182.7}=3.50$; $p<0.001$; Figure 5). Males from non-agricultural sites (NAg Males) had significantly more primary spermatocytes ($F_{3,77}=6.88$, $p<0.001$, Figure 5), secondary spermatocytes ($F_{3,77}=7.41$, $p<0.001$; Figure 5), and spermatids ($F_{3,77}=9.28$, $p<0.001$; Figure 5) than 'normal' males, Bidder's males, and intersex toads living in agricultural sites (Figure 5). Spermatozoa quantity also varied significantly by male type ($X^2_3=9.739$, $p=0.021$, Figure 6) with the sperm rank of 1 (25-50% spermatozoa coverage) occurring more frequently in intersex, Bidder's males, and 'normal' male toads from agricultural sites when compared to male toads from non-agricultural sites. However, a similar percentage of non-agricultural males and intersex toads (from agricultural sites) had the highest sperm ranks (Figure 6).

Discussion

The underlying genetic and endocrine control of gonadal development, growth, and function are highly conserved among vertebrates (Diamanti-Kandarakis et al., 2009). These processes can, therefore, be altered, across diverse taxa, by exogenous exposure to sub-lethal concentrations of endocrine disrupting chemicals (EDCs) causing impaired development, growth, sexual differentiation, and reproduction (Diamanti-Kandarakis et al., 2009; Edwards et al., 2006; Milnes et al., 2006). We have previously shown that primary (e.g. gonadal morphology) and secondary sexual traits, and sex hormone concentrations of toads were altered in agricultural areas where chemicals with endocrine disruptive potential are used (McCoy et al., 2008a; McCoy et al., 2008b). In this study, we show that spermatogenic cell types were reduced in the individuals living in agricultural habitats relative to those living in non-agricultural areas. This finding is analogous to those found in humans exposed to

pesticides through occupational exposure or living in agricultural areas (Chiu et al., 2015; Cremonese et al., 2017; Meeker et al., 2004; Melgarejo et al., 2015; Swan, 2006; Swan et al., 2003a). Although not surprising given the high conservation of hormone receptors and enzymes involved in the synthesis, release, and degradation of hormones (Diamanti-Kandarakis et al., 2009), the fact that we find altered spermatogenesis in toads and humans living in agricultural areas strongly suggests two important points. First, many vertebrate taxa in agricultural areas or exposed to agricultural chemicals across the globe could suffer similar dysfunction. In fact, several studies have found associations between agricultural land-use or pesticides and reproductive system abnormalities in humans (Carbone et al., 2006b; Diamanti-Kandarakis et al., 2009; GarciaRodriguez et al., 1996; Garry et al., 1996; Giulivo et al., 2016; Guillette et al., 2006; Kristensen et al., 1997; Pierik et al., 2004; Roy et al., 2009; Wang et al., 2016) and in other vertebrates (Armenti et al., 2008; Ben Slima et al., 2017; Carson, 1962; Guillette and Guillette, 1996; Manikkam et al., 2012; McCoy et al., 2008a; Milnes et al., 2006; Milnes and Guillette, 2008; Rohr and McCoy, 2009; Zidan, 2009) suggesting that reproductive dysfunction is commonly associated with agricultural land-use. Second, spermatogenesis and spermatozoa function in humans living in the agricultural areas of South Florida, that we sampled, could also be affected. These individuals should be studied in more detail to determine if they are suffering reduced fertility or other reproductive health disorders.

Fetal, or larval, environment is critically important for determining reproductive health, in both humans and other vertebrates. The gross morphological abnormalities within the gonads we reported previously (McCoy et al., 2008a) of these same individuals evaluated here are clearly developmental in origin, so the alteration in spermatogenesis could reflect effects either of embryonic exposure (e.g., altered populations of spermatogonia or Sertoli cell number) or current exposure (e.g., direct effects on mitotic and meiotic events associated with spermatogenesis). In humans, reductions in sperm quantity and motility were associated with current use pesticides measured in urine samples (Meeker et al., 2004; Swan et al., 2003b). In this study, the reduction in spermatogenesis was more closely associated with agriculture (and associated EDCs (McCoy et al., 2008b)) than abnormal gross gonadal morphology. All toads living in agricultural areas had reduced spermatogenesis relative to those living in non-agricultural areas regardless of whether they were morphologically 'normal' (male), had Bidder's abnormalities, or were intersexes. Spermatogenesis was, therefore, reduced even in agricultural animals with no gross developmental gonadal abnormalities. Interestingly, we found similar results for plasma testosterone concentrations in the same toads (McCoy et al., 2008a); all male types from agricultural areas had reduced testosterone relative to non-agricultural males. In fact, testosterone concentrations of agricultural toads were in some cases more similar to females than to non-agricultural males (see Figure 5 in (McCoy et al., 2008a)). All categories of male toads from the agricultural areas also had color patterns that were more similar to females than to non-agricultural males.

Reduced sperm counts, however, can also be a result of abnormal embryonic programming and gonadal development or epigenetic modifications (e.g., reductions in Sertoli cell numbers) (Bay et al., 2006; Ho et al., 2017; Skakkebaek et al., 2001). Indeed, organisms, including humans, developing and living in agricultural areas could be affected during

development as well as childhood and adulthood. Although in this study we do not see sperm levels correlate with developmental abnormalities, clearly agricultural chemicals do negatively impact fetal development. For example, boys born in areas with high pesticide use are more susceptible to developing hypospadias, an abnormality of the penis, than those boys born in low pesticide use areas (Carbone et al., 2006a; Kalfa et al., 2015; Rocheleau et al., 2009). Indeed higher rates of hypospadias are strongly correlated with pesticide metabolites in the placenta (Fernandez et al., 2007).

The physiological mechanisms through which spermatogenesis is altered in toads living in agricultural areas are unknown. We do know, however, that hormonal control of spermatogenesis involves gonadotropin releasing hormones (GnRH), gonadotropins, and androgens (Minucci et al., 1992; Ogielska, 2009). GnRH regulates spermatogenesis, spermiation, and steroid biosynthesis through its direct influence on the pituitary and both direct and indirect actions on gonadal tissue. GnRH and its receptor have been localized in anuran testicular tissue (Ogielska, 2009). In fact, hypophysectomized frogs (*Rana esculenta*) treated with GnRH show a complete restoration of spermatogenesis, including spermatozoa production, showing that these hypothalamic hormones can have direct effects on spermatogenic cells independent of the pituitary gland and its gonadotropins (Minucci et al., 1992). In intact anurans the production of primary and secondary spermatocytes is initiated by the gonadotropin follicle stimulating hormone (FSH) (e.g., *Rana esculenta*, (Minucci et al., 1992; Ogielska, 2009; Rastogi et al., 1976)), whereas differentiation of spermatids is controlled by androgens produced by Leydig cells induced by lutenizing hormone (LH) (Ogielska, 2009). The anterior pituitary secretes both FSH and LH after stimulation from the hypothalamus. The reduction in the number of all spermatogenic cells in the agricultural toads in this study, therefore, suggests that the hypothalamus or pituitary could be altered in toads living in agricultural areas. Indeed, malformed frogs thought to be exposed to EDCs during development have significantly altered concentrations in GnRH, LH, and gonadal testosterone concentrations suggesting that exposure to EDCs can influence the hypothalamic-pituitary axis development in anurans (Efosa et al., 2017; Sower et al., 2000). Sertoli and Leydig cells could also be altered in agricultural toads during development independently of the development of female reproductive tissue such that they cannot appropriately respond to pituitary hormones. These hypotheses need to be evaluated. Regardless of the mechanisms, reduced spermatogenesis is likely associated with reduced fertility and could have important implications for the long-term persistence of affected populations (Diamanti-Kandarakis et al., 2009; Ogielska, 2009; Wang and Zhou, 2013).

Although non-agricultural males had more spermatozoa than individuals from agricultural sites generally, a greater percentage of intersex individuals had high sperm ranks relative to agricultural males. Although unexpected, there is a plausible hypothesis for this apparent conflict. Intersexed individuals might not be sexually active, successful at attracting mates, or able to mate successfully once they are paired with a female. Indeed, intersex toads had higher estradiol to testosterone ratios relative to agricultural males and actually had more estradiol-17 β than testosterone on average (McCoy et al., 2008a). Both androgens and estrogens play an important role in anuran mating behavior, so altered sex hormone ratios could influence many aspects of reproductive behavior. For example, although intersexes had spermatozoa within their testes, they also had reduced breeding glands (e.g. nuptial

pads) and smaller forearms (McCoy et al., 2008a) both of which are critical for maintaining amplexus and reproductive success. There was, therefore a mismatch between various sexual traits that together are critical for reproductive success. If intersex toads do not display breeding behavior, breed as frequently, attract as many mates, or stay in amplexus long enough to fertilize eggs as morphologically normal agricultural males, then spermatozoa could accumulate, and thus appear more numerous in our snap-shot sample.

Our results demonstrate that gonadal function is altered in animals living in agricultural areas and thus implicate agricultural chemicals, some of which are known to function as EDCs. However, it is important to seriously consider the fact that phytoestrogens produced by the crops in the area could also play an important role (Lambert and Skelly, 2016). Corn (*Zea mays*), wheat (*Triticum* spp.), and sugar cane (*Saccharum officinarum*) contaminated by toxic fungal metabolites (e.g., via fungus *Fusarium*) can produce estrogenic mycotoxins and contaminate agricultural surface waters (Kolpin et al., 2010; Lambert and Skelly, 2016). Indeed, the mixtures of phytoestrogens and pesticides could be quite disruptive.

Most EDCs (including phytoestrogens) studied to date have estrogenic, antiandrogenic, or anti-thyroid activity and can influence sex ratio, sexual maturation, gonadal morphology and function, and spermatogenesis, fertility, hormone concentrations (steroidogenesis, metabolism), secondary sexual characteristics and reproductive behavior (Gore et al., 2015; Milnes et al., 2006). All of these characteristics are important for maintaining normal biological and reproductive functions, and could be especially important mechanisms to investigate in taxa that are declining.

A vast amount of work conducted over the last 50 years on diverse vertebrate taxa has shown a relationship between agriculture and pesticides and altered reproductive development and function (Carson, 1962; Edwards et al., 2006; Garcia et al., 2017; Harrison et al., 1997; Lane et al., 2017; Milnes et al., 2006; Milnes and Guillette, 2008; Mizoguchi and Valenzuela, 2016; Rocheleau et al., 2009). Over the last 20 years it has become well established that estrogenic effluent from wastewater treatment plants significantly affect fish reproductive biology (Fuzzen et al., 2015; Purdom et al., 1994). More recent work has shown that endocrine disruption is occurring more generally and is sometimes more severe in suburban and urban areas relative to areas traditionally viewed as point source habitats (Lambert et al., 2015; Skelly et al., 2010). For example, post-metamorphic green frogs (*Rana clamitans*) from suburban landscapes in the Connecticut River Valley had the highest frequency of abnormalities (21%), and there was no evidence of a positive association with agricultural land cover (Skelly et al., 2010). Human land-use patterns and pollutant cocktails will determine the way in which wildlife and humans are affected. The problems induced by pollutants are not restricted to specific sites or single chemicals. Many studies have focused on identifying the land-use patterns that are associated with endocrine disruption. We must now begin to identify the specific drivers or cocktails of chemicals that induce endocrine disruption across pollutant-exposed populations. Comparative experimental approaches that include multiple susceptible populations are essential for improving human and wildlife health.

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Highlights

- Agricultural pollution can be harmful to vertebrate reproduction and populations
- Toads in high agricultural areas have reduced spermatogenesis
- Independent of gonadal defects agriculture negatively impacts sperm levels

Figure 1. Sampling sites. Land-use was determined by estimating percentage of agriculture vs. non-agricultural and urban land within a 5.6 km² grid placed over each site (McCoy et al., 2008a).

Figure 2. Histological preparation of frog testis using H&E. (A) Lobule (20x) circled with a solid white line. (B) Magnified view (40x) within a single lobule showing spermatocysts containing primary spermatocytes (white circle), secondary spermatocytes (black circle), and spermatids (white dotted circle).

Figure 3.

Mean number of spermatocysts of each cell type per lobule across sites of varying agricultural intensities. Agricultural intensity increases from left to right. Manova analysis shows significant differences (see text for statistics) across sites for each cell type. Bars are 95% confidence intervals. Non-overlapping bars denote significant differences within spermatogenic cell type, and black lines are placed to allow comparison back to LW the least agricultural site.

Figure 4. Percentage of toads with a median sperm rank of 0-3 across sites. Ranks represented percentage of spermatozoa within each lobule (0=0-25% of, 1=25-50%, 2=50-75% and 3=75-100%). Agricultural intensity increases from left to right. LW and WT had more individuals with spermatozoa ranks of 2 and 3.

Figure 5.

Mean number of spermatocysts of each cell type per lobule varying across male types including, non-agricultural males (NAgM), agricultural male toads (AgM), agricultural Bidder's males (B), and agricultural intersexes (I). Manova analysis shows significant differences (see text for statistics) across sex groups for each cell type. Bars are 95% confidence intervals. Non-overlapping bars denote significant differences within spermatogenic cell type, and black lines are placed to allow comparison back to NAgMs.

Figure 6. Percentage of toads with a median sperm rank of 0-3 across male categories. Ranks represented percentage of spermatozoa within each lobule (0=0-25% of, 1=25-50%, 2=50-75% and 3=75-100%). Male categories include: non-agricultural males (NAgM), agricultural males (AgM), agricultural Bidder's males (B), and agricultural intersexed toads (I). NAgMs had higher ranks.

Table 1

Sampling site identification, characterization, and agricultural intensity.

Sampling Sites	Abbreviation	Land-use characterization	Agricultural Intensity %
Lake Worth	LW	Suburban- 37 km away from agriculture	0
Wellington	WT	Suburban-14 km away from agriculture	0
Homestead	HS	Mixed land	34
Canal Point	CP	Agriculture	51
Belle Glade	BG	Agriculture	93

Table 2

Male gonad morphology description.

Land-use	Classification	Description
Non-agricultural	Males	Normal organs in non-agricultural sites. No abnormal males were documented at these sites.
Agricultural	Males	Normal organs in agricultural males
	Bidders	Vitellogenin accumulation in Bidder's organ with normal gonads
	Intersex	Testis with ovaries and oviducts